NMR in Pharmacokinetic and Pharmacodynamic Profiling

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1. The Evolving Role of NMR in Drug Discovery

The successful launch of a new drug is a multifaceted, enormously difficult process. In the discovery phase, researchers typically identify a molecular target associated with a disease state, search for small-molecule ligands for this target that have the desired in vitro effect, modify these compounds to improve potency and bioavailability, and ensure that the compounds are safe and efficacious in animal models. It is only then that a candidate compound can enter the significantly more expensive development phase, in which the compound is extensively evaluated for safety and efficacy in man. As a result of the high cost and attrition rate associated with drug discovery and development, a host of new technologies have been developed in order to increase the chances for success. These new technologies span the entire range of drug discovery and development, including siRNA approaches to target identification and validation,[1] ultrahigh-throughput screening methodologies for the identification of lead molecules,[2] combinatorial chemistry and parallel synthesis for the rapid generation of large compound libraries,[3] structure-based drug design for the optimization of lead compounds,[4] and a variety of genomic, proteomic, and toxicogenomic approaches[5] to investigate the safety and potential efficacy of candidate compounds.

In addition to maintaining a critical role in the structure elucidation of small molecules and protein–ligand complexes, solution NMR has continued to adapt to meet the growing demands of the drug discovery process. NMR-based screening has become an increasingly important tool for lead generation and modification, primarily through enabling fragment-based approaches to drug design.[6,7] These fragment-based approaches promise not only to significantly accelerate the optimization of lead compounds, but also to generate leads for protein targets that have previously been intractable. Even more recently, NMR has moved beyond its principal role in the direct analysis of protein–ligand complexes to other phases of drug discovery. Metabonomics is the study of biofluids (e.g., urine, blood) by NMR after dosing of a test compound in order to noninvasively and continuously monitor toxic or other metabolic effects. The universality, speed, and low cost of this approach promises to change when and how we think about toxicity profiling. The recently demonstrated ability to perform solution NMR measurements on whole cells and monitor compound binding in a cellular environment can provide key information about membrane penetration and access to the molecular target.[8] Structural studies of compounds complexed to multidrug transporter complexes can aid in solving problems with cellular penetration and resistance development.[9] All of these developments highlight the power and versatility of using NMR spectroscopy in a pharmaceutical environment.

In this review, we will focus on some additional NMR technologies that can aid in the pharmacokinetic and pharmacodynamic profiling of compounds in the process of lead optimization. It is rare that compounds fail to reach development because of insufficient in vitro potency. As outlined above, structure-based design, high-throughput organic synthesis, and fragment-based approaches are just a few of the technologies that have enabled the rapid optimization of compound affinity for a biomolecular target. Rather, compounds often fail because of unacceptable in vivo properties. This can be because of either what the body does to the drug (pharmacokinetics) or what the drug does to the body (pharmacodynamics).[10] Two of the more common problems are tight binding to

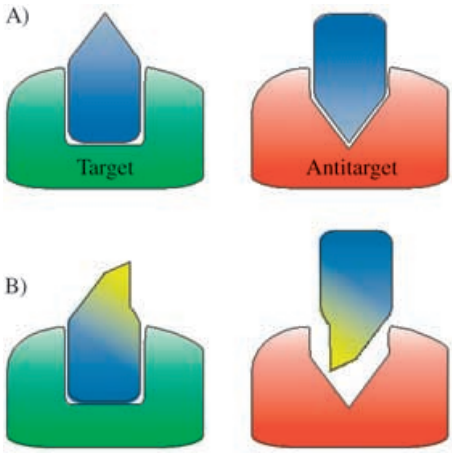

Figure 1. The concept of structure-based antidesign. A) A drug lead (shown in blue) binds with high affinity to both the target of interest (green) and an antitarget (red) such as albumin or a cytochrome P450 enzyme. Based on the structure of the compound complexed to both proteins, the compound is modified in order to abrogate binding to the antitarget, but maintain high affinity to the target of interest. B) In this illustration, the site of the compound modification (shown in yellow) was solvent exposed when bound to the target of interest (therefore not expected to affect binding), but intimately in contact with the antitarget (therefore expected to decrease binding).

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serum albumin and inhibition of and modification by cytochrome P450 enzymes. Both of these problems can potentially be addressed by using the concept of structure-based antidesign (Figure 1), in which structures of the drug lead complexed to both the biological target and the antitarget (either albumin or the cytochrome P450 enzyme) are used to design out affinity for the antitarget but maintain or even increase affinity for the therapeutic target of interest. An additional problem that is well known but often poorly understood is that of compound reactivity. Compounds that covalently modify proteins can have increased risks of low bioavailability (e.g., rapid modification or clearance) or toxicity (e.g., irreversible inactivation of essential enzymes). Recent NMR approaches to measuring and understanding these phenomena and aiding the medicinal chemist in reducing compound liabilities will be described.

