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Monitoring the Ligand Binding Mode by Proton NMR Chemical Shift Differences

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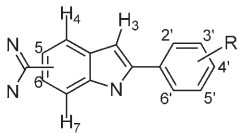
As structure-based drug design involves an iterative process of synthetic chemistry, biological assay, and structural analysis, it is imperative any conformational study not impede rapid progress. To achieve this turnover with NMR spectroscopy, some have used Saturation Transfer Difference (STD)[1] or diffusion epitoping (DE).[2] These techniques provide structural information about ligand orientation within the active site, enabling a more complete understanding of the structure–activity relationship (SAR). These NMR epitope methods work best for fast exchange between the free and bound states but give no information on tight binders. Roberts and co-workers[3] pioneered a novel approach of locating and assigning bound ligand resonances by exchange spectroscopy, a method successfully applied to many other ligand–protein systems.[4–7] To date, the principal use of the bound shift has been to identify intermolecular NOEs to build a 3D complex.[8–11] Here, this work is extended showing that the shift difference itself is valuable in monitoring protein–ligand complexes in the slow exchange regime.

Compounds A–H (Table 1) contain 5-amidino indoles whereas compounds I and J have the amidine at the 6-position.



Amidino-indole scaffold structure

Against bovine trypsin, the IC50s ranged from 1–5 μM depending on the substitution. Application of the CLEANEX[12–13] pulse sequence (see Supporting information), to the trypsin–inhibitor complex generates a spectrum with several free–bound exchange peaks (Figure 1, black circles) in the aromatic region. These cross-peaks are identified by their asymmetric appearance, being narrow on the free side and broad on the bound. The cross-peaks enable a straightforward assignment of the bound shifts from the known free shifts.[3] The CLEANEX experiment removes all of the NOE cross-peaks that are crowded into this region, making the spectra easier to interpret.

Table 1. Substituted indole-amidine inhibitors used in this study.

Compound No.	Amidine position	R group
A	5	H
B	5	4′-Me
C	5	3′-Me
D	5	3′,5′-di-Me
E	5	3′,4′-di-Me
F	5	2′-Me
G	5	2′-CF_3
H	5	2′,6′-di -Me
I	6	2′-Me
J	6	4′ amidino

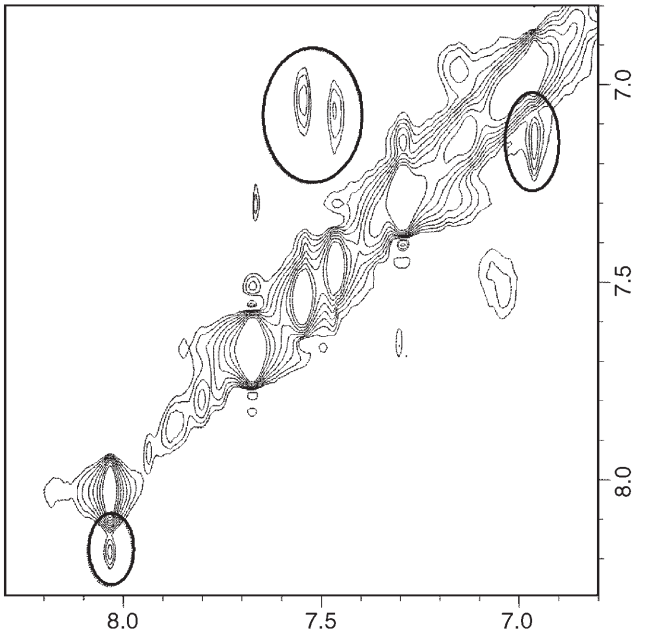


Figure 1. CLEANEX spectrum corresponding to the aromatic region of compound B bound to trypsin. The spectrum was acquired with a mixing time of 100 msec. The exchange peaks of the indole are circled in black. Details are available in the Supporting Information.

A shift map was generated by recording the free–bound differences on the indole 5-amidino protons. Figure 2 depicts as lines the shift differences for the indole protons 3, 6, and 7 on compounds A–H. Proton 4 was not used because in a few compounds no exchange cross peak was observed, presumably due to small shift differences. The resemblance of these patterns to each other suggests a common binding motif for the 5-amidinos. Plotting the differences in this fashion provides a quick way to visualize similarities of binding patterns.

Moving the amidino group from the 5-position (compounds A–H) to the 6-position (compounds I and J) greatly impacts the difference pattern. In Figure 2 the shift differences for the 6-amidino-indoles are shown as filled black symbols. Only protons 3 and 7 are shown as proton 6 is substituted by the amidine group. For proton 3 the shift differences are similar to the 5-amidinos, but for proton 7, the difference has switched from

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Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author: materials and methods, and supporting figures including that of crystallography.

