

Perspectives of NMR in Drug Discovery: A Technique Comes of Age

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Abstract

When it comes to drug discovery, there is currently little consensus on a single technique that alone can tackle all possible drug targets. In the past decade, scientists from both industry and academia realized that Nuclear Magnetic Resonance (NMR) spectroscopy could be very useful and versatile in monitoring intermolecular interactions, making it a potentially powerful and general tool for drug discovery. This essay represents a brief review of our recent discussions and reports a consensus of the current and potential utility of NMR in the drug discovery process.

Introduction

While NMR is often valued for its ability to shed light on structure, its real value in drug discovery probably lies in evaluating molecular interactions at the atomic level. NMR is a multifaceted phenomenon that enables an impact in various aspects of the small molecule drug discovery process¹⁻¹⁸. Every organic chemist is familiar with the chemical shift. This simple parameter is highly sensitive to the exact environment of the atom, and therefore yields information about whether a small molecule binds to a target protein or nucleic acid, what parts of the small molecule are interacting, and to which part of the macromolecular target the ligand is bound. Yet other NMR experiments are sensitive to molecular motions. The variety of readily measurable parameters permits NMR to contribute efficiently to the ligand discovery process by assessing target *drugability*, initial hit identification via fragment-based drug design (FBDD)¹⁹⁻³⁴ with screening of very small molecule (<300 dalton) libraries, pharmacophore identification, hit validation, hit optimization and potentially structure-based drug design. NMR can also be used to determine low resolution structures of target-ligand complexes for natively unstructured proteins or membrane proteins that are not amenable to crystallographic approaches. The combination of advances in instrumentation, the use of orientational restraints³⁵⁻³⁹ in partially oriented media, the use of selective ¹³C,¹H-methyl labeling in otherwise deuterated samples⁴⁰⁻⁴², the simultaneous acquisition of multi-dimensional experiments,⁴³ the use of segmental labeling techniques⁴⁴⁻⁴⁷ and the use of TROSY-type experiments^{48, 49} have made NMR spectroscopy a very powerful and efficient tool for structural biology initiatives⁵⁰. Combined with automated assignment strategies and projection spectroscopy, these approaches promise to significantly reduce the time needed for data collection and analysis⁵¹⁻⁵⁷. Finally, solid-state NMR spectroscopy has also advanced significantly in recent years, making it possible to study proteins such as neurologically relevant GPCRs, transporters or ion pumps⁵⁸⁻⁶⁶. Taking into account such developments, it may be envisaged that new areas of biology may be made accessible for structural research, focusing more on 'systems' rather than on purified material via solid-state NMR.

As mentioned, NMR could also be very useful in monitoring inter-molecular interactions involving macromolecules (proteins or nucleic acids) or a small molecule ligand, making NMR spectroscopy a potentially powerful and general tool for drug discovery.

This essay represents our consensus on the strengths and pitfalls of NMR spectroscopy techniques in the drug discovery and development processes. It is based on a document drafted during the workshop *Perspectives of NMR in Drug Discovery* organized in Florence by Drs. Ivano Bertini and Claudio Luchinat (University of Florence) and Maurizio Pellecchia (Burnham Institute for Medical Research), and financed by the EC-funded Coordination Action *Focusing NMR on the Machinery of Life* (NMR-Life). Our discussion revealed several common views on the usefulness of NMR spectroscopy and its possible future in the drug discovery process, both in industry and in academia. With this perspective article we wish to briefly summarize the general outcome of these discussions.

Drug Discovery and Emerging Technologies

When it comes to developing and introducing novel drug discovery technologies, the response of the scientific and business development communities is often similar (from a communication of Dr. Chris Lipinski at a recent symposium at the Burnham Institute for Medical Research): after a first phase of skeptical resistance whose duration is somehow proportional to the current development stage of other competing techniques, an overwhelming and often exaggerated enthusiasm (hype-phase) is generated around the approach; as time goes by, the overly optimistic predictions turn into distrustful set backs (distrust-phase); eventually, the real value of the new approach emerges and its realistic impact to the drug discovery process is reappraised. This process takes on average about five to ten years, or more. How does NMR fit into this general trend? Even taking into account that NMR spectroscopy is a multifaceted technique that can be used at many different stages and levels of the drug discovery process, one can notice that each and every general NMR approach has followed, to a

certain extent, a similar trend. Two general considerations can be further made: first, only valuable techniques usually survive the hype phase, and, second, when forthright and open discussions can take place evaluating the merits and pitfalls of a given approach, the technique can be appropriately and effectively justified. We believe that many aspects of NMR spectroscopy as applied to the drug discovery and development processes are now mature enough for such critical assessments. Our collective evaluation on past and future of NMR spectroscopy in the hit identification, validation, characterization and optimization processes are summarized in the next paragraphs, hopefully unbiased by innate skepticisms, overrated enthusiasms or over-trusting disappointments.