2. Compound Binding to Human Serum Albumin

In the early 1940s, Bernard Davis published landmark papers in which he demonstrated that sulfonamide drugs bind to plasma albumin and that the bound drug is inactive.[11,12] Since that initial study, the importance of albumin binding in modulating the pharmacology of the vast majority of drugs has become evident.[13] In fact, since 1942, more than 36000 papers have been published that investigate albumin and drug action. The complexity of ligand binding to albumin became apparent in the 1960s and 1970s when numerous biochemical studies were performed on this protein. A metal-binding site was mapped to the N-terminal region[14] whereas a major small molecule-binding site was mapped to a C-terminal domain.[15–17] Further studies showed that there are actually two distinct small-molecule-binding sites in the C-terminal half of albumin[18,19] and that these could be distinguished by binding of specific fluorescent probes.[20] One site had been characterized as early as 1958 as the indole-binding site.[21] The other site, commonly known as the warfarin-binding site, was found to be sensitive to modifications of the lone tryptophan residue in the middle of the protein sequence.[22] When the crystal structure of albumin was solved, the details of these distinct binding sites were clarified.[23] Subsequent crystallography studies of complexes with warfarin,[24] halothane, and fatty acids[25] provided more details of the various binding sites on this promiscuous protein. As summarized in Figure 2, human serum albumin is comprised of three homologous alpha-helical domains that each harbors multiple ligand-binding sites. In total, there are nine binding sites for metals and small molecules on albumin,making it by far the most promiscuous carrier protein in serum.

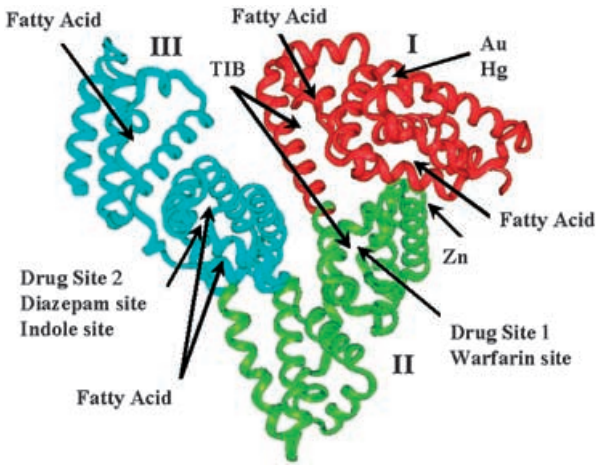


Figure 2. The 3D structure of human serum albumin[80] with known binding sites indicated. The three homologous domains are indicated by color: domain 1 (red), domain 2 (green), and domain 3 (cyan). TIB = 2,3,5-triiodobenzoic acid.

2.1. Measuring compound affinity for human serum albumin

Most drugs have some affinity for human serum albumin (HSA) that results in sequestration of compound in serum.[10] Being the major protein component of human blood, albumin is present at 40 mg mL^{-1} or 600 μM. As a result, if the K_D of the drug for albumin is 600 μM, ~50% of drug in the serum compartment will be bound to HSA. Up to a point, this is an advantageous property as albumin binding increases the drug bioavailability by decreasing the clearance rate. However, when the K_D of binding decreases to the low micromolar range, >99% of drug in the serum is albumin-bound[26] and the in vivo efficacy can be significantly reduced. The other disadvantage of this pharmacokinetic profile is that a highly bound drug is susceptible to severe drug–drug interactions. When the amount of free drug is very small compared to the amount that is protein-bound, any situation that disrupts albumin binding can significantly increase the concentration of free drug. In some cases, this can result in toxic drug levels. Such is the case for warfarin and tolbutamide, which are highly albumin-bound and can be displaced by several other drugs that also bind to domain 2 of HSA. Hemorrhages or hypoglycemia are clinical consequences observed for these types of drug–drug interactions.[13]

Given the importance of understanding and controlling albumin binding in lead optimization, a large number of assays have been developed to measure the affinity of small molecules for HSA. Equilibrium dialysis[27] and ultracentrifugation[28] are standard methods for measuring albumin binding. However, the throughput of these assays is limited because of the need to directly detect the free compound, typically through HPLC or radiometric detection. As a result, alternative methods that make use of chromatography,[29] mass spectrometry,[30] microcalorimetry,[31] and fluorescence spectroscopy[32,33] have been proposed for measuring the affinity constant of a compound for HSA. NMR has also been used to monitor compound binding to albumin, with the earliest report being from Oleg Jardetzky's lab in the study of penicillin binding to albumin.[34] However, most of these methods can neither identify the binding site on HSA involved in the interaction, nor distinguish between high affinity binding to a single site and low af-

finity binding to multiple sites. These limitations can hamper the interpretation of structure–affinity relationships and the design of compounds that have superior pharmacokinetic properties.

New NMR methods have recently been developed to rapidly evaluate site-specific albumin binding. Longitudinal relaxation rates and saturation transfer difference (STD) NMR competition experiments with tryptophan can accurately measure compound binding specifically to the indole-binding site of full-length albumin.[35,36] As shown in Figure 3, the STD NMR-based experiments monitor the reduction in STD NOEs to a probe compound in the presence of a competing ligand.[36] Because an STD experiment can be collected rapidly (typically ~15 min), the throughput of this assay is sufficient to evaluate hundreds of compounds for their ability to bind to serum albumin. Simple extensions of this experiment to include probes for binding to other sites of albumin have the potential to yield a single assay for monitoring site-specific compound binding at multiple drug-binding sites. Another approach to monitor site-specific binding involves 2D NMR on isotopically labeled protein. This has the advantage of monitoring all binding sites on the protein and the ability to quantitatively measure site-specific binding constants. While obtaining isotopically labeled full-length albumin has proven difficult, Mao and colleagues described a method to produce isotopically labeled domain 3 of HSA from *E. coli*.[37] By using this protein, binding to the indole site (Figure 2) can now be rapidly and reliably monitored by chemical-shift changes in the presence of a test compound (Figure 4).

