Drug Design

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Drug Design for G-Protein-Coupled Receptors by a Ligand-Based NMR Method**

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More than 50% of all drug targets are membrane proteins.[1] Recent progress in membrane protein crystallography has made a few of these targets amenable to established structure-based design methods.[2] However, crystallization of a membrane protein target remains a challenge, and medicinal chemists must rely on ligand-based design approaches for the targets that do not crystallize. These approaches can benefit from the knowledge of the bioactive conformation of the ligand conformation and the relative orientation of different chemotypes in the receptor binding site (cross-chemotype alignments). The generation of hypotheses for the bioactive conformation and cross-chemotype alignments depend on the availability of sufficient data on ligand structure–activity relationships (SARs). Herein, we demonstrate that these insights can be derived in the absence of protein–ligand crystal structures by straightforward ligand-based approaches relying on NMR spectroscopy. We show that a qualitative and fast analysis of INPHARMA NMR data[3,4] can be used within the timelines of the drug-discovery process without extended modeling and detailed data interpretation. We performed this study using intact biological membranes rather than reconstituted proteins[5,6] to eliminate solubilization-related artefacts, to expand the applicability to receptors that do not tolerate solubilization, and to significantly enhance the speed of the method. Here we demonstrate that a straightforward ligand-based NMR approach can be used to establish a nonradioactive binding assay for a G-protein-coupled receptor (GPCR) and to give access to the relative orientation of multiple chemotypes supporting ligand-based drug design.

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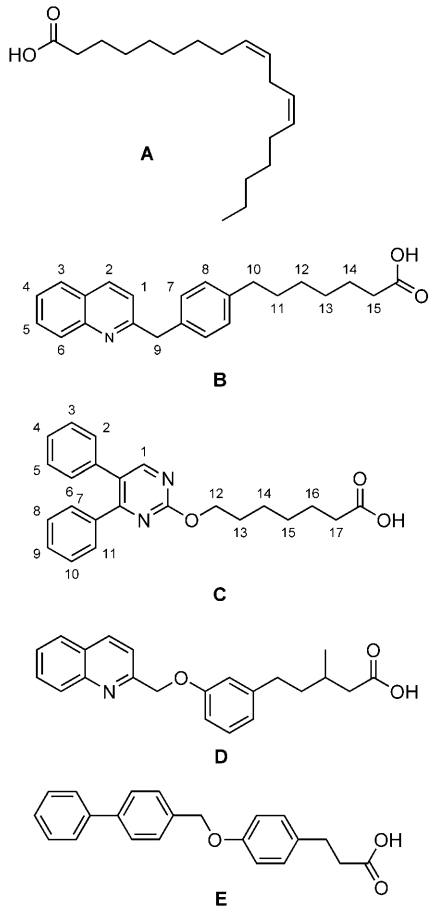
GPR40 is a fatty acid binding receptor from class A of the superfamily of G-protein-coupled receptors and is preferentially expressed on pancreatic β-cells.[7] Long-chain free fatty acids (FFAs) increase glucose-stimulated insulin secretion from these cells by activating GPR40.[8] Reduction of GPR40 expression by siRNA in the murine insulinoma cell line MIN6 led to significant inhibition of FFA-induced insulin secretion.[9] The possible role of GPR40 in insulin secretion has also been studied using GPR40-KO mice (GPR40−/−). These mice have impaired acute insulin secretion after treatment with FFA.[9] All these results underline the important role of GPR40 and its contribution to glucose-stimulated insulin secretion.

GPR40-containing membranes were obtained from baculovirus infected High Five insect cells or mammalian CHO cells overexpressing human GPR40. Cells were lysed by high pressure. The crude lysate was centrifuged ($300 \times g$, 10 min, 4 °C) to remove larger cell debris. Subsequently, the membranes were sedimented by centrifugation ($40\,000 \times g$ to $100\,000 \times g$, 60 min, 4 °C), resuspended twice, and stored at −80 °C.

In a first step we established a saturation-transfer difference NMR (STD NMR) binding assay to detect the specific and concentration-dependent binding of ligands to the GPR40-containing membranes.[5,6] In this control experiment binding specificity is defined by the ability of a given ligand to be replaced by linoleic acid, a natural ligand of GPR40.[9] The study was performed with linoleic acid and several GPR40 agonists obtained from the high-throughput screening of a chemical library (Scheme 1).

