NMR-based screening technologies

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NMR-based ligand screening is now an established field in its own right. In recent years, advances in both methodology and hardware have broadened its range of applications and pushed back practical limitations, leading to the growing importance of NMR screening as a tool in industrial drug research. An overview of new screening methods and applications is presented here, and ways in which NMR-screening is being used in cooperation with other screening techniques are discussed.

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▼ Biomolecular NMR has become a powerful and versatile technique in industrial drug research. Technical and methodological advances have led to an increase in the use of many NMR methods, making true industrial applications possible for the first time (see reviews [1–3]). New examples of NMR in the discovery and design of potent lead compounds are appearing regularly [4–8] and the potential of this technique as a complement to other approaches, such as HTS and *in silico* screening, is also being exploited. The range of NMR applications in drug discovery is increasing and technical advances should see further expansion, as discussed here.

NMR-based screening

One technique that is exemplary of the growing role of NMR in drug research is NMR-based screening. As a screening technique, NMR offers several advantages; first, it is a universal technique for the detection of intermolecular interactions and no target-specific assay or knowledge of target function is required. Second, NMR screening systems directly test the interaction between ligand and target, minimizing the false positives that can plague functional assays. The two key advantages of NMR screening are, however, the superior sensitivity to weak interactions, even with affinities in the millimolar range, and the ability to provide detailed structural information on ligand-binding modes. Together, these advantages make NMR the method of choice in fragment-based screening approaches, where small, low- to medium-affinity binders that provide the seed for structure-based or combinatorial approaches to lead discovery are identified [9–15]. Several reviews have covered general aspects of NMR screening and described the basis of specific experiments [16–26]. Here, we put new techniques into perspective.

Ligand- or target-detected methods

NMR screening can be divided into ligand-detected (i.e. with detection of the small molecule) and target-detected techniques (with detection of the target molecule, usually a protein). Both have their intrinsic advantages and disadvantages (see Table 1). Ligand-detected experiments are well-suited to primary screening; the screening throughput is generally higher, due to the use of fast 1D spectra, and the ability to screen ligand mixtures without the need for deconvolution. Ligand-detected experiments have lower demands on the target molecule, which can be unlabelled, is not subject to upper size limitations and can be immobilized [27]. Because the free ligand is observed, only low protein concentrations are required and ligand-detected NMR experiments can also enable the characterization of the binding epitope of the ligand [28]. Disadvantages of ligand-detected screening include an often limited range of affinities that can be detected and the possibility of false positives due to non-specific binding.

To obtain detailed information on the binding site of the macromolecule, target-based experiments must be conducted. These techniques usually have broad affinity ranges with no upper affinity limit and can distinguish specific binding from non-specific binding and pH effects. With the additional information of the spectral assignment (usually including ^{15}N or ^{13}C), a range of structural information on the binding mode is accessible. General disadvantages of target-based screening are lower throughput, as a result of

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Table 1. Properties of NMR-screening methods (adapted from [16] and [23]).

Method	Limits and Requirements			Enables identification of:		
	Target MW limit	Affinity limits	Labelled target reqd.	Binding site on target	Binding epitope on ligand	Binding compounds in mixtures[a]
Diffusion filtering	Lower	U/L[b]	No	No	No	Yes
Relaxation filtering	Lower	U/L	No	No	No	Yes
trNOE	Lower	U/L	No	No	No	Yes
NOE pumping	Lower	U/L	No	No	No	Yes
Reverse NOE pumping	Lower	U/L	No	No	Yes[c]	Yes
WaterLOGSY	Lower	U/L	No	No	Yes	Yes
STD	Lower	U/L	No	No	Yes	Yes
^{19}F-screening[d]	None	None	^{19}F ligands	No	No	Yes
CSM	Upper	None	^{15}N or ^{13}C	Yes[c]	No	No
Competition screening[d]	None	None	No	Yes[e]	No	No
Competition-based ^{19}F-Screening[d]	None	None	^{19}F ligand[f]	Yes[e]	No	No

[a]without deconvolution of the mixture
[b]U/L: both upper and lower limits exist
[c]at least a partial spectral assignment is required
[d]described in more detail in the text
[e]a specifically binding reporter ligand is replaced
[f]labelling of reporter ligand required
Abbreviations: CSM, chemical shift mapping; NOE, nuclear Overhauser effect; STD, saturation transfer difference; WaterLOGSY, water –ligand observation by gradient spectroscopy.

the more time-consuming multi-dimensional spectra involved, and the need to deconvolute hits from compound mixtures [26], both of which might be offset partially by the lower rate of false positives. The most important disadvantage of target-based screening is, however, the restrictions it places on the target, which must be labelled and available in large amounts (discussed later). Despite this, recent technical advances – in particular, the introduction of cryogenic probes [29,30] and cost-effective ^{13}C labelling of methyl groups – have made target-detected screening runs of over 100,000 compounds practical [31].