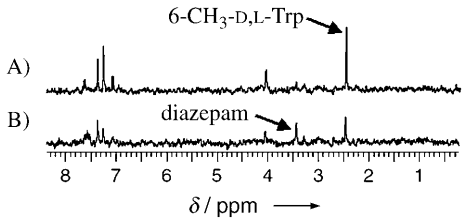


Figure 3. Competition STD-NMR spectra of 6-methyl-D,L-tryptophan (95 μM) and albumin (15 μM) in the absence (A) and presence (B) of diazepam (15 μM). The experiments were conducted by selectively saturating the protein (by using a train of selective pulses at 0.0 ppm) for a period of time (3 s in this example) long enough to transfer saturation to a bound ligand that is in fast exchange between the free and bound states. A reference spectrum in which the protein is not saturated is then subtracted to yield the STD spectra showing only signals from the ligands. The reduction in the STD signal of Trp in the presence of diazepam indicates competitive binding of diazepam for Trp on this binding site on albumin. Adapted with permission from ref. [36].

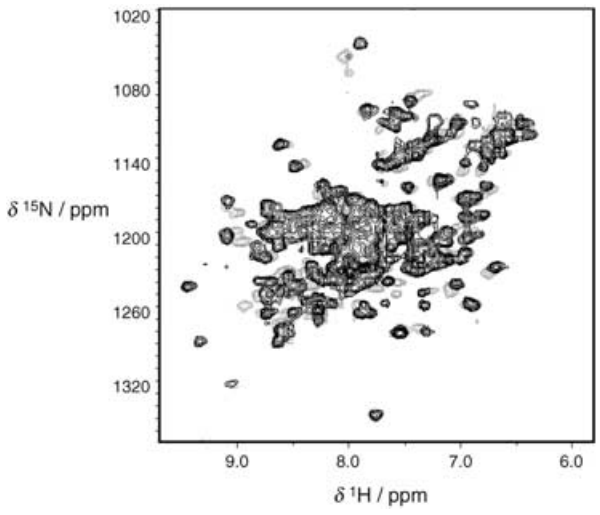
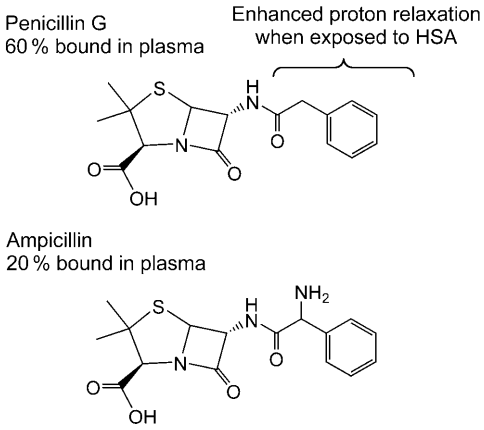


Figure 4. 2D ^{1}H,^{15}N-HSQC spectra of domain 3 of albumin[37] in the absence (black) and presence (gray) of a test compound. The large chemical-shift perturbations in the presence of the test compound indicate binding to this domain of human serum albumin, while the specific pattern of perturbations corresponds to binding at a particular site on this protein.

2.2. Designing out albumin affinity: Where to modify the compound

Once it has been confirmed that albumin binding is severe and compound modification is required for in vivo efficacy, structural studies can be used to identify positions on the compound that, when modified, would reduce the affinity for HSA. This, in fact, was the goal of the first NMR studies by Jardetsky and co-workers when they studied albumin binding of penicillin.[34] To identify the sites on penicillin that contacted albumin, the authors measured the proton-relaxation rates in the presence of increasing amount of albumin. It was observed that the relaxation rates of the benzylic protons were most affected, and it was postulated that this group was in intimate hydrophobic contact with HSA. Their predictions were validated by albumin-binding studies of the penicillin analogue ampicillin (Scheme 1). Addition of a primary amine to the benzyl group (which would be expected to disfavor a hydrophobic interaction with HSA) does indeed reduce albumin binding.[10] This success spawned numerous studies in which ^{1}H- and ^{13}C-relaxation rates and chemical shifts of drugs were measured in the presence of albumin in hopes of designing analogues with



Scheme 1. Disruption of albumin binding of penicillin based on NMR-relaxation data.[34] Benzyl protons exhibited enhanced relaxation rates in the presence of albumin suggesting that this group is buried in an albumin binding pocket. Modification of this region of penicillin (resulting in ampicillin) did in fact reduce serum protein binding.

improved pharmacokinetic parameters (e.g., tolbutamide,[38,39] levamisole,[40] phenylbutazone,[41] ibuprofen,[42] azathioprine,[43] and chloramphenicol[44]).

High-resolution structural studies of the protein–ligand complex can provide the most detailed information for structure-based design of compounds with reduced albumin affinity. By using isotopically labeled domain 3 of HSA,[37] NMR structures of albumin–ligand complexes can be obtained and used in structure-based antidesign strategies to reduce compound binding to this protein (Figure 1). By studying the structure of diflunisal in complex with domain 3, the Fesik group demonstrated how to modify this and related cyclooxygenase (COX) inhibitors to reduce the HSA affinity (Figure 5).[45] By using heteronuclear NMR spectroscopy of ^{13}C,^{15}N-labeled domain 3 of HSA, the 3D structure of the protein was solved in complex with diflunisal. NMR titrations, in which the chemical shifts of the labeled protein were followed as a function of ligand concentration, were performed to determine binding constants for diflunisal analogues. These structure–activity relationships (SARs) in concert with the NMR structure guided the design of COX-2 inhibitors with reduced affinity for HSA. This structure-based antidesign strategy has been successfully used on multiple projects and serves as an excellent complement to concurrent structure-based design projects directed at the therapeutic target of interest.