All ligands exhibit clear STD NMR spectra in the presence of GPR40-containing membranes (Figure 1 and Figures S1 and S2 in the Supporting Information). Addition of linoleic acid resulted in suppression of the STD signals for all heterocycles and is shown as an example for ligands **B** (Figure 1) and **C** (Figure S1 in the Supporting Information). As a control we measured the binding of ligands **B**–**E** in the presence of membrane extracts of the parental cell line without human GPR40. We found either no binding or no specific binding by means of competition with linoleic acid (Figure S3 in the Supporting Information).

Together, these findings indicate a specific binding event between compounds **B**–**E** and the GPR40 receptor. Through quantitative analysis of STD experiments that were performed with increasing concentration of linoleic acid we could determine the binding constants for ligands **B**–**E** (Table 1, Figure S4 in the Supporting Information). We found a good correlation between the biological potency expressed in EC_{50} values from a functional FLIPR (fluorescent imaging plate



Scheme 1. Agonists of GPR40. The natural ligand, linoleic acid (**A**), and ligands **B**–**E**, which were identified by high-throughput screening.

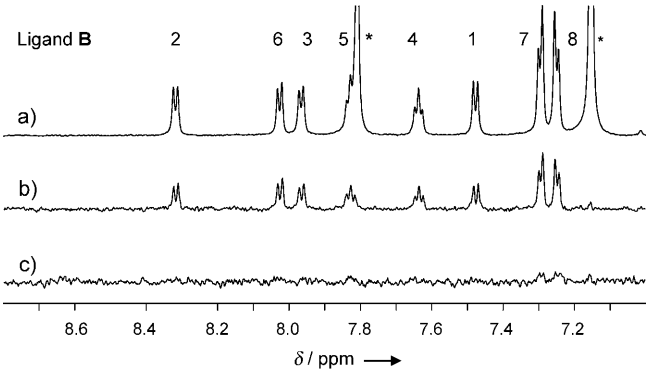


Figure 1. Competition studies by STD NMR analysis for ligand **B**. a) ^{1}H NMR spectrum of 100 μM ligand **B** (128 scans). b) STD spectrum of 100 μM ligand (256 scans) in the presence of GPR40 membranes, and c) after addition of 580 μM linoleic acid to sample in (b). In (c) the STD signal of ligand **B** (256 scans) is suppressed. NMR data were collected at 700 MHz with a cryoprobe (256 scans; 310 K) using buffered solutions with 10% (v/v) crude GPR40 membrane extract, 10 mM perdeuterated tris(hydroxymethyl)aminomethane(Tris)-DCl (pH 7.5), 0.3 mM perdeuterated ethylenediaminetetraacetic acid (EDTA), and 0.01 mM trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSPA) in D_2O. Peaks are assigned according to the position numbering of ligand **B** in Scheme 1.

Table 1: EC_{50} and K_D values for several GPR40 agonists (see Scheme 1).[a]

Compound	EC_{50}	K_D
B	0.34 μM	0.34 μM
C	0.28 μM	0.16 μM
D	1.71 μM	0.57 μM
E	0.13 μM	0.10 μM

[a] EC_{50} values were measured in a functional FLIPR assay.[10] K_D values were determined by STD NMR competition studies with linoleic acid (using $EC_{50} = 2$ μM for linoleic acid).[9]

reader) assay and the K_D values determined by STD NMR experiments. This part of the study demonstrates that STD NMR spectroscopy can be used in a straightforward binding assay that has the potential to replace radioactive binding assays commonly applied in GPCR drug discovery.

Having demonstrated the specificity and dose dependency of the binding events, we set out to investigate whether we could derive data on the bioactive conformation and the relative orientation of two ligands in the binding site of GPR40 using transfer-NOE and interligand NOE methods (INPHARMA).[3,4,11] Ligands **B** and **C** were chosen for these studies as both had been chosen previously for chemical lead optimization.

For the NOE studies, ligand concentrations were adjusted according to the relative affinities of the ligands (200 μM ligand **B** and 100 μM ligand **C**). The concentration of membrane extracts was increased from 10% to 25% (v/v), resulting in a higher fraction of bound ligands, as confirmed by the increased linewidth in 1D spectra. Spectra were recorded with mixing times ranging from 100 to 600 ms (Figure 2). At a mixing time of 100 ms only intraligand NOE interactions are observed. The lack of long-range intraligand NOE interactions is indicative for a stretched conformation of both ligand **B** and ligand **C**. At a mixing time of 300 ms, spin-diffusion-mediated interligand NOE interactions (INPHARMA NOE interactions)[3,4] are observed between protons of ligand **B** and protons of ligand **C** (green boxed signals in green boxes in Figure 2). These INPHARMA NOE interactions are visible if both ligands bind weakly and competitively to the same binding pocket.[3] The interligand NOE interactions decay at higher mixing times and are barely observable at a mixing time of 600 ms.