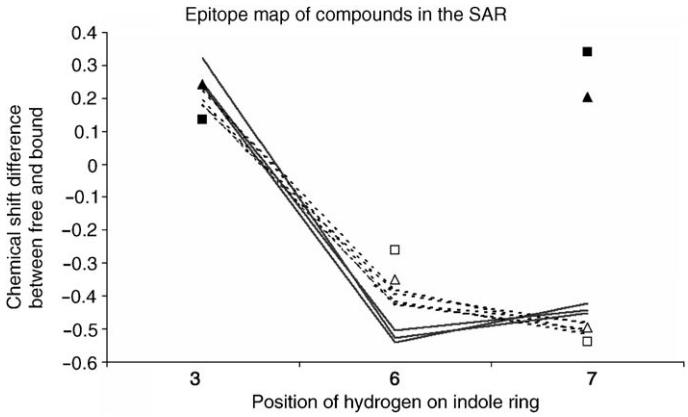

Figure 2. Ligand map plotting the shift differences against the position of the indole hydrogens. The *ortho*-methyl substituted ligands (compounds F–H) are depicted as solid black lines and the *meta*-, *para*-, and unsubstituted ligands (compounds A–E) are depicted as broken black lines. Positions 3, 6, and 7 were utilized to generate the map. The equivalent positions of compound I (triangles) and compound J (squares), representing 6-amidino substituted ligands, are plotted in the indole and amidino binding modes as filled black and unfilled black symbols, respectively.

negative to positive, changing by 0.8 ppm. This large change in shift difference suggests a major change in binding mode for the indole unit.

Figure 3 depicts two possible binding modes for the 6-amidino-indoles when compared to the 5-substitutions. The lower left depicts 6-substituted compounds in the indole binding mode which aligns the indole protons 3 and 7 with the same protons in the 5-series (plotted as filled black symbols in Figure 2). On the right, 6-substituted compounds are rotated by 180 degrees along the indole–phenyl bond into the amidine binding mode which aligns based on that functional group. In this scenario, protons 6 and 7 in the 5-series are co-

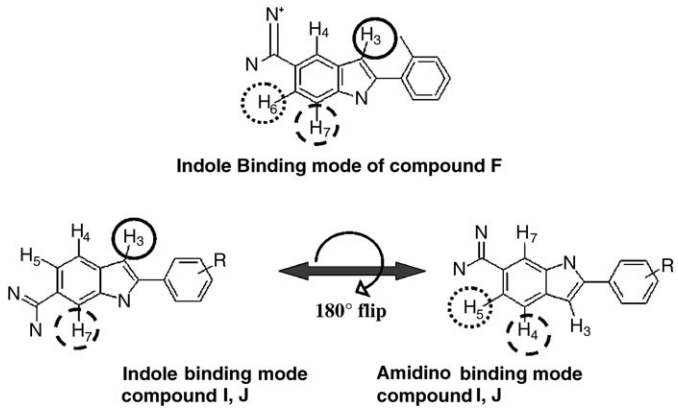

Figure 3. Comparison of equivalent chemical features between compound F in its indole binding mode and compounds I and J in both the indole binding (lower left) and amidino binding (lower right) modes. Protons 3 and 7 (circled in solid and semi-broken black, respectively) in compound F are equivalent to protons 3 and 7 of compounds I and J in the indole binding mode while protons 6 and 7 (circled in broken black and semi-broken black, respectively) in compound F are equivalent to protons 5 and 4 of compounds I and J in the amidino binding mode. The chemical shift differences of the equivalent protons were utilized to ascertain the binding mode.

located with protons 5 and 4 in the 6-series, respectively. The 5–4 shift differences are mapped to positions 6–7 as unfilled black symbols in Figure 2. The shift differences agree more with the amidine mode, suggesting that it is the controlling feature in binding. X-ray crystallography of trypsin complexed with compounds F and I (see Supporting information) confirms the nature of the indole ring, and results in similar placement of the 5–6 and 4–7 protons. Distinguishing the binding modes suggests that the chemical shift differences correlate with the 3D coordinates of the complex.

The shift pattern among the 5-amidino-indoles suggests a common binding motif. However, closer inspection using principal component analysis finds two distinct clusters within the series. *Ortho*-substituted inhibitors (Figure 2 solid black lines) and *meta*- or *para*-substituted inhibitors (Figure 2 dashed black lines) separate into two groups with a confidence of 82% (see Supporting Information). The difference at any single shift is marginal, but combining all three produces this degree of separation.

