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The exploration of interaction studies of smaller size, mostly ignored yet intrinsically inestimable molecules towards BSA; An example of STD and DOSY NMR

Research Article

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Abstract: Larger size or novel structure molecules are always appreciated by all fields of experimental and computational science. Conversely, molecules with smaller size and simple structures are usually ignored with no explanation as to why. However, the vast majority of more diminutive molecules behave as a cornerstone in the synthesis of a bigger structural framework. Subsequently, we planned to uncover the interactions of small molecules towards macromolecules, and successfully presented the binding results of 2-aminopyridine and Isovanillin towards BSA through NMR techniques. STD epitope mapping and also the DOSY results provided evidence that Isovanillin remained closer to the binding cavity of protein. Titration experiments afforded 584 μM (0.584mM) and 487 μM (0.487 mM) dissociation constants for isovanillin and 2-aminopyridine respectively. Furthermore, changes in diffusion coefficient (with and without protein addition in DOSY spectra) were found to be 0.081 log (m^2 s^{-1}) and 0.096 log (m^2 s^{-1}) points for isovanillin and 2-aminopyridine respectively. Docking studies exhibit that these molecules can tie to site 1 (sub-area IIA) through the pi-pi interaction and hydrogen bonding with Trp213. Our results demonstrated that both compounds could be utilized as part of a transporter in the circulatory system and their extension-inspired compounds may be utilized in new drug design.

Keywords: *DOSY NMR • STD NMR • Interaction studies • BSA • Ligand-protein*

1. Introduction

In drug discovery, NMR's long and successful history guarantees its use in a variety of experiments for portraying and distinguishing ligand-based communication. Previously, the harnessing of NMR's capability was just for molecular structure mapping. However, with the disclosure of new high-throughput screening strategy, it became a more delicate and functional device for hit-to-lead drug finding that is far superior than X-ray crystallography in the utilization of small to modest amounts of sample for library screening [1]. By consolidating the structural and additional functional information of ligand inhibitions towards protein, NMR has an edge compared to other spectroscopic and non-spectroscopic strategies. The protein activity hinges upon ligand interaction that forms the cascade of complicated functions within the body. Therefore, the ligand-protein interaction studies are of utmost importance for understanding the functional complexities of the body at the atomic level [2]. The potential capability of NMR to uncover the interactions between the macromolecules

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and small organic molecules in drug discovery has been around since the 1990's, however, has thrived in the most recent decade.

In NMR, ligands and protein both experience perturbations inside the strong magnetic field. Consequently, absolute NMR strategies utilized for interaction studies can be divided into two sub-groups based on perturbations observed, a) receptor-based screening and b) ligands-based screening. Normally, the receptor-based screening strategies demand that isotopically labelled proteins undergo longer experimental time. Nevertheless, even more generally applicable, the most overlooked and an extremely emphasized method for screening purposes is a so-called ligand-based technique. Utilization of minor to moderate amount of protein in ligand-based screening makes it an even more routinely utilized method as part of hit-to-lead optimizations [3,4]. Over the years, a variety of ligand-based NMR strategies have been established to depict the binding affinity of many biological active substances towards protein. Such techniques incorporate STD NMR [4], diffusion experiments [5], NOE pumping [6], Tr-NOESY [7,8], waterlogsy [9], SALMON [10] and INPHARMA [11] and so forth.

The saturation transfer difference (STD) NMR is sensitive to the sub-atomic motions in free and, additionally, the bound state, which is the reason the perturbation created by the radio frequencies (RF) give the information about the free or bound state of a ligand to a protein. Nowadays, STD NMR is the cornerstone for NMR based screening. It demands an extremely low protein concentration, without isotopic labels, no previous knowledge of its structure, and, above all, there is no constraint for protein size. It is likewise plausible to allocate the binding orientation of the ligands in a huge library; purported as group epitope mapping [4]. Very recently, Ferreira and co-workers [12] distinguished the strong ligands from the natural extract with the application of saturation transfer difference NMR together with a LC-SPE-NMR hyphenation framework that makes NMR an immediate device to be connected to the drug discovery process. In this context, one may argue that if the compounds are known inhibitors and/or potent then they can be used for specific targets, but by no means STD NMR infers the bio-activity of the compounds itself.

Moreover, Diffusion order-spectroscopy (DOSY) NMR has been known to be viable in distinguishing weakly bound ligands in a mixture [13,14]. Since, DOSY phenomenon is dependent upon molecular anisotropy; weight, size and shape of the given molecule, is otherwise called NMR chromatography by a few authors [15,16], as it focused on the diffusion based spectroscopic partition (not physically). Imperatively, it is a non-invasive technique [17] in nature and does not disturb the aggregation equilibrium. Despite that, the compound chemical shift sensitivity is a NMR specific characteristic that permits observing the diffusion changes by the addition of macromolecule in a mixture. Thus, it additionally makes discrimination of free ligands from bound ones a simple task.