NMR in Fragment-Based Drug Design: Puzzling Approaches to Drug Discovery

It has been estimated that the number of potential drug molecules is of the order of 10^{10} - 10^{50} ⁶⁷. However, for a given target system it is difficult to imagine high-throughput screening (HTS) performed with much more than 10^6 compounds, especially considering that such endeavors would be very expensive and subject to a sizeable number of false positives and false negatives. The traditional approach of testing variations of known drugs is certainly not going to dive very deeply into this potential pool either, but at least it has the advantage of exploring compound space based on knowledge, so the search will be made more effectively. Of course, our chances of encountering cross-resistance are enhanced if we limit ourselves to compounds similar to those currently in clinical use. Consequently, it would be most useful to find molecules that might lead to development of drugs with novel chemical scaffolds. These statements represent the basic premise of the so-called fragment-based drug discovery approaches (FBDD)¹⁹⁻³³.

In principle, there are in principle several ways to *construct* novel ligands designed for a particular target that could subsequently become lead candidates. These entail a modest exploration of “*drugable*” molecular space, but the efficacy of this approach could be largely enhanced by using knowledge of the target. Sometimes that knowledge may be functional, but more often it is structural. That is, we presume the target protein (or

nucleic acid) assumes a structure, and some aspect of that structure is used to search for small molecule ligands that might bind and be used to develop a drug candidate.

One approach is to build up a drug candidate (with typical molecular weight of 500 daltons) from screening a database of typically 1,000-15,000 compounds composed of smaller molecules (fragments) with molecular weight < 300 daltons and good aqueous solubility, using tethering¹⁹⁻³⁴, X-ray diffraction^{27, 68-72} or NMR approaches³¹⁻³³. These techniques enable one to identify the location of binding of any fragment. Often, a second screen is carried out to find a second fragment that will bind in close proximity to the first fragment. Use of a second screen has an advantage in that binding of the first fragment may select for a particular conformation of the target that can enable binding of a particular second fragment that would not have been found in the absence of the first. Individually, the fragments may bind to the target with K_D values of only 10^{-4} to 10^{-3} M. However, by covalently linking the fragments, the additivity of the binding enthalpy and the favorable entropic contribution may pay off in a big way. In fact, sub-micromolar bi-dentate compounds can be found that are generally novel in structure. These can serve as starting points for further structure-activity studies by synthesizing focused libraries of related compounds or by determining the structure of the linked compound bound to its target and using that knowledge to suggest structural modifications. Several recent manuscripts and review articles report on critical technical aspects of the use of NMR spectroscopy in FBDD¹⁻¹⁸. The fragment-based approach for primary screening has proved to be viable for the identification of lead molecules. The probability of detecting the binding of a low complexity fragment with high sensitivity exceeds that of full-sized ligands with lower screening sensitivity. The functional groups of fragment-based libraries should already include synthetically accessible starting points for chemical linkage. In a follow-up screen, chemical building block fragments with masked linker groups can be utilized, an optimization step in library design called the '*fragment pair concept*'. Key to the success of such a strategy is the quality of the fragment database. Quality, of course, refers to the purity of the compounds, but also to the diversity and chemical nature

of the fragments chosen. Several different laboratories have developed their own fragment databases, some of which are emerging as commercially available libraries (Maybridge Corp., Chembridge Corp., Asinex Corp., Life Chemicals, ActiveSight Corp., Pyxis Discovery, to mention a few).