Requirements for ligands and targets

NMR screening is usually performed in aqueous solution, therefore, the ligands tested must have adequate solubility in water. For ligand-based screening, a concentration of at least 100 μM for each ligand tested should be attained to produce an acceptable signal:noise ratio, using conventional probe head technology. The total ligand concentration can, thus, become an issue for protein stability if larger mixtures are to be tested. The amount of target protein varies between 1 and 50 μM of unlabelled protein per 0.5 mL of screening sample, depending on the technique used. For target-detected experiments (i.e. chemical shift mapping), 100–300 μM of labelled protein are required. The use of cryogenic probes can increase probe sensitivity more than threefold [32,33], with a concomitant lowering of the demands on both ligand solubility and the amounts of protein required.

In most cases, the target molecule is a protein with a molecular weight >10 kDa. To overcome signal overlap in the NMR spectra (necessary for both the spectral assignment and target-detected screening), stable isotope labelling with ^{15}N or ^{13}C is required. For this reason, an overexpression system with a high cell density and high expression rates of the target protein is essential. For screening proteins up to ~45 kDa, global labelling of the target molecule with ^{15}N suffices in most cases. If larger proteins are to be studied, several labelling schemes might be useful. Uniform labelling with ^{15}N, combined with deuteration of all non-exchangeable protons, leads to a dramatic increase in sensitivity using TROSY (transverse relaxation-optimized spectroscopy)-based experiments [34], which can increase the size limit to >70 kDa.

The selective ^{13}C-labelling of valine, leucine and isoleucine methyl groups, which can be achieved by synthesis of the amino-acids from cost effective precursors [35], is also widely beneficial in target-detected screening. The significantly higher signal intensity of such methyl groups, due to the contribution of three protons per signal

(compared to one for ^{15}N-based screening) and advantageous relaxation properties, reduces protein demand significantly, particularly that for larger proteins and the size limit is also extended to >70 kDa [35]. Furthermore, domain-specific or amino-acid-specific labelling can be used to simplify spectra in proteins where a full assignment is not feasible. (For an overview of labelling schemes see [36,37].)

New screening methods

Several new screening methods have combined the advantages of ligand-detected and target-detected techniques and these are described in detail in the following sections. A comparison of their properties in the context of existing screening methods is given in Table 1.

Competition-based screening: beating narrow affinity ranges

The main problem with most ligand-detected experiments is their limited affinity range, which is often restricted to 2–3 decades in K_D, within the range 10^{-3}–10^{-7} M. This is usually a direct result of the need to distinguish the ligand and target signals by exploiting size-dependent effects, for example, via relaxation and/or diffusion filters. Such experiments detect only the free ligand signals and, thus, require rapid exchange between free and bound forms; the affinity range can be shifted towards the detection of higher affinities by decreasing ligand concentrations, with the practical limit being set by the sensitivity of the technique used. Conversely, increasing ligand concentrations to shorten experiment times and increase throughput lowers the affinity range, increasing the likelihood of detecting non-specific binding and missing strongly binding ligands. The sensitivity of a ligand-detected experiment is, therefore, vital in determining how flexibly it can be applied, and is particularly limiting for experiments that rely on intermolecular magnetization transfer, such as waterLOGSY (water-ligand observation by gradient spectroscopy) [38] and saturation transfer difference (STD) [39].

One approach that has recently been used to avoid this problem is competition screening assays [40–42]. Here, displacement of a reporter ligand by the test compounds is observed (Figure 1). The precondition of rapid exchange between free and bound forms only applies to the reporter ligand, meaning that there is no upper affinity limit. The lower affinity limit can be tuned by adjusting the concentration of the reporter ligand, relative to its affinity. Competition-based approaches are ideal for experiments that do not require a separate reference sample (e.g. waterLOGSY and STD) [28,40]. Binding of a test ligand is observed via the disappearance of the reporter signal and the narrow affinity range for these experiments becomes irrelevant.