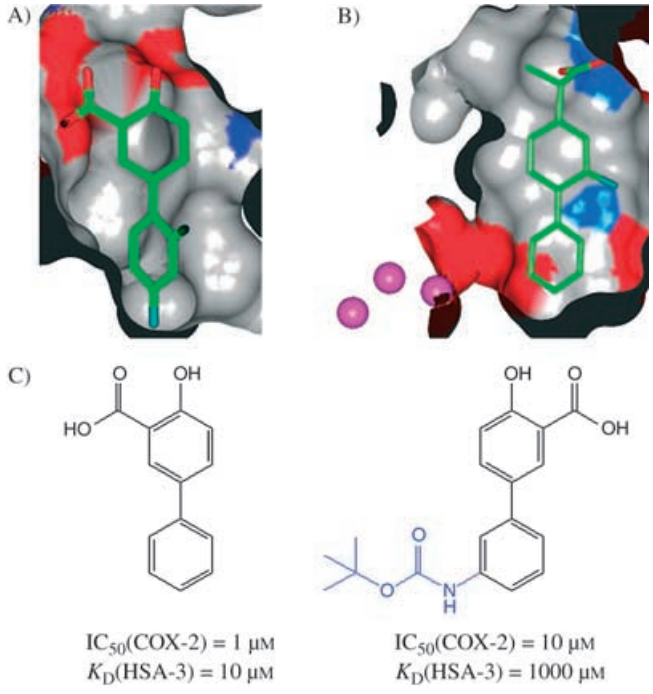
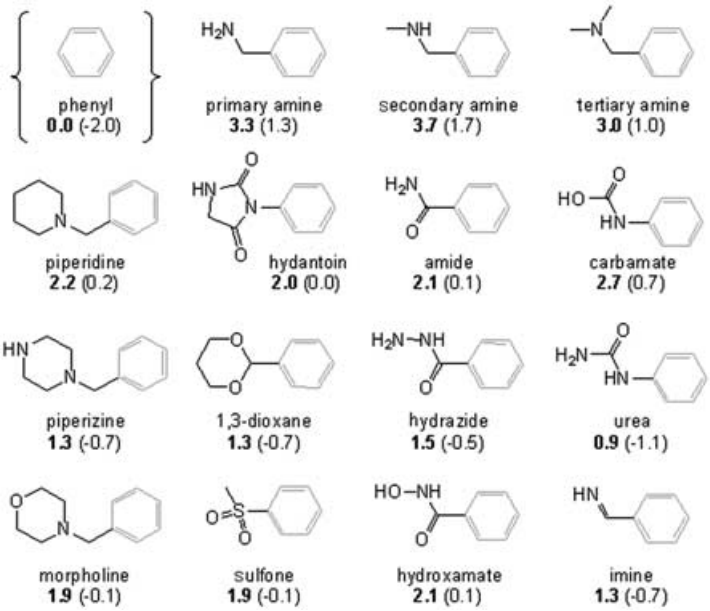


Figure 5. Surface representations of A) diflunisal binding to domain 3 of HSA and B) flurbiprofen binding to cyclooxygenase (PDB accession number 1CQE). Diflunisal and flurbiprofen are shown in sticks. The surface is colored by atom type (carbon in gray, oxygen in red, and nitrogen in blue). Three water molecules near the flurbiprofen-binding site in (B) are shown as magenta balls. C) Biaryl-containing COX-2 inhibitors were modified to reduce albumin binding. In this example, a *tert*-butylcarbamate group (shown in blue) was added in an attempt to access the polar pocket in cyclooxygenase (occupied by the water molecules). This resulted in a more than 100-fold loss in albumin affinity, with only a tenfold loss in COX-2 inhibition. Adapted from ref. [45].

2.3. Designing out albumin affinity: How to modify the compound

By using domain 3 of albumin, the structure–affinity relationships for a set of 889 compounds have been analyzed in order to derive an understanding of binding to this important site on albumin.[46] Using a chemometric approach, the contribution of various substructures to the overall binding affinity of the compound to albumin could be quantitatively estimated. Significantly, 15 different substituents were found to consistently decrease binding to domain 3 of albumin by more than an order of magnitude (Scheme 2). These substituents can be



Scheme 2. Calculated effects of various substituents on benzene binding to domain 3 of human serum albumin.[46] Each substituent is shown in black, and the calculated effect on the binding in pK_D units (the logarithm of the K_D) is shown in bold (the unsubstituted benzene ring is shown in gray for reference). The substituents shown here are calculated to decrease the affinity for albumin by nearly one to three log units. The absolute value of the log of the calculated binding affinity (in M) is given in parentheses.

viewed as anti-albumin fragments, and incorporating these groups into a lead compound has a high probability of significantly decreasing albumin binding. A total of 74 different descriptors that modulate albumin binding were identified using this approach. This short list comprises a convenient look-up table for medicinal chemists to estimate the effects of different substituents on albumin binding.