To determine if this shift clustering was observed in the solid state, co-crystals of trypsin were formed with representatives from each group, compounds C and F. The co-crystal structures (see Supporting Information) demonstrate a high degree of identity, but close inspection of the electron density shows small, but discernable differences in the ligand shape and position in the active site. These changes are likely created by the steric influence of the *ortho*-methyl substituents as opposed to the *meta* or *para*. Thus ligand shift differences demonstrate a sensitivity not only to the large changes in binding mode but to subtle ones as well. The origins of the NMR pattern changes probably result from these small structural changes, though other contributions, such as dynamics, cannot be ruled out. Computing the shifts of the bound and free states could shed light on the origin of the shift patterns and is currently being pursued. The large and small differences in the ligand conformations are illustrated in Figure 4 on the left and right panels respectively.

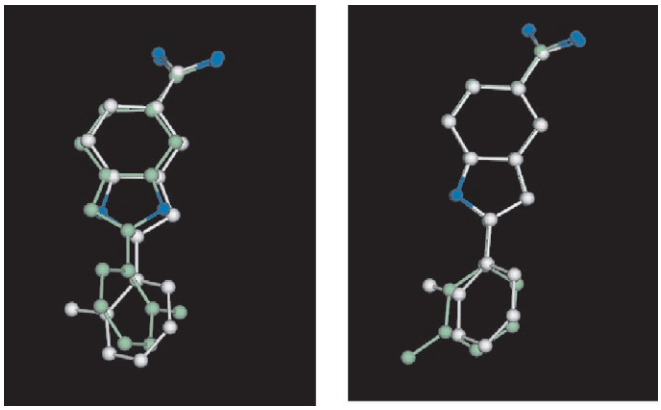

Figure 4. Large and small differences in the binding mode. The left panel illustrates a superposition of compounds F (blue, green) and I (blue, white) in the active site. The nitrogens (blue) of the compounds clearly indicate that the indole ring of the compounds are inverted with respect to each other. The right panel illustrates a superposition of compounds F (blue, green) and C (blue, white). The indoles of the two compounds line up perfectly and the binding modes are only slightly different.

The free–bound shift differences indicate the region of the inhibitor most involved in binding to the protein. The shift differences measured in this study all come from the indole ring. Very few exchange cross-peaks were observed for the phenyl ring protons, and when peaks were observed they were very close to the diagonal. This lack of shift difference between the free and bound state, suggested that the phenyl ring was of less importance to binding. The X-ray structures confirm that the indole has much more protein contact than does the phenyl.

In addition to monitoring the binding mode, there appears to be coordinate information in the shifts. Merz and others[16–17] have shown that the position within the active site can be calculated from the known coordinates. Compared to the solvent, the atoms of the active site have a distinct influence on the ligand protons. This may be the reason a characteristic difference pattern (Figure 2) is seen for molecules occupying the same binding mode but the molecular scaffold need not be identical to see this difference pattern. Clearly H7 of the 5-amidino and H4 of the 6-amidino series have different shifts in the free state due to the difference in their local covalent structure. However, their shift differences between the free and bound shift are similar because of their occupation of the same coordinate space. It is this shift difference which Merz and others[16–20] calculate, and is the basis for comparing shifts and coordinates. While a single proton shift may not be adequate to position an entire molecule in the active site, a more complete shift coverage may be sufficient. This is currently being pursued in the authors' laboratory.

This technique depends directly on the off-rate of the ligand–enzyme complex. If the rate is smaller than the shift difference in the free and bound states (slow exchange regime) and larger than or comparable to the relevant relaxation rate (T1 or T1p), then an exchange peak between the free and bound shifts may occur. If the off-rate is not smaller than the shift differences, then separate resonances will not occur for the two states. The fast exchange regime is adequately covered by the STD experiment. If the off-rate is much slower than the relaxation rate, then no measurable signal will exchange before the magnetization returns to equilibrium. In practical terms, larger shift differences and slower relaxation will give exchange peaks over a wider set of binding constants, than will small shift differences and fast relaxation. The indole-amidines had trypsin IC50 s in the range of 1–5 μM and each showed visible exchange peaks. Some manipulation of the exchange rate might be possible by raising or lowering the experimental temperature. Regardless of whether the binding range is large or small, the exchange experiment covers a range that is difficult for STD and therefore is a complimentary technique.

An advantage in the use of the ligand exchange experiment would be a lack of sensitivity to nonspecific ligand binding. This type of binding often gives saturation transfer and ultimately requires an additional control experiment to determine its presence and extent. Since nonspecific interactions tend to occur in fast exchange, the ligand exchange is not sensitive to these and therefore does not require additional control experiments.

This experiment minimizes costs because it uses unlabeled proteins and inhibitors, and provides reasonable turnover, keeping pace with most SARs. Yet it is sensitive enough to determine when the protein–ligand complex has changed, even subtly. This experiment can impact the ongoing SAR in a timely fashion even without crystallographic support.

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