Competition-based screening: drawbacks

Competition screening effectively combines the advantages of ligand-detected screening with some of the advantages of target-detection. As in other ligand-detected techniques, fast 1D-spectra are used and protein requirements are less restrictive. The broad affinity range, ability to exclude non-specific binding and ease of mixture design, are aspects 'borrowed' from target-detected screening, as is the ability to rank ligand affinities using titration series.

However, also borrowed from target-detected screening is one general disadvantage; the inability to screen compound mixtures directly, without deconvolution. The need to identify a suitable reporter ligand and the large amounts of reporter needed for a full-scale screen are further problems that are intrinsic to competition screening. Also, only ligands with a binding site that overlaps that of the reporter ligand can be detected via competition (although in some cases, binding of the test ligand at a remote binding site can be observed directly, e.g. via WaterLOGSY or STD signals). This might negate one of the general strengths of NMR screening, namely the ability to locate sub-sites neighbouring a binding site, which can be exploited in structure-based or combinatorial drug development. Allosteric effects, caused by binding at a remote site, could influence binding of the reporter ligand; these must also be considered.

Fluorine-based screening

The problem of reduced affinity ranges that occur in ligand-detected screening is absent from target-detected screening techniques, where target and ligand signals are readily distinguished via labelling of the target protein. Attempting a similar approach for ligand-detected screening (i.e. labelling the ligands), seems impractical initially. However, the collation of libraries of fluorine containing compounds is one such promising method [43]. In NMR terms, ^{19}F is an ideal screening nucleus. It has a 100% natural abundance, a high intrinsic sensitivity (0.9 relative to ^{1}H), gives sharp resonance lines and has a broad chemical shift dispersion; the last two characteristics meaning that composing large ligand mixtures without chemical shift overlap is possible. Owing to its large chemical shift anisotropy (CSA), ^{19}F chemical shifts and line widths are highly sensitive to binding to large molecules and screening can be conducted by a comparison of simple, sensitive 1D spectra of ligand mixtures with and without the target [43].

The obvious drawback of ^{19}F-screening is the need to assemble a sufficiently large library of fluorinated

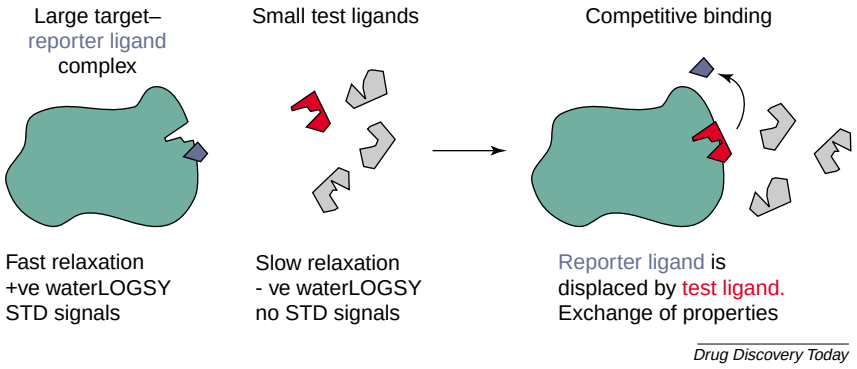

Figure 1. Schematic representation of competitive screening. A low- to medium-affinity reporter ligand (blue) is bound to a specific binding site on the target protein. This reporter ligand is replaced by a test ligand (red) with higher affinity for an overlapping site. Binding of the test ligand is observed via changes in the reporter ligand signals, for example, change in sign of waterLOGSY [38] signals, loss of saturation transfer difference (STD) [39] signals or, for a fluorine-containing reporter, changes in ^{19}F signal intensities as a result of slower relaxation.

compounds and also the intrinsic lack of diversity. It has been pointed out, however, that 12% of compounds in the Available Chemical Directory of Screening Compounds (ACD-SC, http://www.mdli.com/products/acdsc.html) contain fluorine, with ~150,000 molecules containing fluorobenzene and 40,000 containing trifluormethylbenzene moieties [43].

Competition-based fluorine screening

When considering the previous concepts, the combination of competition screening with ^{19}F-screening seems logical. Here, a binding of test ligands is observed via displacement of a fluorinated reporter ligand [43]. The use of ^{19}F avoids overlap between reporter and test ligand spectra, which are potential sources of problems in other competition-based methods.