Usually, interaction studies have been performed on substantial molecular weight compounds or compounds with uncommon (novel) skeletons. Despite that, there are still a large number of bioactive compounds which are modest in size and basic in structure. However, they remain ignored possibly due to the simplicity of accessibility or how easily they are synthesized. We have chosen to tackle such small biodynamic molecules, which are usually neglected for no particular reason. For our studies, we have chosen to track the interaction of 2-aminopyridine and Isovanillin towards the bovine serum albumin (BSA) protein. Both compounds possess a number of biological activities but remain unable to obtain the merit they deserve such as being a lead candidate. Isovanillin has recently been demonstrated as an antimicrobial [18], has antibacterial [19] activities, as a competent substrate of xanthene oxidase [20,21], and a powerful inhibitor of aldehyde oxidase [22]. Aldehyde oxidase is a metabolizing compound whose activity has generally prevailed in the liver [23-25] and plays an important role in metabolizing numerous N-heterocyclic and aldehydic drugs [26,27]. On the other hand, 2-aminopyridine is utilized within the generation of numerous drugs, and is a known inhibitor of Nitric Oxide Synthase (NOS) [28,29].

2. Experimental procedure

NMR spectra were recorded on a Bruker Avance III 600.13 MHz (Hydrogen nucleus) spectrometer at 298K temperature, equipped with a 5 mm cryo-probe TCI (triple resonance $^{13}C/^{15}N/^{1}H$) having pulse field gradients along the z direction. Data acquisition and processing was performed with Bruker software Topspin 3.0 version installed on the spectrometer.

2.1. Sample preparation

Compounds (2-aminopyridine, Isovanillin and BSA) were obtained from Sigma Aldrich Brazil. All samples of ligands (~5 mM concentration) and protein (~50 µM concentration) were prepared in 75 mM phosphate buffer solution having pH=7. 4 and 150 mM NaCl in 100% D_2O, and were stored at 4ºC. Thus, there was existence of a 1:100 molar excess of protein to ligands

respectively. All the samples were used without further purification for the following studies.

2.2. Saturation transfer difference (STD) NMR

STD NMR studies were performed with the 50uM solution of BSA protein in phosphate buffer pH 7.4 in D_2O, and 5 mM of 2-aminopyridine and Isovanillin in (5: 95% v/v) DMSO and D_2O respectively. A series of ten STD NMR spectra were acquired with varying pre-saturation times (0.5-5 s) for the calculation of STD amplification build-up step. Similarly, in competition and titration spectra, eight STD experiments were performed with varying concentration (0.3, 0.50, 0.625, 0.75, 0.875, 1.0, 1.56, and 2.0 mM) of the ligand's solution for the computation of dissociation constant (K_D) for each compound. STD-NMR experiments were obtained with a selective train of Gaussian shaped soft pulses having truncation of 1%, with 49 ms of length and each separated with 2 ms. Bruker standard STD pulse sequence with gradient tailored water suppression was utilized for all these STD experiments for suppression of residual HOD signal. In all spectra, selective irradiation was done at -0.5 ppm for the *on*-resonance and 30 ppm for the *off*-resonance (reference or STD control) with 30ms of spin-lock filter for the protein signal suppression. Subtraction of spectra (*on*-resonance from the *off*-resonance) was performed through phase cycling. The saturation time of ca. 2 s discovered to be more efficient for the STD studies in this case after calculating STD amplification factor at different saturation times (0.5-5 s). Prior to the Fourier transformation, all STD NMR spectra were multiplied by an exponential line broadening function (LB) of 1-2 Hz. The total numbers of scans for acquiring these STD-spectra were 128 with the application of 16 dummy scans for each.

2.3. Diffusion ordered spectroscopy (DOSY)

Bruker standard LED pulse sequence was utilized for these diffusion edited studies. The gradient length used was 1 ms with recovery times of 200 µs for these gradients, and longitudinal eddy-current delay of 5 ms. A total of sixty-four different gradient amplitudes were used for each (with and without protein addition) experiment with an optimized diffusion time of 0.08 s. In both cases (with and without receptor), the acquisition data were collected with gradient strengths starting from 1.07 G cm^{-1} to 53.5 G cm^{-1} with 256 scans to observe these interactions. In every case, the relaxation delay kept to 3 s with the pre-scan delay of 10 µs with automatic baseline correction.

