Assignment: it an indispensable tool in the process

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Assignment: The Use of Protein NMR in Active SiteMapping.

NuclearMagnetic resonance spectroscopy (NMR) is an analytical technique that is basedon using the known chemical constituent of a compound to distinguish it fromother unknown compounds. The ability of this technique to differentiate betweenstructures of molecular substances and the information it provides about thedynamics and interactions of particles in the smallest possible unit of amatter makes it an indispensable tool in the process of drug discovery, development as well as delivery. This chemical analytical method is verysensitive to its environment, so can give very minute information about how thesmallest fragment of a molecule binds to a target molecule, protein or itscomplexes.

Information about the exact binding site or interaction between thefragment and the receptor of interest is also highlighted. Hence, thistechnique is a very vital technique in the Pharmaceutical, forensic, qualitycontrol industry. This analytic technique also has its application in the fieldof research where it is used to determine the purity, quality, quantity andstructure of the unknown while confirming that of the known substance. Thecombination of this analytical chemistry technique to Protein in the biologicalscience is what is known as Protein NMR. IntroductionProteinNuclear Magnetic Resonance has been used extensively to study enzymemechanisms, analyzing structures of proteins, nucleic acid and its complexes Thistechnique is also employed in studying protein interactions with ligand andother protein and the dynamics of proteins in organisms. In the field of drugdevelopment, the study of protein and its complexes are of utmost importance asthey play vital role in physiological and pathological conditions and processhence the

importance of thoroughly understanding their catalytic process andhow they bind to their substrate. Protein NMR in active site mapping thus, isthe application of NMR in the parts of an enzyme where substrate molecules bindand undergo chemical reaction as well as where its remains forms temporarychemical bonds with the substrate. This region in an enzyme is known as theactive site.

The mapping of active sites is quite crucial in the field ofpharmaceutical science or drug discovery. The detailed knowledge of the site ofa target receptor for drug discovery and the understanding of the proteindynamics in the targeted site will maximize the efficacy of the proposed drugby giving a clear and precise understanding of the protein to ligand bindinginformation and also the nature of the existing binding force which existsbetween protein to ligand and also ligand to protein interaction (Yan Li etal, 2017). These interactions aid the design of new drugs for instance enzymeinhibitors, by providing the minutest details of the size on the active sites, how many different sides and auxillary sides are present, their properties, howthey come together and bind chemically. The understanding of this uniqueinteraction is also a tool for comparison in active site mapping, where it isemployed to compare protein active sites and their structures in more detailsso as to design drugs that can exactly match into the enzyme substrate complexusing the key and lock analogue for enzymes.

Thisprotein analytical tool has been used in lots of studies to investigate enzyme behaviors, their mechanisms as it takes less time an effort to acquire structuralinformation of compounds and DNA when compared to

other methods like X-raycrystallography, florescence and IR spectroscopy, hence the ever growingimportance of active site mapping using Protein NMR.(Yong et al. 2012)19FNMRstudies had be done to clearly distinguish structural and functional featuresof protein as seen in its recentapplication in active site mapping out of galactose binding- protein, transmembrane aspartate receptor, the Che – Y protein dihydrofolate reductase , elongation factor-TU, and D-lactose dehydrogenase, that demonstrate the utilityof 19 F NMR in the analysis of protein conformation state even in particles that are so large orunstable for full NMR structuredetermination.(Mark A. D, et al 2010). Thesekind of studies depends on the chemical shift pattern of FNMR as this method isvery sensitive to change in its environment due to the presence of fluorine 19, as well as the existing weak Vander Waal force of bond as well as the presenceof the local electrostatic field. Figure 1.

Overview of applications of NMR in drug discovery NMRspectroscopy can provide critical information at early stages of hit validationand identification. NMR measurements for binding studies can represent a keystep to eliminate false positives from high-throughput (HTS) campaigns, tovalidate putative hits from in silico screensor to identify novel scaffolds in fragment-based programmed. NMR and X-raycrystallography can also provide unique information to subsequently guidehit-to-lead optimization. ADME-tox, absorption, distribution, metabolism, excretion and toxicity (Pellecchia M el at: 2002) Thisreview will mainly concentrate on saturation transfer difference (STD – NMR)method which is a solution state nuclear magnetic resonance spectroscopy techniqueused in target- based drug discovery, hit identification, validation and leadoptimization which is a tool that is

extensively utilysed in drug developmentprocesses as seen in our review of this method in the biological studies of newurease inhibitors. Fig2 flowchartshowing drug discovery process Fig3 showing the process in Protein NMR Process Figure 11. This is a flow chart showing the different level of applicationof NMr in the process of drug discovery from when the target is identifinedthrough the whole complete process and the role it playshighlighted in white and blue; Figure 111, highlights the varous steps involvedin in using protein NMr in active drugin drug discovery and its application.