Mapping of the interligand NOE interactions onto the structures of ligands **B** and **C** suggests that protons 3, 4, and 6 of ligand **B** and protons 3–5 of ligand **C** reside at a similar site in the binding pocket (Figure 2). In addition, protons 7 and 8 of ligand **B** are positioned at a similar site as the protons 12–14 on the alkyl side chain of ligand **C**.

A cross-chemotype pharmacophore model has been derived from diverse GPR40 series (using HipHop module in the program Catalyst, data not shown) and used for the alignment of compounds **B** and **C**. Four different alignment hypotheses were drawn (Figure 3), but the available structure–affinity relationship did not make it possible to distinguish between the four. The experimentally derived interligand NOE interactions, however, are in agreement with only one of four computed alignment hypotheses (hypothesis 2)

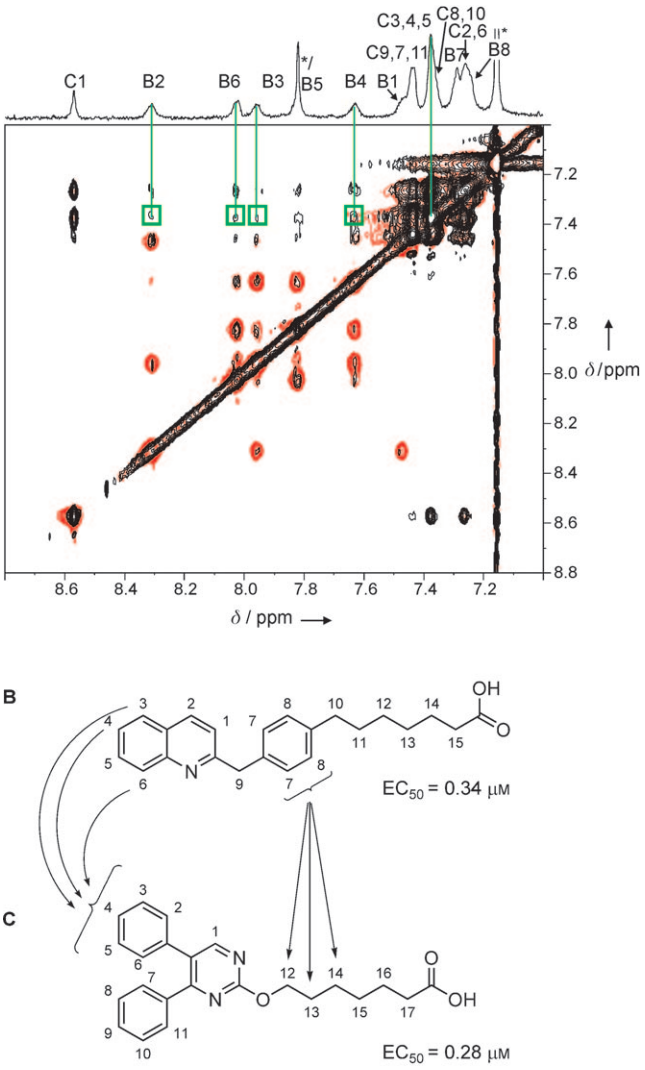


Figure 2. Top: Aromatic region of an $^{1}H,^{1}H$ NOESY spectra of a mixture of ligands **B** (200 μM) and **C** (100 μM) in the presence of 25% GPR40 crude membrane extract, using mixing times of 100 ms (red) and 300 ms (black). The signals are assigned in the 1D spectrum; signals from the crude membrane extract and buffer are marked with asterisks. Signals in green boxes indicate the interligand NOE interactions observed in the aromatic region. (Weak signals right below the signals in green boxes are intraligand NOE interactions within ligand **B**, as proven by NOE measurements in the presence of ligand **B** only.) NMR data were collected at 700 MHz with cryo-probe (128 scans; 310 K), using buffered solutions with 25% (v/v) crude GPR40 membrane extract, 10 mM perdeuterated Tris-DCl (pH 7.5), 0.3 mM perdeuterated EDTA, and 0.01 mM TSPA in D_2O. Bottom: Mapping of interligand NOE interactions onto the chemical structures of ligands **B** and **C**.

and in disagreement with the other alignment possibilities (hypotheses 1, 3, and 4, Figure 3), thereby validating hypothesis 2.