A brief look at a practical set-up for competition-based ^{19}F-screening demonstrates some of the advantages of the technique. For a reporter ligand with a binding constant of 10–200 μM, a concentration of 50–500 μM is appropriate. A 10–50-fold excess of reporter ligand over protein is possible, meaning protein concentrations as low as 1–2 μM can be used. The test ligand concentrations should be somewhat in excess of that of the target – 2–10 μM. The required amounts of both protein and test ligands are, therefore, much lower than for other NMR screening techniques. The demands on the solubility of the ligand are concomitantly lower and the size of mixtures is limited only by the need to identify hits by mixture deconvolution. In the previous example, a typical screening experiment could be conducted in 5–10 minutes on mixtures of ~20 compounds and the introduction of dedicated hardware for ^{19}F detection should increase this throughput further. The ^{19}F-screening of a small library of fluorinated compounds could be used to identify suitable reporter ligands. The main test library need not contain fluorinated compounds, and thus the problems of limited diversity that are associated with fluorinated libraries are avoided.

Extending the range of NMR screening

Conventionally, NMR screening has been applied to isolated target macromolecules in solution. The lack of restrictions on target size in many ligand-detected techniques has been exploited to extend this range to include large, immobilized and *in situ* target systems.

Membrane-bound receptors and in-cell screening

Target proteins are often associated with, or embedded in, a membrane and are therefore difficult to assess with common solution-state NMR methods. The ligand-detected STD technique has recently been used to characterize the specificity of RGD peptide ligand-binding to an integral membrane protein (integrin $\alpha_{IIb}\beta_3$) [44]. The protein target was embedded in liposomes prepared from detergent-solubilized phospholipids; efficient saturation transfer could be achieved by the restricted mobility of the liposome-embedded protein. A similar situation exists for intact virus particles: the use of STD techniques to detect ligand binding to intact, functional viruses has recently been demonstrated [45].

In-cell NMR spectroscopy offers a wide range of potential applications in drug discovery, including characterization of metal ion binding, phosphorylation, post translational modifications and the uptake of drug candidates in cells, which is fundamental because, in many cases, compounds with high affinity *in vitro* show drastically weaker effects *in vivo*. In-cell NMR would clearly reveal whether compounds are able to permeate the cell membrane. Serber and co-workers have demonstrated the potential of in-cell NMR by recording spectra on a bacterial slurry with conventional NMR methods [46–48].

Applying solid-state techniques

Systems with restricted mobility display NMR signals with large line widths, which render the spectra uninterpretable

in most cases. High-Resolution Magic Angle Spinning (HR-MAS), a technique adopted from solid-state NMR, in which the sample is rapidly rotated around the magic angle, reduces line widths dramatically, leading to well-resolved NMR spectra. Wieruszeski *et al.* investigated the cyclic osmoregulated periplasmic glucan of *Ralstonia solanacearum in vivo* using HR-MAS and conventional liquid-state NMR techniques [49]. They found that the spectral quality for liquid-state techniques strongly depend on the sample preparation and the sample conditions, i.e. cell density and so on, which are hard to control and which yielded spectra of irreproducible quality. By contrast, HR-MAS techniques gave spectra of good quality with high reproducibility. HR-MAS NMR techniques have also been applied to STD-based screening, where the target molecule was immobilized on controlled-pore glass, facilitating its recovery [27].

Screening for macromolecular interactions

The screening for macromolecular interactions represents a special case, in which the binding partners have molecular weights of the same order. Experiments that rely on the apparent difference in molecular mass are, therefore, difficult to use, and chemical shift mapping has been the conventional approach. Recently, a method that combines STD with a TROSY-type HSQC (heteronuclear single-quantum coherence) has been used for determination of the interface of protein–protein complexes [50]. This principle is referred to as 'cross-saturation' and uses different labelling schemes for both macromolecules. One protein is unlabelled, whereas the other protein is uniformly ^{15}N-labelled and all non-exchangeable hydrogens are replaced by deuterium. Magnetization is transferred from the protonated protein to amide protons of the labelled protein and subsequently detected in a HSQC-type scheme. The same approach was used in a slightly modified version for mapping of the interface of a protein–nucleic acid complex [51], where well-resolved protons of the nucleic acid were saturated. Such techniques raise the possibility of screening for small molecules, which act as modulators of macromolecular interactions.

Obtaining structural information

Residual dipolar couplings (RDCs) are generally used for structural refinement [52]. If subjected to a system in the fast exchange regime, RDCs are transferred from the bound to the free state of a binding ligand. RDC data are not prone to spin diffusion [in contrast to NOE (nuclear Overhauser effect) data] and are thus precise even for immobilized targets. So far, RDC data have been used to derive angular information about the structure of a ligand in its bound state [53], and to characterize its orientation with respect to the target [54]; however, no screening applications using RDCs have been reported to date.