2.4. Designing out albumin affinity: Putting all the tools together

Given the variety of tools to study albumin binding of drug candidates, one is faced with choosing techniques that will most rapidly provide the information needed to reduce albumin affinity. Initially, one is presented with a compound that has high albumin binding and whose activity in in vitro assays is markedly attenuated in the presence of serum. The first step

in a structure-guided antidesign strategy is to identify which site(s) on albumin is involved in binding. As described above, this can be done by NMR with 1D competition experiments using full-length albumin or with 2D experiments using domain 3 of albumin. Generally, we consider a compound to have "problematic" binding to albumin when the site-specific dissociation constant is significantly less than 10 μM (>99% protein binding to whole serum). If it can be determined that a specific site is (or sites are) problematic ($K_D < 10$ μM), then structural studies are pursued on the compound in complex with albumin using either NMR on domain 3 of HSA or X-ray crystallography on full-length albumin. Structures of the compound complexed to HSA and the target of interest are then compared, in the context of the known SAR, to identify regions of the compound that can be modified to disrupt albumin binding but not significantly affect binding to the target. Ideally, regions of the molecule that are solvent-exposed when complexed to the target but buried when bound to albumin should be modified (Figure 1). Based on the available chemistry, groups known to decrease albumin binding (Scheme 2) can then be incorporated into the compound. This cycle of testing for binding to HSA, structural studies, and chemical modifications to the lead is continued until the overall affinity for albumin falls to an acceptable level.

3. Drug Interactions with Cytochrome P450 Enzymes

Cytochrome P450 enzymes (CYPs) are mixed-function monooxygenases that can oxidize a large variety of both exogenous and endogenous compounds, including most drugs.[47–50] In humans, at least 57 different CYP isoforms have been identified, which have broad and overlapping substrate specificities.[51] It was recognized as far back as the 1960s that CYPs play a role in the oxidation of steroid drugs.[52–54] Ongoing research has documented the nearly ubiquitous involvement of CYPs in the metabolism of small organic molecules. In fact, it is currently estimated that the cytochrome P450 enzymes are responsible for ~90% of the phase 1 metabolism (e.g., oxidation, reduction, and hydrolysis) of drugs.[55]

There are two major outcomes of compound interaction with cytochrome P450 enzymes that can have a major impact on drug discovery efforts. First, as is often the case, the compound is itself a substrate for at least one of the CYP isoforms, resulting in rapid oxidation of the parent drug. If the oxidized product is inactive, this leads to rapid clearance and low bioavailability of the active drug. It can sometimes be the case that the oxidized product retains activity, or even that the administered compound is inactive until acted upon by the CYP enzymes (a process known as bioactivation).[48] A second major outcome of compound binding to CYP enzymes is that of CYP activation or inhibition. This can result in potentially undesirable drug–drug interactions by enhancing or preventing the metabolism and changing the pharmacokinetic (PK) profile of a second drug that is acted upon by that particular CYP isoform. While CYP inhibition is generally undesirable, there are examples of CYP isoforms that are intentionally inhibited in order to boost the PK profile of a second drug, as is the case with the coadministration of ritonavir, a known CYP3A4 inhibitor, with other HIV-protease inhibitors.[56] Despite serendipitous positive outcomes of drug interactions with the CYP enzymes, compound binding as either substrates or effectors/inhibitors of the CYPs is generally avoided or minimized. This requires rapid and reliable means of measuring compound binding to these enzymes, as well as rational approaches to designing out compound affinity.

3.1. Measuring compound binding to cytochrome P450 enzymes

Predicting phase 1 metabolism by cytochrome P450 enzymes in man is complicated. As mentioned, at least 57 different isoforms of CYP enzymes have been identified in humans. Among these, CYP1A2, CYP2C9, CYP2C19, CYP2C6, and CYP3A4 are the primary isoforms involved in the metabolism of current clinical drugs.[51] The most abundant enzyme in liver microsomes is CYP3A4, and this isoform is responsible for over 50% of drug metabolism.[57] CYP2D6 accounts for over 30% of drug metabolism, and the variation of CYP2D6 levels in patients combined with numerous genetic polymorphisms (in which different individuals carry genetically distinct copies of the gene) can significantly modulate in vivo drug concentrations and hamper the ability to predict clinical outcomes.[58] Thus, drug candidate interactions with these two enzymes are of particular interest. As CYP activity is relatively straightforward to monitor in vitro, a number of high-throughput assays have