The experimentally supported superposition of the quinoline group of ligand **B** with the phenyl group II of ligand **C** (hypothesis 2, Figure 3; see labeling in Figure 4) indicates an essential pharmacophoric feature in the region around this phenyl group (ligand **C**). To test the validity of this hypothesis

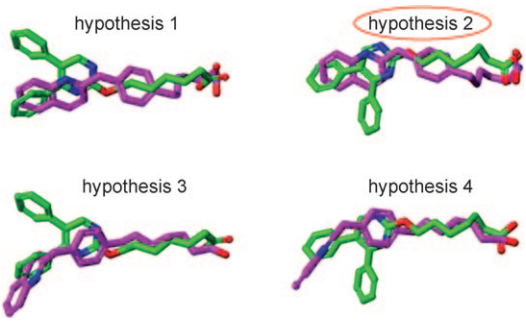


Figure 3. Pharmacophore-based alignment hypotheses for ligands **B** (magenta) and **C** (green). Both compounds are flexibly aligned on a cross-chemotype pharmacophore model derived from diverse GPR40 series (using HipHop module in the program Catalyst, data not shown). Each compound can be mapped on the pharmacophore in two different ways, resulting in four different alignment hypotheses: hypotheses 1 to 4. The NMR data are consistent with hypothesis 2 only.

a set of related ligands were synthesized[12] and tested in the functional GPR40 FLIPR assay (Figure 4). In line with the NMR data, elimination of phenyl ring II (ligand pair **I**, **J**) results in a functionally inactive compound, whereas elimination of phenyl ring III (ligand pairs **C**, **F** and **G**, **H**) retains activity in the low micromolar range.

In summary, we have demonstrated that ligand-based NMR data on membrane proteins can be used to devise a nonradioactive binding assay and to determined NOE-based structural constraints on both the ligand bioactive conformation and relative orientation of different chemotypes in the receptor binding site. Medicinal and computational chemists can apply these constraints as the experimental basis to derive ligand-based models and design hypotheses on membrane proteins for which no crystal structure is available. The prerequisite for both the STD and INPHARMA methodologies is a fast k_{off} rate of the ligands and the absence of unspecific interactions with the membrane. For INPHARMA, ligands with similar affinites to the receptor provide optimal signals. The method is fast and relies on the availability of only small quantities of crude membrane extracts containing the receptor in its native surrounding. When a model structure of the receptor is known, atomic structural models of the ligand–receptor complexes can be obtained using a combination of transfer-NOE data, which define the bioactive conformation of the ligands, and INPHARMA data, which define the relative orientation of pairs of ligands in the receptor binding pocket.[3,4] This procedure requires a large computational effort and a model for the structure of the apo-protein. However, if the data analysis is not intended to determine a structure of the ligand–receptor complexes but rather at discriminating between two different pharmacophore models, the INPHARMA data can be interpreted successfully in a qualitative manner, as demonstrated here. In this case, the method is fast and does not rely on the availability of any structural model for the receptor. This makes our approach a very valuable tool for the investigation of membrane protein–ligand interactions,

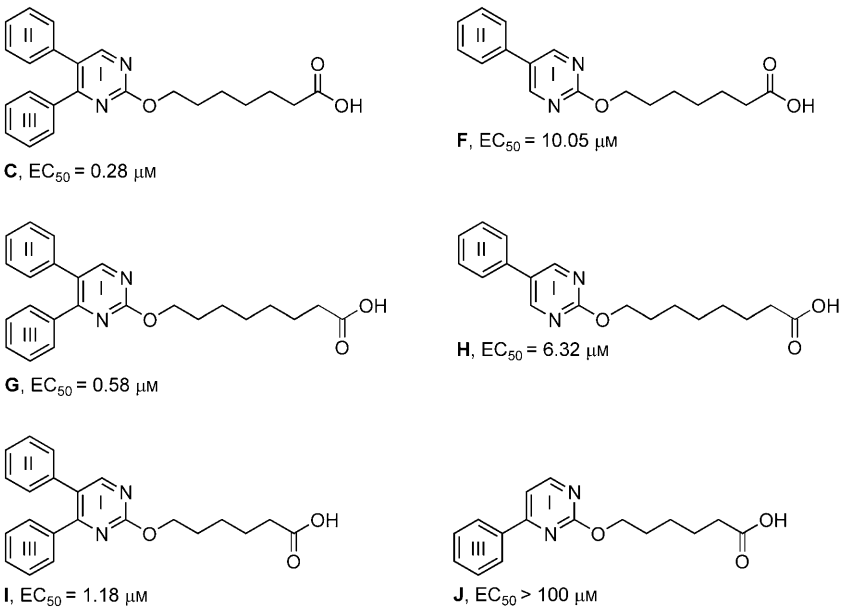


Figure 4. Structure–activity relationship for some derivatives of ligand **C**.

where a structure of the receptor is in most cases not available.

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