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Editor Invitation

NMR methods in fragment based drug discovery

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Abstract Nuclear magnetic resonance (NMR) spectroscopy, owing to its ability to provide atomic level information on molecular structure, dynamics and interaction, has become one of the most powerful methods in early drug discovery where hit finding and hit-to-lead generation are mainly pursued. In recent years, drug discovery programs originating from the fragment-based drug discovery (FBDD) strategies have been widely incorporated into academia and industry in which a wide variety of NMR methods become an indispensable arsenal to elucidate the binding of small molecules onto bimolecular targets. In this review, I briefly describe FBDD and introduce NMR methods mainly used in FBDD campaigns of my company. In addition, quality control of fragment library and practical NMR methods in industrial aspect are discussed shortly.

Keywords FBDD, HTS, NMR, Fragment library quality control

Introduction

The first goal of all drug discovery organizations including pharmaceutical companies and academic institutions is to find novel drug-like molecules. For the past few decades, high-throughput screening (HTS) campaign has been a prevalent mainstream to achieve this goal and yielded successful results.[1] However, to pursue the HTS programs, in which tens of thousands to millions of lead-like molecules are typically screened, tremendous amount of investment is needed. This high cost, relatively low hit-finding rate and difficulties in targeting more challenging biomolecules, such as those involved in protein-protein interactions (PPIs), have driven researchers to find and develop alternative ways. Since Fesik and his co-worker's pioneering work was published in 1996[2], the principle of FBDD[3], which suggests the binding affinity can be enhanced by tethering together very small chemical compounds dubbed 'fragments', has made a marked impact on the way of drug discovery. Currently, although it may still be regarded as a novel approach in Korean pharmaceutical industry, FBDD seems to gain its position as a primary alternative to or a complement for the traditional HTS.

Fragment-based Drug Discovery

Fragment (by general acceptance, molecular weight less than 300 Da)-based approaches offer the following advantages compared with traditional HTS in which lead-like compounds of ~500 Da[4] are screened. First, with the far fewer number of compounds (1,000

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~ 2,000) than that (100,000 ~ 1,000,000) used in HTS, a fragment library can cover broader range of chemical space, because as molecular size decreases the number of molecules required to represent chemical diversity decreases exponentially. As a result, cost and time for hit screening can be greatly reduced, hence even academic laboratories or small companies like pharmaceutical companies in Korea can set up the FBDD programs easily. Second, thanks to its smaller molecular size, more atoms on fragment can be engaged in direct contacts with target molecules, resulting in a more efficient binding with a lower binding energy. Third, hit-finding rate in FBDD is generally higher than in HTS because fragments relatively smaller in size have better chances to interact with biomolecular targets. This is why FBDD is patronized to find starting binders to tough targets that have been presumed undruggable.[5, 6] Lastly, if the binding mode of hits is structurally elucidated, a subsequent hit-to-lead optimization is more easily accomplished by expanding from the direct binding moieties with only a few compounds. Generally, hits from FBDD have low functionality and correspondingly weaker binding affinity (mM ~ 10 uM) than that of most hits from HTS (10 uM ~ nM). The screening methods employed in FBDD, therefore, should be so sensitive as to detect the interaction in mM range. Whereas less stringent bioassays can be applied to HTS, several highly sensitive biophysical techniques[7] need to be implemented in FBDD. Among these, NMR has been extensively incorporated into FBDD campaigns from a front line of hit finding to hit-to-lead generation. According to recent survey at FBDD blog (http://practicalfragments.blo
-gspot.kr//2015/11/nmr-poll-results.html) hosted by Dan Erlanson, the president of Carmot Therapeutics, and Edward R. Zartler, the president of Quantum Tessera Consulting, 87 % of the respondents said that they use NMR for finding and validating fragments. What makes NMR as a powerful tool in FBDD comes not only from its robustness of identifying binding hits but also its ability to provide structural information on both ligands and targets.[2, 8]