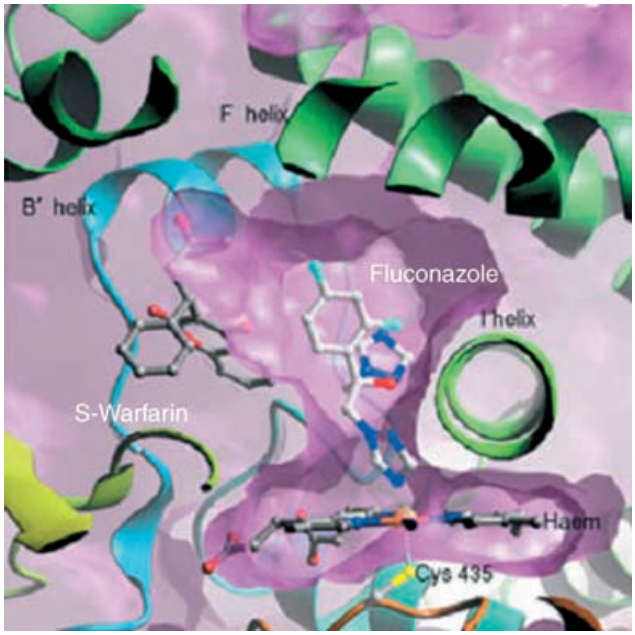


Figure 6. Model of fluconazole bound to the X-ray structure of the *S*-warfarin–heme–CYP2C9 complex.[61] *S*-Warfarin is labeled and rendered in stick with gray carbons. The binding surfaces for fluconazole (white carbon atoms) and heme (gray carbon atoms, bottom) are shown in pink. Notice in this model that warfarin provides some of the binding surface for fluconazole, potentially explaining the observed positive cooperativity between *S*-warfarin and fluconazole. Adapted with permission from ref. [61].

been developed to measure CYP inhibition or activation, including fluorescent, LC-MS/MS, and radiometric assays.[57,59] However, interpreting these data is not at all straightforward due, at least in part, to the atypical kinetics exhibited by many CYPs (including heterotropic positive and negative cooperativities) and substrate-dependent inhibition profiles.[50] Many of these peculiarities of the CYPs can be explained by the presence of multiple drug-binding sites within the active site of the enzyme, wherein compounds can act as effectors, inhibitors, or substrates. For example, mutagenesis studies on CYP3A4 suggest that this isoform potentially contains three subpockets in the active site, including one noncatalytic effector site.[60] The existence of multiple drug-binding sites is supported by the recently solved crystal structures of CYP2C9 and CYP3A4 that reveal exceptionally large active sites capable of simultaneously binding to more than one compound (Figure 6).[61–63] Thus, as with human serum albumin described above, interpreting structure–affinity and structure–activity relationships on the CYP enzymes is significantly hampered in the absence of site-specific information.

NMR can potentially play an important role in monitoring site-specific compound binding to the CYP enzymes, regardless of whether they function as substrates, inhibitors, or effectors. 1D NMR approaches are particularly well suited to the study of CYP enzymes as they contain a natural paramagnetic center at the iron atom of heme. This induces relaxation effects on compounds that bind near this group. Since the relaxation induced by unpaired electrons is proportional to the inverse sixth-power of the distance to the iron, not only can the occurrence of binding be deduced from the relaxation effect, but a precise distance to the paramagnetic center can also be derived.[65] An example of this is shown in Figure 7, in which paramagnetic effects on the relaxation rates of the protons of diclofenac allowed for a precise measurement of distances to the iron atom.[66] This paramagnetic effect confirms binding in the active site and also allows models to be derived for the orientation of this compound with respect to the heme group. Similar work has been performed on CYP2C9 with flurbiprofen and dapsone.[67] Activation of flurbiprofen hydroxylation is observed in the presence of dapsone, and biochemical data suggested that these two compounds bind simultaneously in the active site. NMR T_1-relaxation studies confirmed that both compounds bind in the active site, and conformational changes induced by concurrent binding were therefore detected. These types of studies are rapid and sensitive enough to analyze large numbers of compounds against multiple CYP isoforms for the development of SAR and pharmacophore models for drug design.

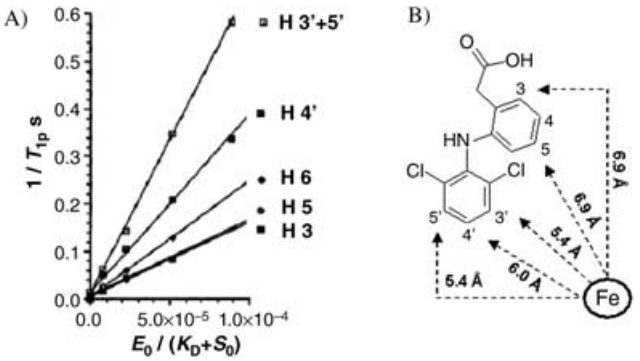


Figure 7. A) Effects of CYP2C9 on the proton longitudinal-relaxation rates of the substrate diclofenac. E_0 and S_0 are the concentrations of enzyme (CYP2C9) and substrate (compound), respectively, while K_D is the equilibrium dissociation constant between the protein and the compound. B) Distances between each proton and the iron atom as derived from the relaxation data. Adapted with permission from ref. [66].

By its very nature, 2D heteronuclear NMR spectroscopy provides information not only on the occurrence of compound binding, but also on the location of the binding site. To this end, Atkin and co-workers recently reported the monitoring of allosteric binding using isotopically labeled CYPeryF (a soluble bacterial CYP450 enzyme).[68] In this study, the Phe residues of CYPeryF were ^{15}N-labeled and ^{1}H,^{15}N-HSQC spectra were acquired in the presence and absence of a test compound (Figure 8). By monitoring the spectral changes throughout the addition of two equivalents of compound, two sets of spectral perturbations were observed (suggesting two distinct binding sites), and the affinity for the second equivalent of compound was higher than that for the first; this indicates cooperative binding. While such work on soluble bacterial proteins is relatively straightforward, this task is much more challenging with human microsomal CYP enzymes, which are membrane-bound. However, much progress is being made in producing isotopically labeled, recombinant CYP enzymes. For example, CYP2C9, CYP2D6, and CYP3A4 have all been recombinantly produced in *E. coli* and/or baculovirus.[61–65] Clearly, additional