(Yan Li et al, 2017;). Materialsand Sample preparation STD-NMR ExperimentJack bean (Canavaliaensiformis) urease (EC 3. 5. 1. 5), urea, Dulbecco’s Modified Eagle Medium (DME), cycloheximide, di-sodium hydrogen phosphate, mono-sodium di-hydrogen phosphate Unichem(India). Mous, and phenol were obtained from Sigma-Aldrich (USA). Deuteratedmethanol (CD3OD), and deuterium oxide (D2O) were purchased from the Armar Chemical (Switzerland) Methods/ExperimentalThemeasurement of urease inhibitory activity by STD-NMR technique was done usingthe afore mentioned technique, that is very popular in drug discovery andpossess high sensitivity hence often used for ligand –observed NMR screeningmethods. In this experiment, Gaussian RF pulse was applied to the most up fieldprotons of the target protein which when saturated is then transferred throughoutthe molecule by spin diffusion.

At the final stage of this process the boundligands received magnetisation through cross relaxation and enhanced signalintensity is displayed (Atia-tul_Wahab et al. 2013:). The sample for thisexperimental process is prepared with Jack bean (Canavalia ensiformis, EC3. 5, 1. 5) using deuterated NMR buffer toprepare(20uM) of urease solution, which is then stored at 4 °C ligands. The reaction mixture was in excess of100folds of urease concentration.

They were dissolved in 13. 3% of CD3OD, and 86. 7%deuterated phosphate buffer (4 mM, pH 6. 8). This was followed by STD-NMR screeningexperiment performed on Bruker 400MHZ NMR spectroscopy at 298K Stddiffgp19pulse program was used for STD-NMR experiments. Saturation time was 1. 0–2.

0s, while interpulse delay (D1) was the same as D20 or D20 + 1. Loop counter was8. 0 and 4. 0. STD-NMRspectra were recorded with 32 scans (NS), and eight dummy scans. For eachexperiment, 90° pulse was calibrated separately. Gaussian selective pulses of48ms length with an excitation bandwidth of 140 Hz, separated by 1 mms delayswere used. To saturate the protein selectively, on-resonance irradiation wasprovided from 0 to ? 1 ppm (protein resonances), while off-resonance irradiationwas provided at 30 ppm.

Difference spectrum was obtained by subtracting theon-resonance irradiation spectrum from off- resonance spectrum. This wasfollowed by docking studies that involve the study of the molecules present andhow they interact with each other so as to establish their identity, molecularstructure and how they bind to the proteins present. These facts highlight thekind of inhibition and the kind of interaction that is existing between theligand and

the protein at the atomic level(Scopes 2002;)(Meng et al, 2011;). Experimental For F-NMRTechnique Purification of thetarget protein is usually the first step, followed by the modification of theprotein of target by using compounds containing fluorine like 2 bromo-N-(-4 – trifluoromethyl) phenyl)acetamide(BTFMA) at cysteine residue which results in the presence of a protein withactive " F spin (Horst et al, 2013;) (Kitevski et al, 2012:) (Liu J, J et el, 2012;) making it possible forchemical analysis to be carried out , which is normally the last step beforethe process of Hit identification. (Nortonet al, 2016;)Hit identification iscarried out at this stage to for thepurpose of screening F- labeled compound using ligand – observed experience known as FBDD, that usually has anexisting library or in the absence ofthis library one can easily be made-up by adopting similar rules to those use in usual fragment library to sustainligand size and chemical variations.

F- NMR as a target based proteinspectroscopy can be used to affirm the hit screening from HTS campaigns inwhich a chemical assay has being used as the primary screen (Gee C. T et al, 2016:). The proteins of targets, which are normally close to the active site, arelabeled with Fluorine atom. This technique is then preceded with theidentification and validation of the targeted resonance in the presence of thefluorinated substrate. Results: In this review we havelooked at the use of protein NMR in active site mapping by using biochemicalassay, then followed by the use of STD-NMR which is a ligand resonance based technique, for the primary identification of urease inhibitors. Then followed by moleculardocking studies to validate the biochemical experiment as well as to estimatethe relative binding affinity

between the ligand and receptor. F-NMR which is atarget based resonance, coupled with hit identification methods were also usedto observe targeted ligand, screening were carried out, confirmation of theprimary screen with the use of the F atom and its identification and validationin the presence of the fluorinated substrate was achieved in this experimentDiscussion; Themeasurement of urease inhibitory activity by STD- NMR technique was done using Saturationtransfer differential NMR which is a ligand resonance based spectroscopicmethod that is undoubtedly one of the most widely used NMR Spectroscopic techniquedue to it's ability to establish a binding relationship between the inhibitorsand protein as seen in this experiment. This technique uses the advantage of theability of the protons of the inhibitors which are in close contact to thetarget protein so receive high value of Rf saturation hence promotingdifferential signal in STN-NMR spectroscopy hence displaying this signalreceived from the environment with great intensity between receptor protein andligand molecule.