Several NMR strategies, which follow the initial screening trials, have been proposed (**Tables 1 and 2**), ranging from the more traditional chemical shift mapping to ligand-based techniques monitoring changes in ligand nuclear spin-relaxation properties upon binding, to measurements of diffusion, etc. Some of these approaches are better suited as screening methods and/or to validate hits coming from HTS campaigns (**Table 1**), whereas others are better suited to guide their optimization into more potent, selective and *drug-like* compounds (**Table 2**). It should be also possible to extend some of these approaches to *in-cell* NMR experiments to provide, for example, mapping information from chemical shift perturbations for serially expressed protein systems⁷³⁻⁷⁵. Other applications could include possibly deriving novel compounds with reduced serum albumin binding and/or cytochrome P450 enzymes interactions by designing out unacceptable properties during the iterative optimization process⁷⁶. The detailed description of these methods can be found in the reported citations within Tables 1 and 2 as well as in recent review articles cited throughout this manuscript.

NMR in structure-based drug design

The identification of new possible targets or of possible “*druggable*” sites on known targets can also begin with structural studies. However, many multi-domain proteins show considerable flexibility in the organization of their components during interactions with multiple ligands, and allosteric modulation of activities is of considerable significance in their activity. In contrast to a structure determined in a crystal, where the inter-domain interactions accommodate the need for the lowest crystal packing energy, structures in solution reflect a more physiological milieu and can characterize the dynamic inter-conversions available⁷⁷. NMR methods to characterize these interactions, using relaxation properties and special isotopic labeling can be

applied to complex systems like protein tyrosine kinases, widely identified as significant targets, but where plasticity of interaction with ligands (or known drugs) is evident⁷⁸. Magic-angle-spinning solid-state NMR entered the scene in the past five years as an additional alternative method for protein structure determination, and offers new perspectives for structural investigations on samples that could not easily be analyzed before, such as native membranes, fibrils and cytoskeletal complexes. Recently, models of a potassium channel-toxin complex, of various fibrils, and of receptor-agonist complexes were published as a result of the constant advance of the field⁵⁸⁻⁶⁶. Projects aiming at well-determined structures of membrane proteins are underway in several laboratories.

However, how does this structural information materialize into new potential drug candidates? An obvious strategy is to employ *in silico* approaches for the initial screening of a compound database to predict those that should bind to a (usually static) target protein structure. This has the apparent advantages that the search should be less expensive and faster (if time to develop an experimental assay is considered) than HTS. Computational “hits” from this virtual screening will need to be tested experimentally, but a relatively small number of top-ranking binders can be selected, e.g., 1%, for NMR screening; while certainly not high-throughput, a pleasant feature of NMR is that a system-specific assay needs not be developed. Currently, it is feasible to screen $>10^6$ compounds computationally; for the money spent on a single HTS screen of a pharmaceutical company’s in-house database of compounds, a computer cluster could be constructed to screen all of the pure compounds extant in the world (assuming this number is *ca.* 10^7).

Numerous programs have followed the initial DOCK⁷⁹ algorithm and have been used on many systems⁸⁰. For successful prediction of hits that are subsequently verified experimentally, the actual search algorithm used at this point in history seems to matter little. However, challenges for the field largely center around two general aspects: (a) most molecules adapt their conformation upon binding to another molecule, i.e., both the ligand and the target are malleable such that an induced fit occurs; and (b) the scoring functions used to rank the ligands

for binding affinity are highly imperfect. There is work in the field on both points, promising an improved future, but computational predictions of molecular recognition are today at a state where predictions of protein folding were about fifteen years ago.

With either of the two approaches mentioned above, one must be cognizant that any promising binders are only that: promising. The vast majority of compounds that look promising at this stage will fail in further development, chiefly from lack of bioavailability, i.e., not getting to the site of action, and from toxic side effects from insufficient selectivity. It is a challenge to increase the probability of any promising preclinical compound making it through the drug development pipeline to become a clinically viable drug. With the realization that ADME-Tox problems are an expensive limitation in drug development, scientists are working to develop tools to identify compounds or classes of compounds early that may engender these problems. One school of thought in recent years has been to filter the databases to be screened such that criteria largely comply with Chris Lipinski's "Rule of Five", an experiential list based on clinically successful drugs with good bioavailability^{81, 82}. However, as Lipinski reports, there are numerous examples of drugs that do not abide by these rules, so it is not really a rule to be followed blindly, e.g., the rules of Veber et al. are suited as well and provide an alternative way to look at potential drugs.⁸³ Likewise, methods to predict toxicity are now being developed. While computational toxicology is still in its infancy, it will undoubtedly improve with time. There is a fairly new initiative (DSSTox) aiming at creating a common format for chemical structures and searchable data files for toxicity databases that will be available to the public. This worthwhile venture is described at <http://www.epa.gov/nheerl/dsstox/>.