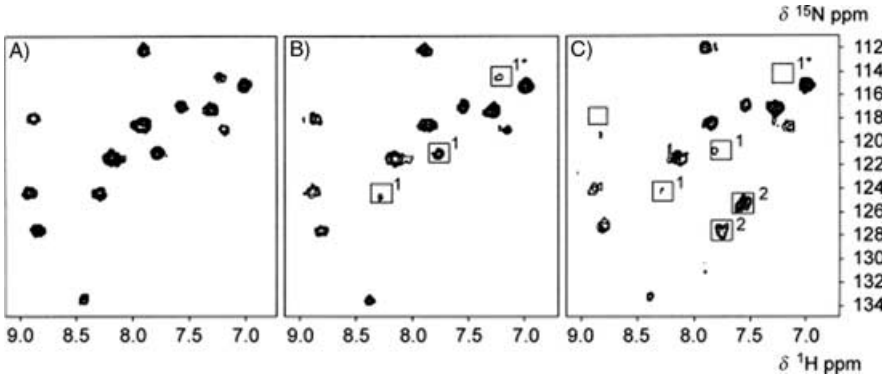


Figure 8. ^{1}H,^{15}N-HSQC spectra of ^{15}N-Phe-labeled CYPeryF in the presence of A) 0.0, B) 0.75, and C) 1.5 M equivalents of 9-aminophenanthrene (9-AP). In spectrum B three peaks showed significant broadening (boxed and labeled as 1). Upon addition of 1.5 equivalents of 9-AP (C), one additional peak broadened, and two new peaks appeared (boxed and labeled as 2). These differential effects in the presence of excess 9-AP provide strong spectroscopic evidence for two compound-binding sites (denoted as 1 and 2). Reproduced with permission from ref. [68], copyright: Marcel Dekker, 2004.

progress in this area would open new doors for screening and characterizing CYP-ligand binding by using 2D NMR approaches.

3.2. Designing out CYP450 binding

Similar to designing out albumin binding, the first step in an antidesign strategy against the CYP enzymes is to identify what constitutes a "problem." With HSA, the typical cutoff is a K_D value less than 10 μM, as drug affinities significantly better than this lead to total protein binding in excess of 99%. An IC_{50} (inhibitor) or EC_{50} (effector) value of 10 μM also seems to be appropriate for compound interaction with CYP enzymes, as most drugs with good PK achieve C_{max} values of 10 μM in the blood or liver and can affect CYP function. Until recently, when presented with such a "problem" compound, the medicinal chemist had to rely on serendipity or intuition to reduce compound affinity. While chemometric models for substrate or inhibitor binding to CYPs have been developed that can aid the chemist in this process,[69–71] these are of greatest utility with large databases of structurally disparate compounds, and do not perform particularly well with highly similar compounds within a given series. The recently described crystal structures of CYP2D5 and CYP3A4 have enabled structure-based antidesign against these important enzymes. Ideally, the crystal or NMR structure of a lead compound in complex with the target CYP should be used in this process, as described for reducing compound affinity for human serum albumin. However, as mentioned above, 1D NMR-relaxation data can be used to derive site-specific, 3D QSAR-descriptor models for compound binding. This approach has recently been formalized by Sem and co-workers in what has been termed the heme-based coordinate system.[72] This protocol integrates the experimental NMR data on the compound(s) of interest with known crystal structures to produce protein–ligand complexes that can be used in antidesign strategies.

4. Bioactivation and Protein–Drug Adduct Formation

As described above, compound binding to human serum albumin and cytochrome P450 enzymes can both reduce bioavailability (through sequestration in the plasma or rapid compound modification) and increase the risk for drug–drug interactions (by releasing albumin-bound drugs or affecting the normal oxidative modification of other drugs). However, another consequence of compound modification through oxidative enzymes is the creation of bioactive compounds that can form protein–drug adducts and result in organ toxicity, particularly in the liver.[73] Evidence for adduct formation being involved in organ toxicity goes back to the 1930s,[74] and the "covalent-binding theory" of chemical-induced hepatotoxicity was formulated during the 1970s.[75,76] Since then, a number of molecules have been implicated in organ toxicity through bioactivation of the parent molecule and subsequent inactivation of critical cellular proteins or immune-mediated adverse events. While no consistent link exists between the formation of protein–drug adducts and organ toxicity (as some apparently nontoxic compounds can also form covalent adducts), the avoidance or minimization of adduct formation is standard operating procedure at several pharmaceutical companies.[73] This has led to the development of a number of methods for assessing and measuring protein–drug adduct formation both in vitro and in vivo.[73]

4.1. NMR methods for assessing the propensity for adduct formation

Glutathione (GSH) is routinely used in the detection of compounds that can form covalent adducts with thiol-containing molecules, such as cysteine amino acids in proteins.[73,77] In particular, the reversal of adduct formation with the compound of interest in the presence of GSH is a strong indicator of covalent modification of cysteine thiol groups. This has led to the identification of a number of structural groups that are either inherently reactive with proteins[78] or that can be bioactivated to form reactive compounds.[73] However, while widely used in metabolism studies, GSH is relatively unstable and might not be appropriate for assessing large numbers of chemically diverse compounds. To address this issue, an alternative assay called ALARM NMR has been described that can rapidly and reliably assess the propensity for compounds to covalently modify protein thiol groups.[79] The assay utilizes isotopically labeled human La antigen (a protein that stabilizes human RNA transcripts against exonucleolytic digestion) as the surrogate for covalent modification and heteronuclear NMR as the assay read-out (Figure 9). Both cysteine residues in the La protein exist as free sulfhydryls and can react with organic compounds, as confirmed by both NMR and mass spectrometry. Specifically, the cysteines can form direct adducts with the organic compounds, or the oxidation state of the cysteines can be changed by the presence of the compound. In NMR experiments, both of these effects induce large chemical-shift pertur-