This is an edge that the ligand resonance spectroscopictechnique have over the target based NMR technique as this method explores theproximity of the inhibitors to the protein and the intense signals generated tomake deduction we were able to established from this experiment that the wholemolecules were interacting with the enzymes (Jalaluddin A. et al 2017:). ligandNMR as seen in this study, tend toobserve signals from ligands, no isotopic labeling is required for targetprotein, thus experimental method takes less time than target based NMR methodand can be used to determine dissociation constant either by the use oftitration experiment or be observation of changes in the width of a ligandinduced by protein binding (Yan Li et al.

2017;). The Docking studies was able to affirm enzyme inhibitoryactivities F-NMRexperimental on the other hand is a target based method employed for theinvestigating of protein-ligand binding interactions in drug delivery mainlyuse in fragment screening, as the 19F nucleus has a natural abundance of100%(83% of the sensitivity of 1H) and a massive chemical shift of dispersion(Didenko, J t et al, 2013;). Since " F- atom is not naturally present in biologicalsystems, which means there will not be any background signal observed ordetected (Horst .

R. et al, 2013:) (Kitevski – LeBlanc J. L et al, 2012:) (Liu J. Jet al, 2012:). So atarget protein was first labeled in the bacteria system by adding 19F–labeledamino acid in the culture medium, then purified after which it is modified byusing 2-bromo-N-(-4-(trifluoromethyl) phenyl) acetamide (BTFMA) resulting in avery rapid 19F spin and because it is ligand resonance spectroscopy a 19f atomwas introduced in the ligand to enable its observation through chemical analysis due to the 19F atom's chemical shift being very sensitive to itsenvironment and the changes that occurs in it as a result of the weak Dan Der Waals bond and the presence of electrostatic field (Didenko T. et al, 2013:) Hit- identificationsteps is then adopted to identify, screen and validate the inhibitor as it is avery sensitive technique that is able to break down compounds with similarstructures to aid their detection by comparing the chemical shift change. Thehit identification step was carried out using F-NMR method as this technique isalso use for this purpose in fragment base drug delivery in three differentways, which are; the comparison of the 19f-labelled compound with libraries ofavailable ligand –observed experiment with the aim of screening the19F- labeledcompound

against libraries of available screened compounds to establish theligand size and chemical diversity with the view of using it for furtherdevelopment. More so, as biomedical assays are mainly use for primary screen inprotein NMR active site mapping, this method is then employed to confirm hitsscreens from HTS campaigns (Gee C.

T, et l 2016:) as the 19f-labeled target isdistinguish from every other compound present in the normal HTS library as theyall do not possess 19F-labelling and in this system of identification theresidue from the labeled atom is usually close to the active site to enable structuraland biochemical characteristic to be studied, the presence of a fluorinated compound makes ease of studyof substrate by the use of F-NMR methodThisassay is design in such a way that the changes of the substrate on breakingdown must be carefully observed to monitor the disintegration of the targetprotein, so as to be able to record and determine its ability to test ascreened compound accurately as this is used for the hit identification andconfirmation of the fluorinated substrate. Theadvantage of this method over the other is that even though ligand-observed experimentscannot be use for the identification of binding site this method can be used attimes due to the presence of the 19F labeled atom that aids in identificationof residue that are vital for binding in the presence of 19f assigned atom. This methods of identification and confirmation also tends to produce positivefalse results in ligand – observed experiments due to the problem ofnon-specific interaction and aggregate effects (Zega . A, 2017;)Conclusion: Conclusively, protein NMR spectroscopy in active site mapping is an indispensable tool withwide range of application in early stage of drug discovery, through all thephases of

manufacturing till it is displayed on shelf owing to its methods, suchas STD-NMR spectroscopy, and its ability to adapt molecular docking techniques toits advantage. This characteristics of this technique aids its precision in drugscreening and the ease of its application as well as the fact that it does notrequire a lot of data and its less time consuming when compared to other NMRmethods employed in this field. Furthermore, the knowledge that this methodprovides about the presence and the kind of enzyme present in a target site asseen in the study of the new urease inhibitor, the intensity of the bondbetween the active site and the inhibitor is very important for the formationand design of new drug, hence aids in producing drugs that binds to its receptor and exert a physiological effect as well ashighlighting Professionals on pathological issues.