NMR spectroscopy in the drug discovery process: a critical assessment

Penetration of NMR into drug discovery remains rather limited when compared to HTS and X-ray crystallography, despite repeated large scale investments, and, as reported above, the fact that various NMR

techniques can be considered essential tools in a vast array of academic and industrial research. One problem is that the utility of NMR as a structural biology tool in the hit to lead stage has fallen far short of original expectations. Clearly X-ray crystallography can provide higher resolution structures much faster and, as a result, it is far more widely used. The only exceptions are companies that have been setup based on NMR expertise, certain large pharmaceutical companies where NMR has proven itself over the long term, or in academia where individual research groups may focus on NMR. The question becomes: are there other areas of drug discovery where NMR information is clearly superior or for which there are no alternatives? In our admittedly biased opinion, the answer to the question posed above is a resounding yes! One very important application that we foresee is extending the current principles of fragment based drug discovery (FBDD) to membrane proteins. Although membrane proteins represent something like a third of our genome, more than 2/3 of all marketed drugs target them. Further, there is great underutilized potential in membrane proteins as pharmaceutical targets. Advantages of targeting small molecules to cellular membrane proteins include the fact that the compound needs not traverse the outer cell membrane to reach its target. However, a major hurdle to overcome is that membrane proteins are, by and large, very challenging in terms of biochemical manipulation. At present, most drug discovery efforts targeting membrane proteins (mostly GPCRs and ion channels) utilize cell-based assays and high throughput screening of large corporate compound collections. FBDD is having tremendous success in developing orally bio-available drugs to soluble targets via NMR³³ and we anticipate making a similar impact in the future in targeting membrane proteins.

However, FBDD has had as much success also by using other techniques. Therefore, one has to ask the question: what unique insights does NMR actually bring to drug discovery? Currently, NMR techniques provide some information about the binding epitope on the ligand and map the binding site on the target quickly. For the future, increasing numbers of membrane proteins can be recombinantly expressed and solubilised. These protein samples are often not suitable for crystallography yet can be used to perform NMR-based ligand

screening. Based on the SAR of hits, follow-up focussed libraries could be synthesized to jump start the drug discovery process. NMR can also be used to determine structures of the ligands in the bound state and, using data from paramagnetic labels or traditional nuclear Overhauser effects (NOE), low-resolution structures of target-ligand complexes can be determined. No other biophysical technique can provide this sort of information for membrane proteins. On the strengths side, NMR can very quickly deliver information about ligand binding properties even if the receptor cannot be characterized at high resolution: it can provide a detail picture of the bound ligand, even if the receptor cannot be characterized; as mentioned, ligand binding to membrane-integrated proteins can be analyzed; if the receptor can be characterized, a limited set of NMR data can provide information on the location and orientation of ligands^{84, 85}; and last but not least, as mentioned in this article, NMR is well suited for fragment-based screening because it allows characterization of weak binding (see another recent example in reference⁸⁶).

Clearly, however, drug discovery is an immensely complex venture, requiring a multi-disciplinary effort. One issue that we recognize is that each of the intervening disciplines (e.g., HTS, crystallography, cell biology, protein NMR, medicinal chemistry) is usually poorly integrated. Fragment-based approaches such as the SAR by NMR strategy require excellent integration with medicinal chemistry and possibly biology. Effective use then implies some degree of centralized organization, and specialization of labor. In an academic setting, this must come from collaboration. We envision that a possible solution would be to engage in collaborative programs that would pull together the state-of-the-art design of new drugs using NMR and other technologies to optimize the speed and quality of lead optimization. Large NMR-based infrastructures worldwide could play a role in these programs. Another major need is for laboratories of mainly synthetic chemistry groups that would be willing and able to collaborate in such an effort. Research groups are also needed that would be willing to perform biological and functional testing of intermediately generated new compounds in order to combine more efficiently and rapidly binding studies with functional assays. In the

United States, there are several screening centers that may provide such support (Examples are the Molecular Libraries Screening Centers Network initiative, <http://nihroadmap.nih.gov/molecularlibraries>; the NIAID's Antimicrobial Acquisition and Coordinating Facility, <http://niaid-aacf.org>; the NCI's Developmental Therapeutics Program, <http://dtp.nci.nih.gov>).