NMR in FBDD

In NMR, the only two directly observable parameters that one can obtain from molecules of interest are chemical shift and peak intensity, regardless of the origins of NMR phenomena used in various NMR methods; therefore, all binding events can be determined qualitatively or quantitatively by measuring changes in these two parameters. NMR methods used in FBDD can be categorized into two groups, ligand-based and receptor-based, depending on which molecules are detected. In ligand-based NMR, as indicated by its name, detected molecules are fragments and hence, binders are distinguished by the differences of fragments in resonance patterns observed in the absence and presence of target molecules. Advantages of ligand-based assays for a screening are attributed mainly to their relatively high throughput, easiness of sample preparation and no limitation on maximal size of target molecules. In ligand-based NMR screening, 6 to 10 fragments are simultaneously screened in a form of cocktails, which can reduce the experimental time 6- to 10-fold compared to repeating an analysis of individual fragments. Once the resonance of each fragment is recorded beforehand as a part of fragment library quality control program, hit fragments can be easily assigned. In ^{1}H signal observing experiments such as saturation transfer difference (STD)[9] and Water-LOGSY[10], the two most widely adopted techniques using transfer-NOE, combination of fragments in mixture must be designed carefully to prevent resonance of each fragment from overlapping, and this maneuver as well as fragment solubility issue set the limit to the number of fragments that can be integrated into one cocktail. In a recent example of NMR screening against fluorinated fragment library, 12 ~ 13 fragments were combined together into each cocktail[11] and more than 1,000 fragments can be screened within 24 hours, demonstrating fluorine NMR can be another attractive tool in FBDD. In the light of my experience, if well designed, 40 ~ 100 ^{19}F-fragments mixture can be seemingly screened at a time without solubility and DMSO tolerance issues because the ^{19}F-fragments have very simple resonances and their dynamic range of resonances is wider than that of proton by more than 20-fold. Generally, medicinal

chemistry for lead generation starts after hit finding. One of the most important roles of potency evaluation for synthesized compounds at a drug discovery company is to impart guidance to medicinal chemists for an accurate analysis of structure activity relationship (SAR). For this purpose biochemical assays are preferred in a hit-to-lead process to biophysical assays as it can be performed in a relatively high throughput manner. However, as a lot of synthesized compounds in initial stage of lead generation still have low to medium binding affinities, biochemical assays do not often work as intended. In our K-Ras project, we had a trouble with a biochemical guanine nucleotide exchange assay due to a narrow dynamic range of read-out signals and to a compound precipitation issue. Fluorine-NMR competition binding experiments[12], exploiting fluorine's ultrahigh sensitive line-broadening feature upon target biding events, could be an effective solution to overcome the aforementioned problems. In our fluorine-NMR competition binding experiments, we were able to successfully measure the dissociation binding constants of tested compounds, minimizing the precipitation issue by lowering the concentrations of K-Ras binding 'spy' molecule and synthesized compounds to 20 μM and 200 μM, respectively, which are one-fifth of 1 mM used in our biochemical assay.

Receptor-based NMR methods detect ligand binding by monitoring the changes in resonance frequencies or peak intensities of the corresponding protein residues. As a ligand-protein binding almost always leads to changes in chemical environments surrounding the binding residues, one can easily detect binding events by measuring alterations in chemical shifts and peak intensities. Due to its ability to explicitly detect a ligand binding and to concomitantly provide structural information on the binding site, 2D heteronuclear correlation experiments, such as heteronuclear single quantum coherence (HSQC) and heteronuclear multiple quantum coherence (HMQC) are preferentially used rather than 1D proton NMR in receptor-based NMR. Despite the drawbacks that 2D heteronuclear NMR needs ^{15}N- or ^{13}C- labeled proteins in ~mg quantity and that it is difficult to assign the resonances of relatively large proteins (MW 〉 35 kDa) by routine NMR methods, receptor-based NMR has advantages that cannot be easily obtained by any other techniques. Once the resonances in 2D spectra are correctly assigned, ligand binding sites on protein surface can be quickly determined by interrogating the chemical shift perturbation (CSP) caused by ligand binding. Moreover, if the ligand of various concentrations is titrated with protein, its binding affinity is directly measured by fitting the shifted peaks in non-linear manner. Receptor-based NMR is currently the sole method that can simultaneously provide structural information and binding affinities. To the present, the most reliable technique to define a binding mode of ligand onto protein is arguably X-ray crystallography. Nevertheless, solving a protein-ligand complex structure by X-ray crystallography is quite often unsuccessful, particularly for weak binders such as fragments.[13] Understanding binding modes between ligands and their targets to the atomic level are crucial to medicinal chemists in trying to make more potent compounds with the help of SAR in FBDD. Furthermore, whether the high-resolution structure information on protein-ligand complex could be obtained sometimes determines a success or a failure of the whole project. Krimm and coworkers showed that when X-ray crystallography trials to get complex structure are not productive, NMR methods combining 1D STD and 2D HSQC can back up an FBDD campaign.[14] In this very recent research, binding modes of catechols onto the target protein PRDX5 were identified from the calculation of 'epitope mapping'[15] based on STD results and of CSPs in ^{15}N-HSQC, exhibiting a good accordance with the corresponding crystal structures.

