Pharmacophore Mapping

The INPHARMA Method: Protein-Mediated Interligand NOEs for Pharmacophore Mapping**

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In structure-based drug design, the relative orientation of two competitive ligands A and B in the receptor binding pocket plays a central role in the design of high-affinity drug candidates from weakly bound lead compounds. In this communication we report on the observation of interligand spin-diffusion-mediated transferred-NOE data, which have the potential to be used for the determination of the relative orientation of two competitive ligands in the receptor binding pocket. In many cases, the orientation of one ligand in the binding pocket is known, for example, from crystallography or fluorescence resonance energy transfer (FRET) data. In these cases it is desirable to develop a methodology that allows the determination of a unique binding mode and pharmacophore model for a second competitive ligand. We anticipate that the interligand transferred NOEs described here could provide the experimental basis for a methodology that is applicable to any combination of ligands weakly bound to a common receptor.

The conformation of a ligand in the binding pocket of a macromolecular receptor can be accessed by NMR spectroscopy in solution by two approaches. If the molecular weight of the complex is of the order of 50 kDa or smaller and $^{13}C/^{15}N$-labeled receptor can be produced in vitro, a detailed three-dimensional picture of the ligand and of its interactions with the receptor binding pocket can be obtained with standard NMR spectroscopy methods.[1] However, in other cases the macromolecular receptor is too large for NMR studies in solution or it cannot be synthesized in vitro to introduce NMR-active isotopes. In these instances, NMR spectroscopy

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can be applied to determine the receptor-bound conformation of the ligand, provided the rate constant for the dissociation of the complex (k_{off}) is much larger than the relaxation constants of the ligand in the complex and the difference between the resonance frequencies of the free and bound ligand forms (a fast-exchange regime). Under this condition, which implies a dissociation constant (K_d) value in the low micromolar to millimolar range, information on the ligand structure is derived from transferred NOEs (tr-NOE)[2] and transferred cross-correlated relaxation rates (tr-CCRR).[3,4] The determination of the receptor-bound conformation of the ligand leaves open the question of its orientation in the receptor binding pocket.

The relative orientation of two competitive ligands weakly bound to a common receptor could be determined by the simple and novel approach that we propose here. A NOESY spectrum of a mixture of the two ligands in the presence of the common receptor is recorded. Under the fast-exchange condition described above, and provided that the two ligands A and B bind competitively to the macromolecular receptor T, intermolecular tr-NOE peaks between the two ligands A and B can be observed in the NOESY spectrum. A NOE peak between a proton H_A of ligand A and a proton H_B of ligand B in the presence of the receptor T originates from spin diffusion involving a proton H_T of the receptor. During the NOESY mixing time, ligand A binds to receptor T and H_A transfers its magnetization to H_T (Figure 1). The complex AT

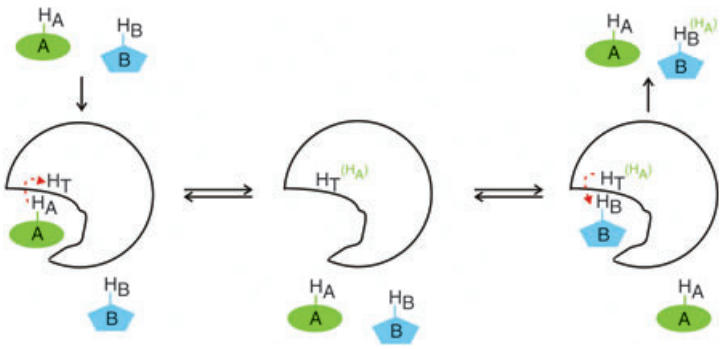


Figure 1. Schematic representation of the principle of the interligand NOEs observed between two competitive ligands A (in green) and B (in blue), which bind consecutively to the same target receptor T. For details see text.

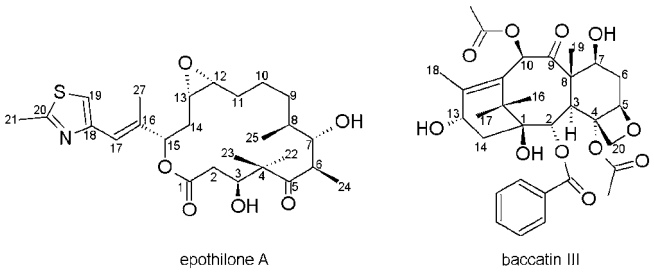
then dissociates and ligand B binds to receptor T. The magnetization of H_A, which had been transferred to H_T, can now be transferred to H_B; this results in an intermolecular peak H_A–H_B. Clearly, this peak can only exist if both H_A and H_B are close to H_T in the AT and BT complexes, respectively. Therefore, a number of such intermolecular NOE peaks will describe the relative orientation of the two ligands in the receptor binding pocket. Such interligand NOEs have never been observed so far and should not be mistaken for the interligand NOEs that occur for two ligands binding simultaneously in adjacent or partially overlapping binding pockets.[5] Here, the two ligands A and B are never either close in space or bound to the protein simultaneously; instead, the two ligands bind to the protein competitively and consecutively and the observed NOEs are a consequence of spin diffusion mediated by the protons of the protein.