Another issue that we discussed at length is the use of macromolecular NMR as a structural tool to complement X-ray crystallography. That paradigm is not fully deployed in industry for a variety of reasons, but especially because it is not rapid. It may still be viable in academic research, where the choice of protein targets is less constrained by immediate therapeutic relevance. It is possible that a structural focus can re-emerge with the growing interest in integral membrane proteins, particularly with emerging solid-state NMR techniques, but this is not yet certain. NMR-based structure determination of protein-ligand complexes should also gain more weight in the future. But it must become fast and should take advantage of synergies with X-ray crystallography and computational tools⁸⁵. To be viable in a fast-paced industrial setting, structure refinement via NMR must be stream-lined. Another option that may pay off with more research is computational modeling using a limited amount of NMR structural data. One issue we recognize is that there is a lack of suitable NMR structure determination software for such industrial purposes; a suggestion is to establish a consortium to develop new compatible, easy-to-use software, much as it was done by and for the X-ray community in the past. NMR screening, on the other hand, *via* several of the briefly mentioned methods for lead generation, optimization, and 'rescue' is a newer paradigm. It provides quicker turn-around for information of more immediate impact on medicinal chemistry. This application seems to have reached a plateau now, and it is unclear as to what further room for expansion remains. Again, possibly applications to 'non-traditional' targets is the best venue, e.g., RNA¹⁰⁰⁻¹⁰² or membrane proteins. A renewed interest in NMR as a metabolomic tool for predictive toxicology⁸⁷ seems to be a powerful contributor to drug development, permitting forays into systems biology⁷⁶. The general impression is that this field is not yet saturated.

In addition to monitoring ligand binding and to determining macromolecular structures, NMR is among the most powerful methods for profiling biomolecular motion⁸⁸⁻⁹³. There is potentially great value in learning how to ascertain and ultimately exploit intrinsic motion to guide drug discovery and delivery. A huge challenge, however, is that dynamics studies are of similar or slower pace than structural studies. Moreover, there is not yet a consensus on how dynamics information can be best used to advance ligand design. A promising area is in estimating configurational entropy changes associated with binding, and coupling to calorimetry⁹⁴.

Therefore, at least in theory, NMR spectroscopy should find a significant role in each step in the drug discovery process (**Figure 1**). However, it seems that NMR suffers from a sort of Leibniz syndrome, in that it is often the “second best” at so many things. The diversity of problems it can attack is extremely alluring to its practitioners, leading to a state of mind: “All I have is a hammer, so everything is a nail”. This attitude is, of course, unhealthy for NMR in drug discovery. Macromolecular NMR works best in drug discovery when its data can be quickly integrated with those from other analytical techniques. It has to be comprehensible, portable, and available on a time scale compatible with medicinal chemistry. No matter how unique NMR information may be, it will not be used unless it meets these criteria. Some years ago, the “word around the campfire” was that researchers in modeling and bio-informatics eschewed solution NMR structures (as opposed to crystal structures) because one gets a series of structures, thus calling into question the accuracy and meaning of the data. This may be myth; nevertheless, if true, it underscores the importance of integrating NMR data with the world-views of other disciplines that are the stronger driving forces of drug discovery.

One important issue is that training researchers able to translate basic discoveries to new drugs is not at all established in academia. In the US, medicinal chemistry is predominantly taught at the employer, with no focus on professional degrees in the specific areas. Only recently, several Schools of Pharmacy have either instituted or increased their investment in educating research scientists relative to professional pharmacists. Within these educational programs, there is controversy attending the issue of how much spectroscopy is

required for its effective use, but with few exceptions, there is little educational effort in NMR relative to other means of lead generation/optimization. Defining the necessary steps for effective training is a valuable exercise. For example, for the use in screening and FBDD, medicinal chemists rather than physicists, biologists or even organic chemists would be preferred 'users' of the techniques. For NMR, the current educational focus still has a strong structure-determination bent, which appeals more to biologists or biophysicists than medicinal chemists. Yet, many of these students are interested in entering drug-discovery in industry. What do we tell them? What kinds of jobs await them? An increasing number of students want "turn-key" biophysical methods. There is less interest in mastering the underlying theory of a given technique, and much more on fast downloads to rapidly summarize the results of multiple techniques. This is natural, given our relentless emphasis on the urgency and competition in drug discovery. That is all fine, but what does this imply in terms of designing an appropriate curriculum for such students? Maybe a curriculum which includes more detailed studies whereby NMR is used to decompose the overall thermodynamics of binding for a given ligand-protein interaction into enthalpic and entropic contributions from the ligand, protein and solvent, could be a good compromise between basic and applied research in this area.