Processing of 2D DOSY spectra with or without the addition of protein were performed with the help of Topspin 3.0, by the line broadening function 1 Hz and 0.03 Hz along the F_2 and F_1 dimensions respectively. The DOSY macros were run by using 64 K data points and with noise sensitivity factor of 20. The Gaussian apodization function of 0.01 Hz was put only in the F_1 dimension. Zero filling up to 4 K points along the F_2 and 1 K points along the F_1 were employed in both cases for the 2D spectra before performing Fourier transformation. The resultant DOSY was pseudo two-dimensional spectra with NMR chemical shifts (in ppm) along one axis and calculated diffusion coefficients (in log (m^2 s^{-1})) along the other. Comparison of diffusion coefficients in the presence or absence of protein was measured by taking the water signal as a standard.

2.4. Docking simulations

Docking is a technique that ties the ligand with protein by different types of interactions. Docking simulation is conducted to validate the binding affinity and interacting mode of ligands within the target's binding cavity. For this purpose, docking studies of selected small molecules (2-aminopyridine and Isovanillin) were performed against bovine serum albumin (BSA) by using Molecular Operating Environment (MOE) 2011.10. In principle, both compound structures were constructed using ChemDraw and, later on, converted to 3D form by Babel program incorporated in OpenEye. Atom type correction, charge application and minimization of ligands were done on MOE 2011.10. On the other hand, a PDB file of BSA protein was taken into consideration from the protein data bank (pdb) with the pdb code number 4F5S. As BSA molecule is a homodimer of two chains, so, chain B was prepared for further docking analysis. Protein preparation was done by using auto-correction option in MOE. Furthermore, protein residues were protonated and minimized according to use as a receptor input for docking. Selection of active site for the construction of a map around the active site residues was taken from the literature [30]. Default MOE docking parameters were used for docking with the Triangle Matcher algorithm. London dG was selected as rescoring function 1 and GBVI/WSA dG as rescoring function 2 with the generation of 10 conformations of each ligand to better fit within the binding pocket. The docking results were clustered in a mdb output file that were then analyzed for evaluation of ligand-protein interactions and their binding affinity.

3. Results and discussion

As a rule, scientists, organic chemists and biologists are enraptured by bigger and complicated structural compound such as kibdelomycin, vancomycin, and

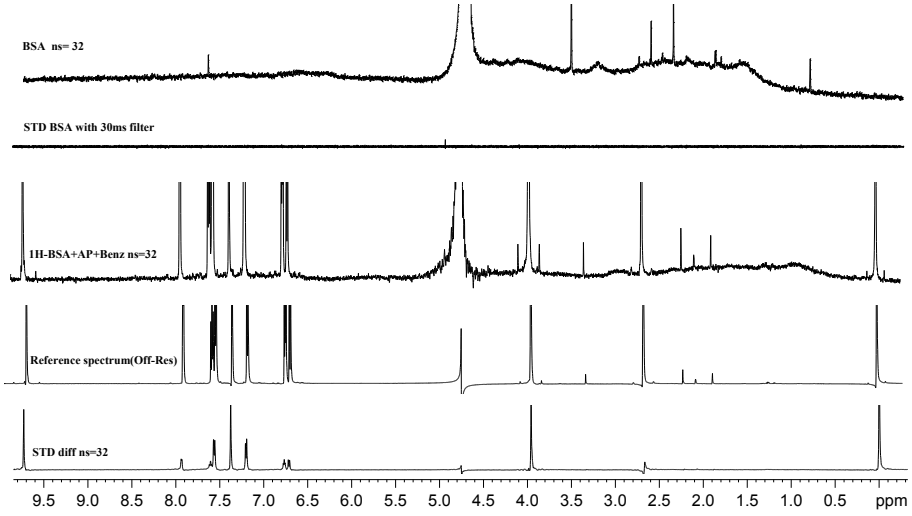

Figure 1. A) The top, ^{1}H-NMR spectrum of 50 μM BSA in phosphate buffer with the pH 7.4 solution, showing a characteristic broad signal obtained by using three mm NMR tube. B) STD Spectrum of protein with 30 ms of Spinlock filter, showed a complete removal of the protein signal from the spectrum. C) A ^{1}H-NMR spectrum of 50 μM BSA with 5 mM of each, 2-aminopyridine and isovanillin. D) A reference spectrum (*off*-resonance) obtained with a cascade of 40 Gaussian shaped soft pulses, 2 seconds of saturation time and by applying 30 ms of spin lock filter ($T_{1\rho}$). For STD spectra, *on*-resonance saturation pulse was placed at −0. 5 ppm, while the *off*-resonance at 30 ppm. E) A STD difference spectrum obtained by internally subtraction *via* phase cycling. All above spectra were produced on Bruker 600MHz AVANCE III spectrometer with a cryogenic TCI probe at 298 K temperature.