Analogous to RDCs, cross-correlated relaxation (CCR) can also be used for structural refinement [55]. Here, cross-correlated relaxation rates of the ligand are measured and converted into angular restraints. CCR has been used for the determination of sugar-pucker bound to the bacterial elongation factor Tu–GDP complex [56] and Blommers *et al.* have used CCR data to characterize the structure of a bound ligand in a case where NOE data was insufficient for a structure calculation [57].

Integration of NMR with other techniques

NMR in fragment-based drug design

The ability of NMR to provide information for fragment-based drug design is one of the main reasons for its growing industrial importance. NMR not only provides the initial fragments from which high-affinity leads are assembled, but can also direct the assembly process via knowledge of the spatial relationships of the fragments in the bound state [9–13]. In some cases, it is possible to obtain atomic-resolution structures of the target–fragment complexes. If the structure and assignment of the free target are available, complex structures can be solved rapidly by NMR – within 2–5 days per ligand, including measurement time – making it an attractive alternative to X-ray crystallography. In other cases, important information can still be obtained, albeit at lower-resolution; for example, from residual dipolar couplings and cross-correlated relaxation, as described previously. A quantitative extension of chemical shift mapping, using calculations of ligand electron current densities to derive binding locations and orientations has also been described recently [25,58].

The spatial relationships of fragments can also be obtained through second-site screening (see Figure 2) [7]. In this method, a ligand that is known to bind to a binding site of interest (the first site) is used as a probe to detect ligands that bind to neighbouring sites (the second site). Chemical shift mapping, or observation of direct interactions, is used to establish the relative orientations of the two fragments. One powerful method of second-site screening is the use of paramagnetic labels (spin labelling). Interactions of an NMR-active nucleus with an unpaired electron follow the same rules as the interactions between two nuclei; however, due to the vastly higher gyromagnetic ratio of the electron, the induced effects (line broadening) can be observed over distances up to 20 Å.

Jahnke *et al.* have used a spin-labelled ligand known to bind to the target as a probe for second-site screening [59]. This method is analogous to the observation of transferred inter-ligand NOEs [60], although it offers higher sensitivity

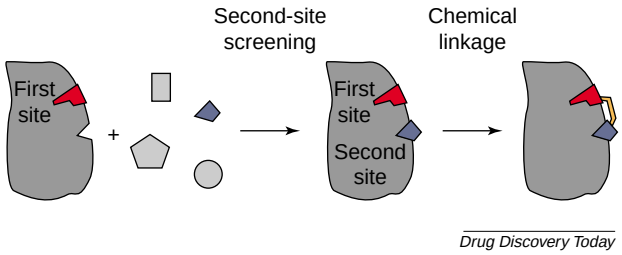

Figure 2. Second-site screening in fragment-based ligand design. A ligand (red) is identified that binds to the primary binding site (the first site). This ligand is used to saturate binding at the first site, while ligands binding to a second site are identified. Second-site ligands binding close to the first site can be identified by chemical shift mapping or directly via interactions with the first-site ligand. Linkage of the fragments leads to ligands of improved affinity.

(therefore lowering the amounts of target and ligands needed), and the ability to identify binding sites at greater distances. False positives as a result of competitive binding of the test ligand at the first site are also eliminated. As a prerequisite, spin-labelled ligands have to be synthesized without altering the binding properties. Furthermore, a blind test must exclude interactions between the ligands in the absence of the target. A different approach is based on spin-labelling of the target molecule in close proximity to the binding site, enabling the identification of ligands via line broadening. The so-called SLAPSTIC method (spin labels attached to protein side chains as a tool to identify interacting compounds) [61] reduces the amount of both target and ligand needed for an acceptable throughput by as much as two orders of magnitude. It is, however, challenging to spin-label the target near the binding site without altering binding properties. Several examples of the use of second-site screening in lead design are given by Jahnke *et al.* [7], and several other examples of fragment-based design have appeared in the recent literature [4–6].

NMR screening with other screening methods

The interaction of NMR with other screening methods is likely to be of increasing importance. Particularly promising are methods that combine a high-throughput primary screen with NMR as a secondary screen (reviewed in [31]). A good example is the use of affinity-selection MS (AS-MS) as the primary screen [62]. NMR is used to assess AS-MS hits, confirm binding to the target protein and exclude false-positives as a result of non-specific binding. Owing to the ability of NMR to locate the binding site of a ligand on the protein surface, binding to specific binding sites or subsites can be confirmed, thus providing extra information for lead optimization. Another use of NMR is in designing the HTS systems themselves, identifying compounds that can be used, either directly or as fluorescent or radiolabelled analogues, in competitive binding assays [31].