Concluding remarks

When all facts are considered, NMR remains a multifaceted and unique technique that is sensitive to both structure and dynamics and that can monitor the binding of low molecular weight ligands to biological macromolecules in the *early stages* of drug discovery due to its ability to detect even very weak binders. One common pitfall of the implementation of NMR in the industrial drug discovery pipe lines is that it is often brought in too late. On the other hand, while many examples do exist of successful drug discovery projects that are entirely jump-started by NMR-based approaches, it is clear that when applied in isolation, these methodologies, much like any other technique, cannot be fully effective. The successful implementation of NMR in the drug discovery process is often based on the early and effective integration of medicinal chemistry,

computational approaches and biology. Training the scientists of the future based on these observations may be the long-term solution of these problems; establishing large collaborative efforts in the academic setting or the coordination of technologies in the industrial setting may represent the short term solutions.

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Table 1: MOST USED NMR METHODS FOR COMPOUND SCREENING AND HIT VALIDATION

Approach (original references)	Observation	Used for	Description (references to recent applications)
Chemical Shift Perturbation (Reviewed for example in reference ¹)	Target (protein or nucleic acid) resonances	Primary screening/ Hit validation/ Site of binding	Identifies binders by means of chemical shift perturbation of resonances of target (⁹⁵⁻¹⁰⁰)
Saturation Transfer Difference (STD) NMR (¹⁰¹)	Ligand	Primary screening/ Hit validation	Identifies weak binders, build-up curve identifies interacting functional groups (^{100, 102-106})
WaterLOGSY (¹⁰⁷)	Ligand	Primary screening	Identifies binders by water mediated NOEs (^{108, 109})
SLAPSTICK Using Spin-labeled protein (¹¹⁰)	Ligand	Primary screening	Highly sensitive detection of fragments (^{32, 110})
TINS (¹¹¹)	Ligand	Primary Screening/Hit validation	Identifies small molecule compounds by screening libraries against immobilized protein targets (¹¹¹)
T _{1ρ} and T ₂ relaxation; Line broadening (¹¹²)	Ligand	Primary screening/ Hit validation	Binding enhances relaxation, affinity estimate, build-up curve identifies interacting functional groups (¹¹³)
<i>Transferred</i> NOEs (¹¹⁴)	Ligand	Hit validation/ Conformation of flexible ligands	Interaction of binders with the target (^{4, 115}) Also helpful to determine bioactive conformation of flexible ligands such as peptides (¹¹⁶)
FABS (¹¹⁷)	Substrate of cofactor	Primary screening/ Hit validation	Utilizes reference substrates or cofactors to monitor enzymatic reactions (^{14, 118-123})
FAXS (¹²⁴⁻¹²⁶)	Reference Ligand	Primary screening/ Hit validation	Utilizes reference substrates or cofactors to monitor enzymatic reactions (^{14, 118-123, 127, 128})
Diffusion measurements (^{129, 130})	Ligand	Primary screening/ Hit validation	Measures the difference in diffusion rates for ligands in the bound versus free state (¹³¹)

TABLE 2: REPRESENTATIVE NMR METHODS FOR HIT/LEAD OPTIMIZATION

Approach (original references)	Observation	Used for	Description (references to recent applications)
SAR by NMR (^{31, 132})	Ligand/Target	Structural information FBDD Screening/Compound optimization	Design bi-dentate compounds (^{133, 134}).
SLAPSTICK with first-site spin- labelled compound (¹³⁵)	Ligand	FBDD Screening/ Compound optimization	Highly sensitive detection of fragments and weakly interacting second site compounds (¹³⁶)
SAR by ILOEs (^{137, 138})	Ligand-to-Ligand	FBDD Screening/ Compound optimization	Detects protein mediated ligand-ligand interactions (compounds occupying adjacent sites) (¹³⁹)
Pharmacophore by ILOEs (¹⁴⁰)	Ligand-to-Ligand	FBDD Screening/ Compound optimization	Detects protein-mediated ligand-ligand interactions and uses information for pharmacophore-based search of bi-dentate compounds (¹⁴⁰)
H ₂ O/D ₂ O exchange rate measurements	Target	Compound characterisation	Identifies binding epitope (¹⁴¹)
INPHARMA (¹⁴²)	Ligand-to-Ligand	Compound characterization	Detects protein mediated ligand-ligand interactions (competition for the same binding site)