others for the bioactivities or inhibition recognized as useful for interaction studies. Conversely, smaller sized structures, such as those that come to be a starting point of most drugs, are generally overlooked. Moreover, modest size compounds such as sugars andamino acids are convincingly picking up much importance by playing additional imperative roles in diverse disease curing and also in the infection aetiology by easily reaching the site of action after ingestion. Thus, keeping these favourable points of interest of the simpler structures and, in addition, the bioactivities [18-29] of the selected molecules (2-aminopyridine and Isovanillin) attracted our focus on the interaction studies.

3.1. STD NMR Studies

Nowadays, large numbers of high-throughput screening methods are undoubtedly being utilized for the screening of huge libraries joining the spectroscopic, non-spectroscopic and computational methodologies simultaneously. Consequently, we took advantage of STD NMR and diffusion ordered-spectroscopy (DOSY) to describe the binding of 2-aminopyridine and Isovanillin to bovine serum albumin. STD-NMR analyses were done by utilizing 1:100 ratio protein to ligand concentration respectively, at 25°C in D_2O, buffer (having pH 7.4) and 5% DMSO-d_6. A freshly prepared solution of ligands and, in addition, the protein was utilized as a part of this characterization, and sample was irradiated at -0.5ppm to accomplish the protein saturation (*on*-resonance) and at 30ppm of unbound ligands (*off*-resonance). The signal presence in the STD NMR spectrum is a sensible proof of the binding of compounds to protein as indicated in Fig. 1. Conversely, the signal absence in STD NMR spectrum reveals that the particular ligand is not suitable as inhibitor for this protein. In the present study, we displayed the technique for tying of ligands to serum albumin. The epitope mapping by STD-NMR portrayed here describes to the atomic binding level of ligands (2-aminopyridine and Isovanillin) with BSA. The result of STD spectrum demonstrates that the signal shows clear ties of both structures (2-aminopyridine and isovanillin) to serum albumin.

3.2. Group Epitope Mapping (GEM) studies

In the STD NMR spectrum, the signals from Isovanillin (4-Hydroxy-3-methoxybenzaldehyde) were readily recognizable, which showed that this is an active ligand, whereas the little signals of 2-aminopyridine along these lines, is a less dynamic compound. The methoxy signal from isovanillin provided a larger integral value and was given a 100% STD effect, and whatever remains of the signals were given a relative STD effect to methoxy signal as indicated in Fig. 2. Indeed, the methoxy group occupies closest to the binding cavity of the protein, thus, accepted more saturation transfer from protein because of cross relaxation. Similarly, the second large integral values were discovered for an aldehydic proton of isovanillin with 73% STD effect, guaranteed a closer contact likewise. The ring protons (H-2 and H-4 of isovanillin) contributed almost equivalent STD Effect with 50% and 51% respectively, while the proton H-5 of isovanillin indicated a 63% STD effect.

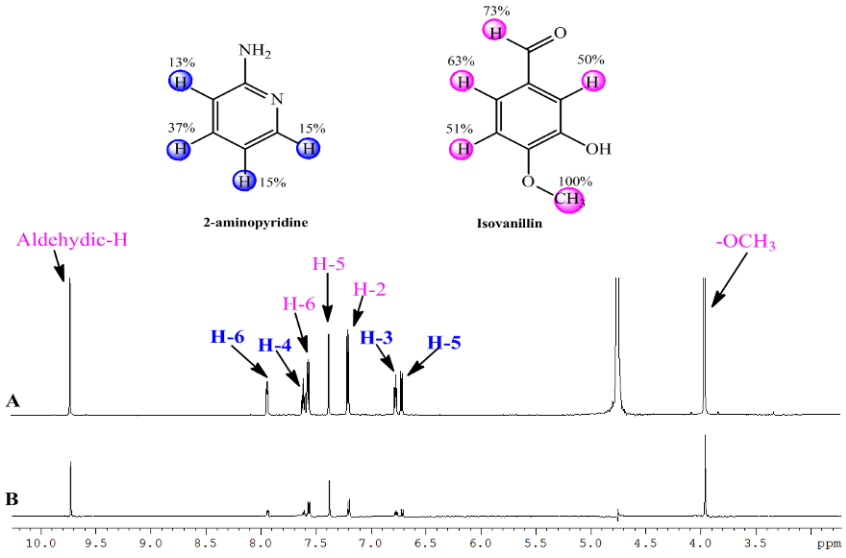

Figure 2. STD-NMR of 2-aminopyridine and isovanillin with BSA: (A) reference ^{1}H spectrum of 2-aminopyridine and isovanillin with BSA and (B) STD difference spectra of 2-aminopyridine (5mM) and isovanillin (5mM) with BSA(50 μM in phosphate buffer with pH 7.4). The relative STD amplification factors as calculated from STD difference spectrum are shown on the top of the spectra.