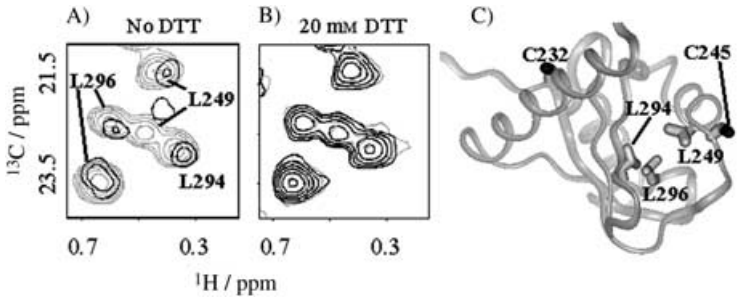
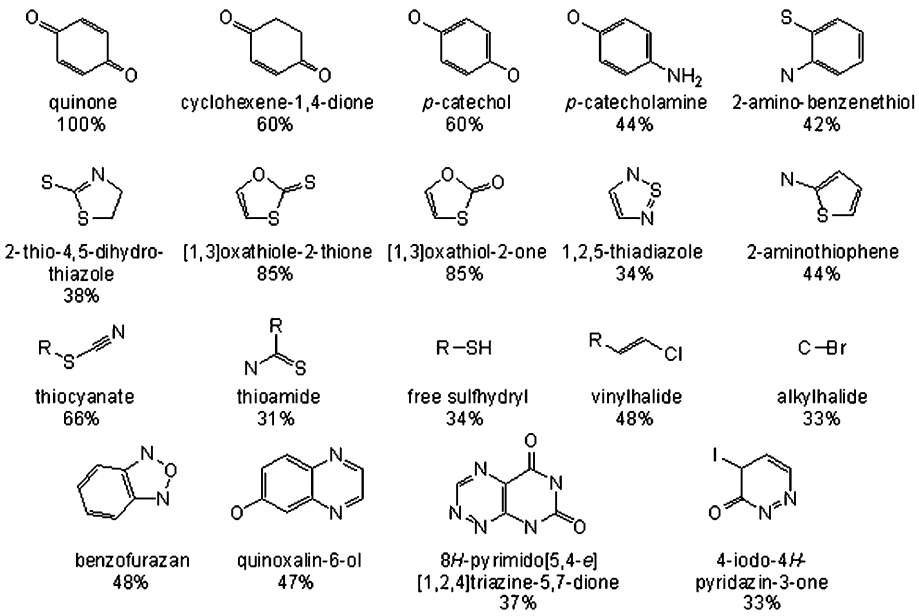


Figure 9. ALARM NMR data.[79] A) Subset of the 2D ^{1}H,^{13}C-HSQC spectra of the La protein showing cross peaks for four methyl groups of the human La antigen in the absence (gray) and presence (black) of a known oxidizing agent and no dithiothreitol (DTT). Large spectral perturbations are observed. B) Same spectra for samples to which 20 mM DTT had been added. The reversal of chemical-shift changes in the presence of reducing agent is evidence for a thiol-mediated modification of the La protein. C) Structure of the C-terminal RNA recognition-motif (RRM) domain from the human La antigen protein.[81] Cysteine and leucine residues whose methyl chemical shifts are shown in A and B are indicated. Adapted with permission from ref. [79].



Scheme 3. Substructures that impart high reactivity (oxidation or adduct formation) with the La protein. Shown are the substructures and the percent of compounds tested in the ALARM-NMR[79] assay that contained this substructure and were found to be reactive. As reactivity hit rates for general compound collections were on the order of 10%,[79] hit rates greater than 30% were flagged as potentially reactive moieties.

bations in the resonances of nearby residues, thus enabling facile detection. While ALARM NMR is used primarily to identify false positives from biochemical screens, an analysis of the NMR data resulted in the identification of dozens of structural groups that have a high propensity for covalent modification of proteins (Scheme 3)—which significantly increases their likelihood to induce adduct-related toxicity in vivo. While specific examples of drugs that act through covalent modification of the target protein can be cited (e.g., cefaclor and omeprazole),[79] these groups should generally be avoided or carefully evaluated for chemical-induced organ toxicity when incorporated into drug leads. The successful use of the La antigen as a surrogate for assessing adduct formation has led to the investigation and development of ALARM-based assays to assess the bioactivation of organic compounds.

5. Future Perspectives

As illustrated with the above methods and examples, the versatility and rich information content of NMR spectroscopy has allowed scientists to continue to break new ground in the analysis and understanding of protein–ligand interactions and have a significant impact on the drug-discovery process. As with NMR-based screening and fragment-based approaches to ligand design, we expect the techniques described here to become established tools in the struggle to find new therapeutics. We look forward to the next round of innovations in NMR that will continue to enable this process.

Keywords: albumin · cytochrome P450 · NMR spectroscopy · proteins · screening

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