'Good' Fragment Libraries ensure successful finding.

One of the most important things that are sometimes overlooked in FBDD is executing a definitive quality control of fragment library. Fragment libraries, whichever they are purchased or made in-house, contain a few 'bad actors' such as pan-assay interference compounds (PAINS)[16] and aggregators which give rise to false positives. These wrong hits impose

futile tolls on research groups, wasting considerable amount of their resources and time. Information about PAINS is relatively well established[16] and researchers can examine their compounds preliminarily with PAINS server (http://cbligand.org/PAINS/). As almost all fragment screenings are conducted in aqueous buffer, fragment solubility in the buffer has to be tested to meet the criteria of internal cut-offs. NMR itself is an adequate method to assess not only the solubility but also purity, stability and identity. By examining the concentration-dependent peak intensity and chemical shift, fragment aggregation is readily determined.[17] One of more practical strategies to conduct fragment quality control is measuring simple 1D proton spectra and comparing peak intensities of fragments with an internal standard. In our laboratory, we measure 1D proton spectra of 1 mM fragments mixed with 0.1 mM internal standard 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in phosphate buffer. If chemical shifts of the fragments are not matched to their own predicted structures or peak integration values are less than half of DSS, the corresponding fragments are excluded from library as failures of the quality control. Through careful visual inspection and computer-based analysis of recorded spectra, mixtures of 8 to 10 fragments can be constructed without peak overlaps. Afterwards, NMR spectra of fragment mixtures are recorded and compared with those of each component to investigate any fragments exhibiting unexpected profiles in the mixture. Once the sets of fragment mixtures are firmly constructed with chemical shifts of individual fragments pre-assigned, they are recurrently applied to various hit-finding projects.

Concluding Remarks

Taken together, I briefly described FBDD and NMR methods mainly used in my company. Certainly, there are many other useful NMR techniques for drug discovery not mentioned here[18] including TINS, SLAPSTIC, INPHARMA, and SAR by ILOEs. Some NMR methods that take advantages of paramagnetic relaxation enhancement (PRE) or residual dipolar coupling (RDC) provide undoubtedly valuable structural information in FBDD.[19, 20] However, NMR sample preparation for these techniques are quite often labor-intensive and time-consuming, limiting their usage in practical FBDD. As I mentioned previously, assays for hit-growing have to be fast enough to help medicinal chemists to decide their synthesis directions in time. In this regard, aforementioned ^{19}F-NMR competition assay is very attractive. Nonetheless, ^{19}F-NMR does not appear to be deployed as widely as expected, which may attribute to instrumental limitation rather than to limitation of ^{19}F-NMR assay itself. ^{19}F-NMR needs fluorine tunable probes and an additional high frequency channel for proton decoupling, meaning that one has to set up a 4-channel system (2 high channels plus 2 low channels) instead of a conventional 3-channel system. In addition, regarding a lot of research groups have installed cryogenic probes for biomolecule studies, it is likely that people don't want to use probes in a probe-exchanging manner. It is worthwhile to emphasize that a pivotal prerequisite for successful fragment screening is a complete quality control of fragment library. Although, it is a very laborious and time-consuming work, the quality control must be done for efficient FBDD and minimization of resource waste. Up to now, pharmaceutical companies in Korea have seemed to concentrate their resources on low risk researches such as incrementally modified drugs or 'best in class' targets rather than 'first in class' targets. However, in these difficult times when revolutionary drugs are required for the survival of companies, discovering novel chemical entities for challenging targets is critical. In this sense, FBDD may be one of the most efficient approaches to adopt for small companies like Korean pharmaceutical ones, and NMR can be utilized as a very powerful tool in FBDD.

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