Conversely, 2-aminopyridine provided significantly less intense signs, thus, less interaction with the protein. The greatest STD impact was seen from the proton number H-4 of the 2-aminopyridine with the 37% effect, while the proton number H-5 and H-6 showed up with equivalent effect of 15%, deliberately showing less contact to binding cavity of protein. Whereas, the proton number H-3 remained further along the way from the contact surface with the STD effect of 13% and hence, more solvent exposed. In the example, which we displayed here, the STD saturation time of ca. 2 s discovered to be more efficient after evaluation of series of experiments with varying saturation times from 0.5 s to 5 s, and consequently further experiment including STD titration experiments were performed with this presaturation time. On the bases of screening outcome presented in Figs. 1 and 2, it is evident that the isovanillin ties tightly to protein while the 2-aminopyridine is more towards the solvent side (a long way from protein's binding cavity).

3.3. STD Build-up studies

In the STD, build-up experiments (varying STD saturation time from 0.5s-5s) were performed for explicit evaluation of the STD amplification factor. By definition, the STD amplification factor is the fractional saturation received appropriated from the protein for a given proton multiplied by the molar excess of the ligand over the protein, as indicated below in Eq. 1

$$A_{STD} = \frac{I^{\circ} - I_{STD}}{I^{\circ}} X \frac{[L]}{[P]} \quad (1)$$

Where A_{STD} is an enhancement element of the given proton (I_{STD}/I^{o}), and [L] and [P] is given concentrations of particular ligands and protein separately. Indeed, the amplification factor is the power of the STD signal (average number of molecules) saturated for every atom of protein.

In principle, more extended saturation time or more ligand utilized, led to the biggest STD signal intensity (A_{STD}) and higher turnover rates. The confirmation about the saturation time came to be clear when different STD spectra were acquired by varying saturation times (0.5 s to 5.0 s) (Fig. 3), and A_{STD} plotted against these saturation times (Figs. 4a-4b). A_{STD} came to be exceptionally imperative by quantitative analysis at different saturation time, as the A_{STD} of giving hydrogen is very different from the other one clarifying the proximity to the protein surface. From the saturation profile as demonstrated in Figs. 4a-4b, it is self-evident, at maximum saturation time, the receptor sites become saturated and no more ligand saturation takes place resulting in a flattened intensity curve.

3.4. STD titration studies

Similarly, the increase in ligand concentration likewise is answerable for larger STD intensities until the saturation of all binding sites of the protein. A_{STD} is also supportive for titration ponders. Dissociation constant value of these ligands (2-aminopyridine and isovanillin) might be computed with the assistance of the following mathematical Eq. 2:

$$A_{STD} = \frac{\alpha_{STD}[L]}{K_D + [L]} \quad (2)$$

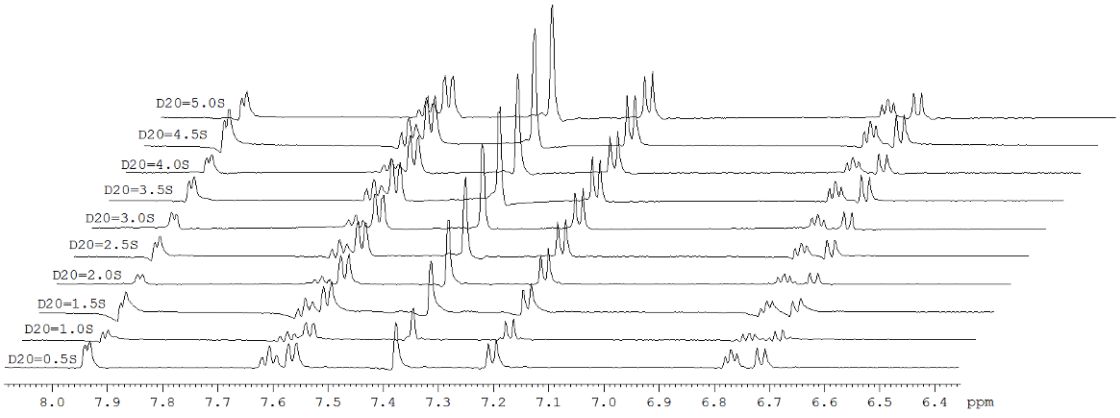

Figure 3. Stack plot of STD-NMR (2-aminopyridine and isovanillin with BSA) spectra at varying saturation times (0.5 s to 5.0 s). The STD-NMR spectra were performed with 50 μM BSA (~67-KDa) in phosphate buffer pH 7.4 solution, with 5 mM solution of 2-aminopyridine and isovanillin each in 5% DMSO with 95% D_2O. Further details can be found in experimental section.