If the orientation of ligand A in the binding pocket of receptor T is known, the possible binding modes of ligand B, resulting from docking calculations, could be ranked by evaluating their compatibility with the experimental interligand NOEs. The expected H_A–H_B intermolecular NOEs could be computed for each orientation of ligand B in the BT complex and compared with the experimental ones. The structures that give rise to intermolecular NOEs in good agreement with the experimental ones would be retained, while the others would be rejected. Work along these lines is currently being carried out in our laboratory to develop a computer program for the evaluation of interligand NOEs by using the full-relaxation-matrix approach.[6] A qualitative interpretation of the interligand NOEs, based on the simplified model shown in Figure 1, should not be attempted, as spin diffusion among protein protons can generate intermolecular NOEs between ligands that are in contact with nonneighboring sites of the protein binding pocket. The interligand NOEs should be interpreted exclusively with the help of theoretical calculations by using the full-relaxation-matrix approach. We call this approach INPHARMA (interligand NOE for pharmacophore mapping).

Herein, we show for the first time that such interligand NOEs can be observed. Our system is a mixture of the protein tubulin and two of its ligands. Tubulin is the target of several drugs that interfere either positively or negatively with the tubulin polymerization process. Disturbing microtubule dynamics and, therefore, the cell-division process represents one of the major therapeutic strategies against human solid tumors. Among the small organic molecules that promote tubulin polymerization, paclitaxel is routinely used in hospitals to treat mammalian and ovarian carcinomas. Epothilone and discodermolide,[7] which compete with paclitaxel for the same binding pocket,[8] are more potent than paclitaxel in the promotion of tubulin polymerization and will most likely enter the pharmaceutical market in the next decade. Two low-resolution structures of Zn-induced tubulin sheets stabilized by paclitaxel and epothilone, respectively, have become available by electron crystallography.[9,10]

Recently, thanks to a combination of tr-NOE and tr-CCRR data, we determined the structure of epothilone A (Epo A) weakly bound to soluble tubulin.[11] The tubulin-bound structure of Epo A determined by NMR spectroscopy[11] is not in agreement with the tubulin-bound conformation of Epo A determined by electron crystallography,[10] a fact suggesting that the binding mode of epothilone to tubulin can be dependent on the protein state. Therefore, it is relevant to investigate the binding mode of Epo A to soluble tubulin, namely in conditions that are as close as possible to the physiological ones.

We applied the INPHARMA method to a mixture of epothilone A and baccatin III[12] in the presence of tubulin. The complex Epo A–tubulin is only transiently formed during the mixing time of a NOESY experiment and tr-NOEs can be observed for a 100:1 mixture of Epo A and tubulin, thereby



making the system suitable for application of the INPHARMA method (epothilone A = ligand B; tubulin = target T). A model of the complex of tubulin with paclitaxel is available by electron crystallography. Unfortunately, paclitaxel is poorly soluble in water and therefore not suitable for NMR studies in solution. However, the precursor of paclitaxel, baccatin III, which lacks the C13 side chain, is water soluble, binds to tubulin in a transiently formed complex, and delivers tr-NOEs. Thus, although the biological activity of baccatin III is lower than that of paclitaxel, we chose baccatin III as ligand A for our experiments. To confirm that baccatin binds in the same pocket as paclitaxel, namely, that it competes with epothilone for the taxane-binding pocket, we evaluated the change in the tr-NOE rates of baccatin in the presence of tubulin upon addition of epothilone. For a concentration ratio of epothilone A/baccatin of 0.67:1, the tr-NOE rates of baccatin decrease to 60 % of their original value, a result confirming that the two ligands compete for the same binding pocket with a similar K_d value.

We recorded NOESY experiments at different mixing times (20, 40, 70, 100, and 200 ms) for a mixture of epothilone A (0.6 mM), baccatin III (0.6 mM), and tubulin (12 μM) in D_2O. Intermolecular NOE cross-peaks between the aromatic protons of baccatin III and several methyl groups of Epo A are clearly observable in the section of the NOESY spectrum recorded at a mixing time of 70 ms and shown in Figure 2.