As demonstrated here, the coupling of NMR screening with a HTS system can exploit the benefits of both techniques. This is equally applicable to the combination of NMR and *in silico* screening [63]. Here, the synergy extends beyond confirming *in silico* hits and the location of their binding sites; the information from NMR can be used to select sub-libraries for further screening and to refine the scoring functions used for ligand selection. The iterative use of cycles of *in silico* screening and NMR-based hit validation (see Figure 3) promises to be a powerful emerging method in lead discovery.

NMR in the analysis of drug metabolism

The detection of intermolecular interactions by NMR also has applications later in the drug development process. The use of NMR in evaluating binding of a drug candidate to human serum albumin (HSA) is one such example; strong binding to HSA is one reason for lower efficacies of drugs *in vivo*, in comparison to *in vitro* assays. Target-detected methods, which require labelled protein, have been used to localize HSA binding to specific sub-sites [64] and ligand-detected methods have also been applied. Dalvit and co-workers have described a fast competition assay for HSA binding to Sudlow´s site II using L-tryptophan as a reporter ligand [65]. Other reporter ligands with specificity to other sites are feasible, as is the extension of the principle to other proteins that are important in drug metabolism. (The broad use of NMR in drug metabolite analysis and in the emerging field of metabonomics is beyond the scope of this review.)

Conclusion

Recent developments in NMR screening technologies are contributing to its increasing importance in industrial drug research. The limitations of the technique are constantly being challenged and the range of applications is broadening. NMR screening is now established as a field in its own right, and one that benefits from both novel applications of well-established methods and the adoption of new techniques emerging in general NMR research; both of which will provide future innovation in the field. The development of dedicated hardware will also be fundamental, but, perhaps most importantly, the interplay of NMR with other screening techniques will yield opportunities for an increasing industrial role.

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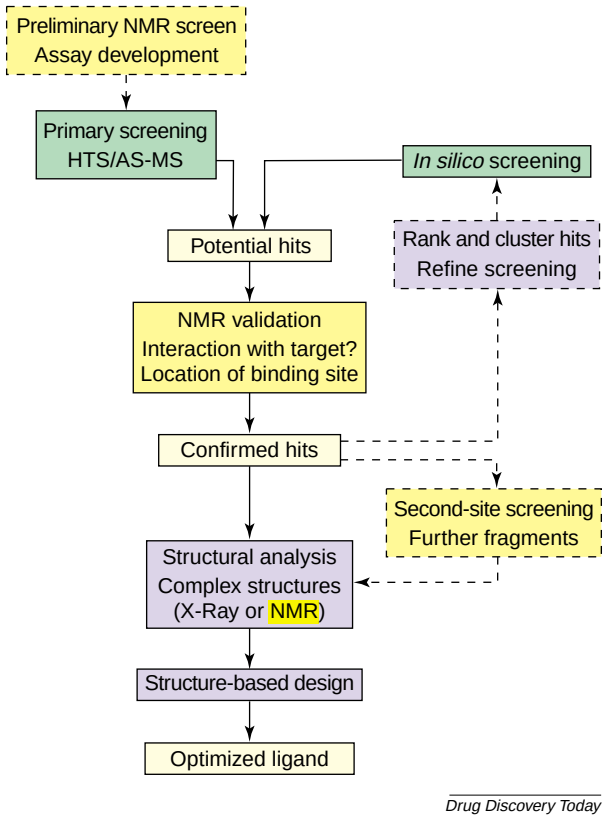

Figure 3. A generalized flowchart showing the involvement of NMR-based methods (highlighted in yellow) in an integrated screening programme. Optional steps are indicated by dashed lines. NMR screening is used to validate the hits obtained from a high-throughput or *in silico* primary screen. Methods which can confirm binding to a particular binding site, for example, target-detected or competition-based ligand-detected screening, are best suited to this. Confirmed binders can be used as the seed for structure-based design, to refine and focus further rounds of *in silico* screening, or as first-site ligands for second-site screening for additional binding fragments. Preliminary NMR screening could also be used to develop competition-based HTS assay systems. Figure based on those in [31] and [63]. (AS-MS; affinity-selection MS)

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