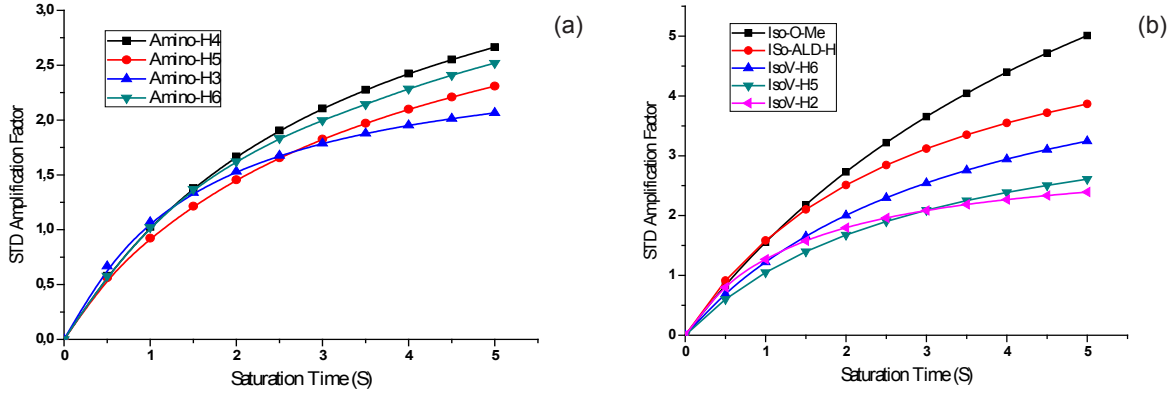

Figure 4. STD amplification factors plot of 2-aminopyridine (A) and Isovanillin (B) as a function of saturation time, as a result of integral values obtained from different STD spectra (shown in Fig. 3). Methoxyl signal with largest intensity of Isovanillin was given the 100% STD effect and rest signals were normalised with rfrence to this signal.

Where K_D represents to dissociation constant, and α_{std} to maximum amplification factor, calculated at a given saturation time. For the K_D constant examination, we expected three things; 1) system (ligands and protein) is rapidly and reversibly exchanging, 2) all the binding interactions have the same equilibrium and 3) binding surface (binding sites) of protein is independent. Unique STD examinations were performed with varying ligands concentration (see the experimental section) while, the saturation time for all experiments were kept the same and was equivalent to 2 s. From the stack plot (see the ESI†) it came to be clear that A_{STD} in addition got higher with an increase in ligand concentration. A parabolic curve obtained when the amplification factor (A_{STD}) was plotted against the different concentration of ligands, as demonstrated in Fig. 5. The K_D values as estimated from Eq. 2 by the use of Microsoft Excel (see ESI†) were 584 µM (0.584mM) and 487 µM (0.487 mM) for the isovanillin and 2-aminopyridine respectively.

3.5. Diffusion ordered spectroscopy studies

Furthermore, we have also performed the diffusion-ordered spectroscopy of the selected molecules with (Fig. 6) and without (Fig. 2 ESI†) the addition of protein. A quick inspection of 2D DOSY spectrum showed that both compounds diffusion coefficient was laid between -9.0 log m^2 s^{-1} to -9.8 log m^2 s^{-1}, as seen in the spectrum of Fig. 6. The calculation of the diffusion was very easy, as NMR chemical shifts (in ppm) stays in 2D DOSY spectrum along one axis and figured diffusion coefficients (in log (m^2 s^{-1})) along the other. By gathering all straight-line signals, one can obtain the diffusion coefficient of a compound. Thus, the top five signals in a single line (Fig. 6) represented to the diffusion coefficient of Isovanillin on diffusion axis and chemical shifts on separate axis, as shown from the 1D spectrum in Fig. 1. Similarly, the second four signals in one line were acknowledged to 2-aminopyridine atom. The molecule having affinity towards the macromolecule shows the