19 intermolecular NOE peaks can already be observed in the NOESY spectrum with a mixing time of 40 ms. The build-up curves of the intensities of six of these NOE peaks (Figure 3) show the characteristic damped-parabolic shape for spin-diffusion-mediated NOEs. Additional intermolecular NOEs become visible after 100 ms or at higher mixing times. As a control, we recorded a NOESY spectrum of the mixture of Epo A and baccatin III in the absence of tubulin with a mixing time of 400 ms; this spectrum showed no intermolecular NOEs. Thus, we can conclude that the intermolecular NOEs between Epo A and baccatin III, observed in presence of tubulin, are mediated by protons of tubulin. We are in the process of developing a full-relaxation-matrix analysis of the observed interligand NOEs to determine the binding mode of epothilone to soluble tubulin, with the assumption that baccatin III binds tubulin with the same pharmacophore as paclitaxel.

Herein, we have reported the first observation of protein-mediated NOEs between two ligands that bind competitively and consecutively to the same target molecule. The inter-

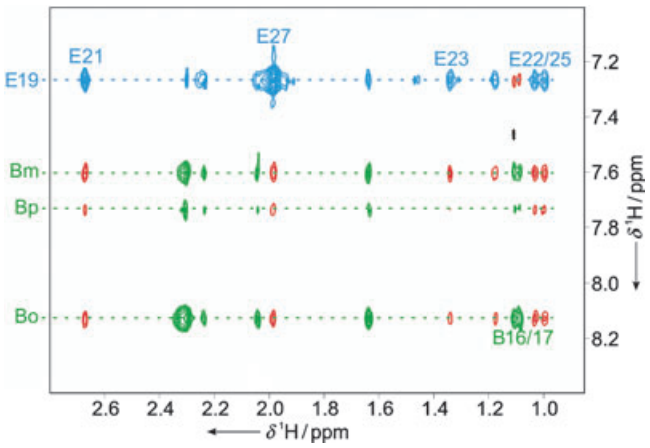


Figure 2. NOESY spectrum of a mixture of tubulin, epothilone A, and baccatin III with concentrations of 12 μM, 0.6 mM, and 0.6 mM, respectively. The spectrum was acquired on a 900-MHz spectrometer equipped with a cryoprobe with a mixing time of 70 ms. The blue and green peaks are intramolecular transferred NOE peaks of epothilone A and baccatin III, respectively. The red peaks represent the interligand transferred NOEs mediated by the protein protons. The numbering of the atoms corresponds to that shown in the compound structures for epothilone A (E) and baccatin III (B). Bm, Bo, and Bp indicate the protons in the *meta*, *ortho*, and *para* positions of the benzene ring of baccatin III, respectively.

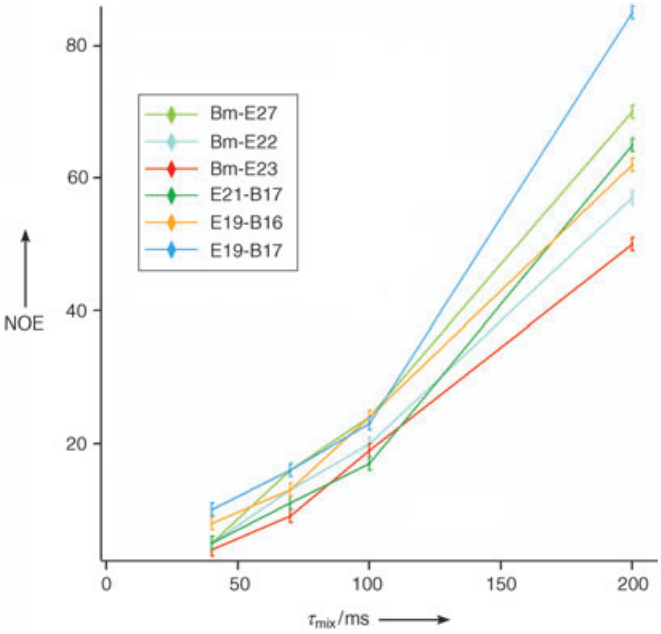


Figure 3. Build-up curves of 6 out of the 19 most intense interligand tr-NOE cross-peaks. The typical damped-parabolic behavior can be observed for all peaks, thereby confirming that the interligand NOEs arise from spin diffusion mediated by the protein protons.

ligand NOEs observed by us should not be confused with the interligand NOEs already reported in the literature,[13] where the two ligands bind simultaneously to the target. Our INPHARMA methodology could be used to distinguish among different binding modes of a ligand to a macro-

molecular target, if the binding mode of a second competitive ligand is known. This novel approach should be particularly attractive for the pharmaceutical industry, where there is a strong need for the rapid determination of the binding epitope of different ligands to a common target.

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