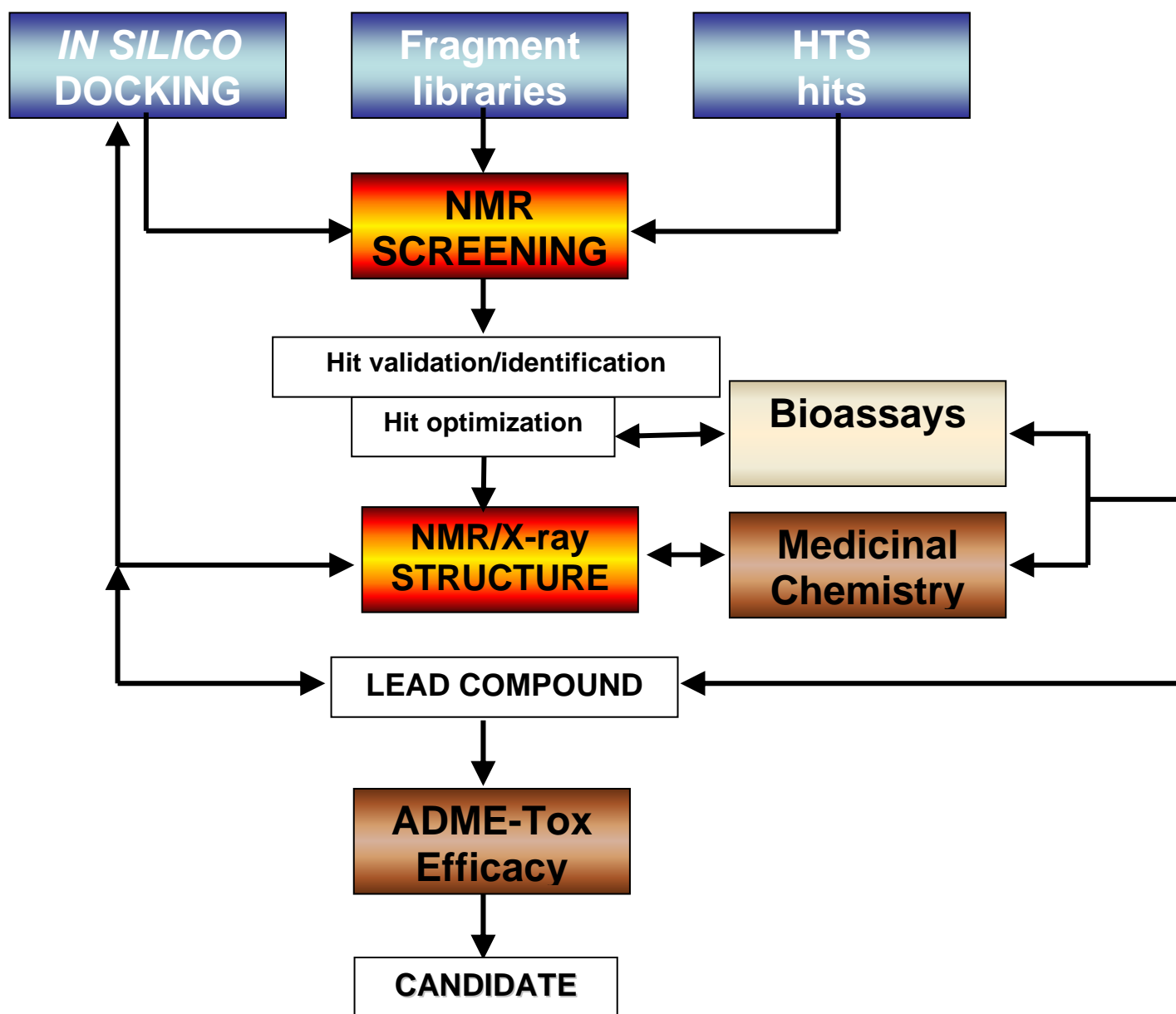


FIGURE 1

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