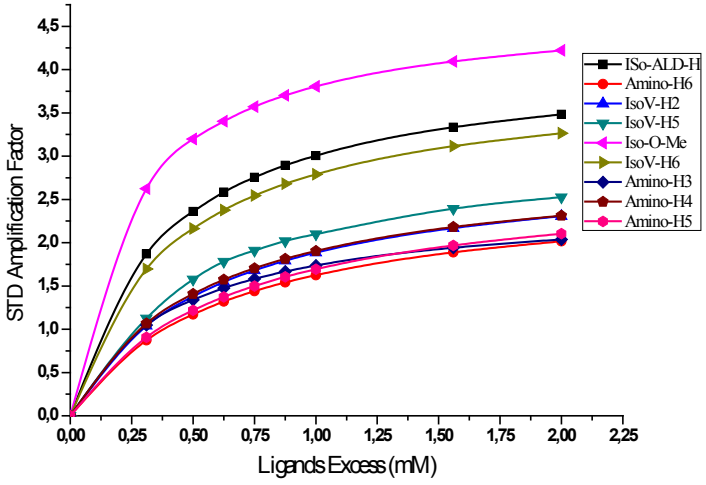

Figure 5. STD amplification factor curve plot of 2-aminopyridine and Isovanillin as a function of varying concentrations ranging from 0.25 mM to 2.00 mM for each single parabolic line that determined from the STD titration spectra as shown in ESI†.

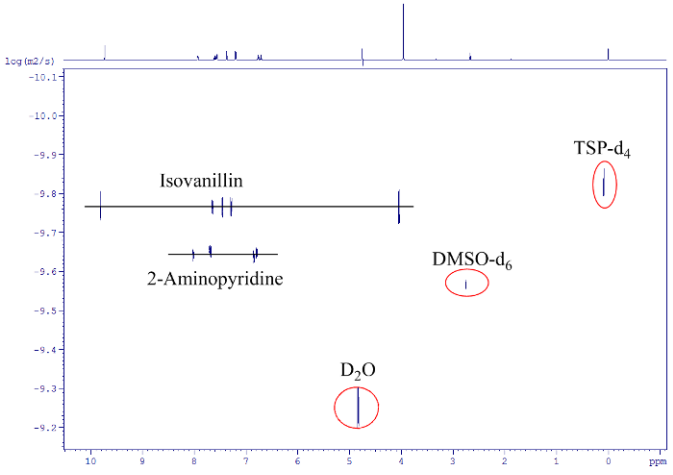

Figure 6. 2D-DOSY NMR spectrum of 2-aminopyridine and isovanillin with BSA, where the X-axis contains the standard ^{1}H-NMR chemical shifts, while the y-axis contains the diffusion dimension. Spectrum produced as a result of total diffusion time (Δ) of 80 ms, 5 ms of eddy current and 1000 μs of small delta (∂). The area in a circle represents the diffusion coefficient of reference solvents (D_2O, DMSO and TSP-d_4) while, the solid straight lines represent the molecules of interests.

change in diffusion coefficient with and without the addition of the macromolecule [13,14]. By comparing both spectra (with and without protein) it is quite clear that both molecules indicated a change in diffusion. Isovanillin in the presence of the protein showed a -9.75 log (m^2 s^{-1}) while in the absence -9.50 log (m^2 s^{-1}) (ESI†). Moreover, 2-aminopyridine -9.643 log (m^2 s^{-1}) (Fig. 6) and -9.365 log (m^2 s^{-1}) was observed in the presence and absence of protein respectively. Adjusting HOD signal at -9.28 log (m^2 s^{-1}) as reference for both spectra, the diffusion coefficient of Isovanillin indicated 0.081 points while the 2-aminopyridine showed a 0.096 points change (Fig. 2 ESI†) in diffusion by the addition of protein (Fig. 6). These effects prompted the conclusion that both molecules were involved in the interaction, but the compound with little structure or less atomic weight indicated a bigger diffusion change with the addition of protein.

3.6. Docking studies

For further consolidation of our results, we initiated the help of docking simulation studies. In principle, docking is a strategy that ties the ligand through diverse interaction within the binding pocket of the target protein. To perform the docking studies, we have chosen the sub-space IIA of site 1 to see the different conformational adaptations with lowest possible ligand-protein complex energies for these compounds, as illustrated in the experimental section. Docking results uncovered the fact that both compounds were small

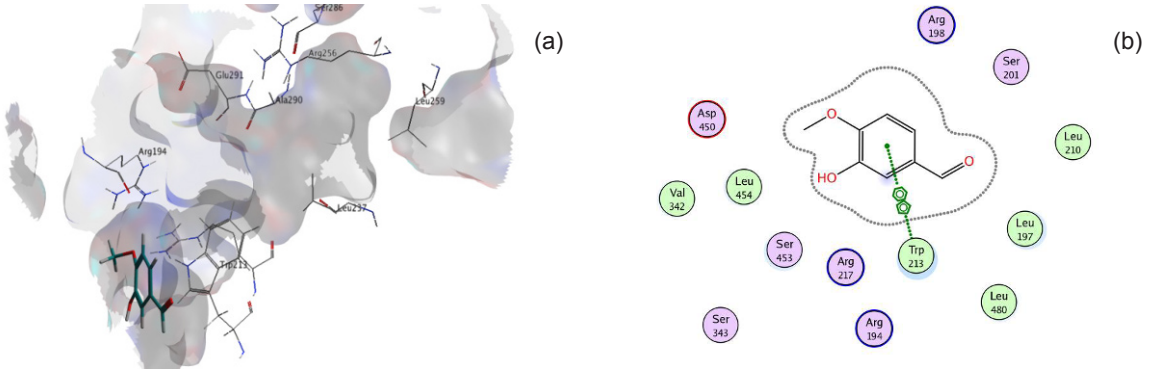

Figure 7. Molecular Docking model of interactions between the Bovine Serum Albumin (BSA) and isovanillin, generated by using Molecular Operating Environment (MOE) 2011.10. The BSA PDB file was taken from protein data bank (http://www.rcsb.org/pdb/explore.do?structureId=4F5S) (4F5S). The classical binding sites were marked in corresponding subdomain cations. See more details in the experimental section. Fig.7b representing a 2D scheme for interaction between the BSA binding sites and Isovanillin molecules. Where, the ϖ-ϖ interactions between the isovanillin and Trp213 residues of BSA binding sites are shown with green dashed lines.

in size, and could only occupy a part of the active site and interacted with residues. Isovanillin, being a small molecule, interacted with only Trp213 through pi-pi interaction (Fig. 3 ESI†) leaving all other residues vacant (Fig. 6a). Correspondingly, 2-aminopyridine is likewise a small molecule that cooperated progressively because of amino aggregation and upheld both hydrophilic and hydrophobic sorts of interactions. Arg217 donated its bond to NH_2 whereas Asp450 received hydrogen bond from NH_2 and pi-pi interactions were favored by Trp213 (Fig. 4 ESI†). Atomic Operating Environment (MOE) 2011.1 stacked up these molecules (2-aminopyridine and Isovanillin) as an active ligand but, being a smaller size, they could not fill the cavity properly. Based on this study provided through MOE, we inferred that the small molecules interacted with BSA through hydrophobic (pi-pi interaction) and, in addition, hydrophilic (Hydrogen bonding) interaction that stabilized this ligand-protein complex.

4. Conclusion

We have presented an STD NMR method that permitted the identification of high-affinity ligand based on fast exchange process (turnover rates) on the NMR time-scale. This study was represented with reference to the BSA (Bovine Serum Albumin: a model protein) –ligand complex framework. Affinity towards the receptor might be promptly understood from the ligand signal intensities in the STD difference spectrum. Larger signals in STD spectrum implies a greater affinity to the specific targets and, thus, stronger will be the saturation gained from the receptor. In this study, we utilized Bovine Serum Albumin as a model, however this approach could be connected to different targets, and it was used to uncover the approximate value of the dissociation constant of dynamic ligands. By utilizing a low protein to ligands concentration (50 μM: 5 mM respectively), we have successfully demonstrated that isovanillin is a stronger ligand than 2-aminopyridine, as it is clear from the signal intensities (Fig. 2). The estimated dissociation constant values as a result of STD NMR at different concentrations ranked isovanillin a potential ligand compared with 2-aminopyridine with the corresponding values 584 μM and 487 μM respectively. Moreover, diffusion ordered spectroscopy provided the same outcome as acquired from larger changes in the diffusion coefficient value of isovanillin as well. Likewise, the docking simulation also discovered to be in magnificent concurrence with these NMR results; announced isovanillin as potential ligands that can tie through hydrogen bonding and additionally by means of pi-pi interaction. Effective STD NMR, DOSY NMR and docking depicted that both compounds may be utilized as a transporter as a part of the circulatory system. Thus, on the premise of the combined results from the NMR techniques and docking simulation, there is no obvious reason for why these smaller compounds should be ignored in drug discovery.

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Abbreviations

STD - saturation transfer difference;
NMR - Nuclear magnetic resonance;
DOSY - Diffusion -ordered spectroscopy;
BSA - Bovine serum albumin;
Tr-NOESY - Transfer nuclear overhauser effect spectroscopy;
STD-AF - STD Amplification factor;
MOE - Molecular Operating Environment;
LED - longitudinal eddy current delay;
ESI† - Electronic supporting information.

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