

Small molecule HTS hit validation with biophysical / label-free SPR binding assays in lead finding for an orphan nuclear receptor

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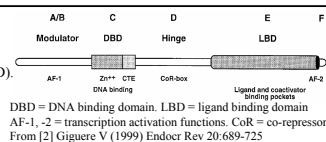
Abstract

Hormone nuclear receptors (NR) comprise a large ligand-inducible transcription factors family. They are involved in nearly all aspects of development and adult physiology by regulating complex gene programs in response to hormones like steroid and thyroid hormones, retinoic acid, and vitamin D [1,2]. A large number of the NR family also includes receptors with no identified ligand and therefore are denominated orphan receptors. These orphan receptors not only provide opportunities to characterize novel signal transduction pathways but also may be new potential drug targets.

A biochemical co-regulator recruitment screening approach was launched to identify agonists and antagonists of an orphan NR. In order to distinguish between real and false actives, to further increase the value of the hit compounds from the screening, and to further characterize those agonists and antagonists, label-free binding assays were applied. Surface plasmon resonance (SPR), thermal stability and mass spectrometry (MS) were applied to this NR target and are described in this poster.

Introduction and Approach

NR have several highly conserved domains known to be important for transcriptional regulation such as a DNA-binding domain (DBD) containing two zinc fingers which bind to DNA sequences called hormone response elements (HRE), and a ligand-binding domain (LBD). The LBD is critical for transcriptional regulation by providing the interaction surface between receptors and co-activator or co-repressor complexes. A transactivation function 2 (AF2) domain is also found at the LBD of this orphan NR.



In response to ligand binding, nuclear receptors undergo conformational changes leading to recruitment of transcriptional co-activators and resulting in transcriptional activation of target genes. The most important domain for drug interaction is the LBD. Therefore we used the LBD for the HTS as well as for all label-free biophysics experiments.

Through a "PamGene" chip approach 15 out of 48 co-regulator peptides tested onto this orphan NR were found to bind to the target. A suitable co-regulator peptide was selected to develop the biochemical HTS assay measuring the interaction between the NR and co-regulator peptide (TR-FRET; donor 340/620 nm, acceptor 620/670 nm). Agonists and antagonists can be identified and distinguished. Over 1.2 million compounds were screened in a 1536 well assay format. Following confirmation testing and hit list triaging, 3192 hits were validated as antagonists with $IC_{50} < 30 \mu M$ and 179 of them with nanomolar potency.

Many antagonists were found, but how about the hit binding mechanism knowing that this NR is prone to unfolding? To answer this question the label-free approach was a logical approach to get ride of artifacts inherent to HTS and the nature of the NR target. The objective of this label-free process following the HTS campaign is not only the hit confirmation of the biochemical screen to distinguish real binders (SPR) or structure stabilizer (thermal stabilization) from non-binders, but also to elucidate the mechanistic action of the compounds using competitive MS binding.

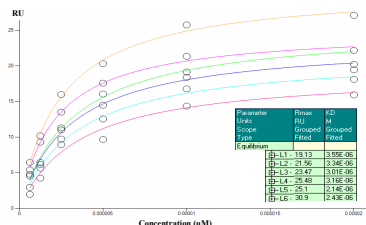
Biophysical / label-free binding assays results

Surface Plasmon Resonance: SPR

The BioRad ProteOn XPR36 SPR technology [3] was used to characterize compound binding events on the target protein. Several experiments and modes of immobilization were performed:

1. chemical non-directed NR protein immobilization (direct compound binding assay)
2. directed NR protein immobilization with an "avi-tag biotinylated" protein (direct compound binding assay)
3. co-regulator peptide immobilization. (indirect competition assay)

Only approach 2 was successful, binding of the co-regulator peptide on the protein has shown some binding, but not at the expected level (10 % of calculated theoretical binding). An apparent K_D of $\approx 3 \mu M$ for this peptide could be determined at equilibrium pointing to only partially active protein on the sensor chip. The true K_D is in the range of 40-100nM.



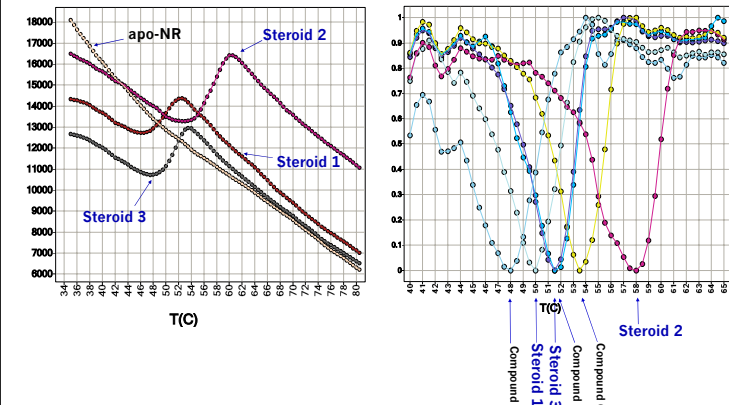
The SPR assay does not work very well in this case, but this NR is a difficult target protein and was found to be extremely environmental sensitive.

Protein stability assay

The protein stabilization assays measures binding affinity by detecting ligand-dependent changes in the thermal folding of a target protein.

The thermal protein stability assay is an opportunistic "quick-win" assay for compounds and a positive shift of protein unfolding temperature is used as sensor of compound binding to target.

The figure below shows the temperature shift of different steroid compounds tested compared to the apo-NR:



Until now ca 200 compounds were tested and validated in this system but work is in progress.

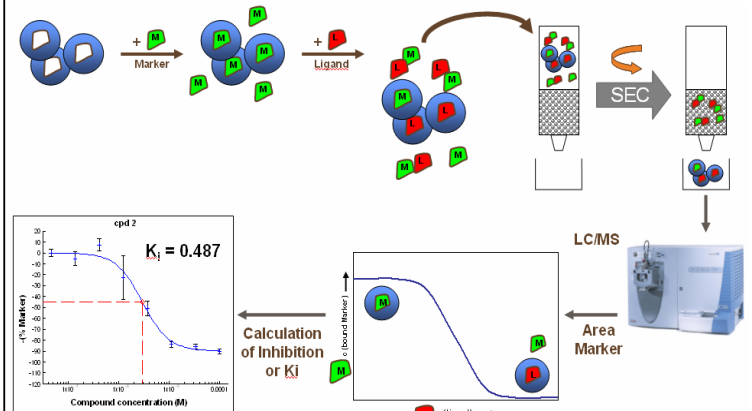
Mechanistic competitive affinity MS binding

The competitive assay principle is based on the displacement of a marker/reporter (ligand which addresses the binding site of interest) by a test compound.

MS allows direct detection of unlabelled components even in complex reaction mixtures. The proof of "active-site" binding for hit compounds can be confirmed and quantified through this competitive MS binding assay.

After incubation of the protein/marker/compound mixture the marker or compound bound to the target is separated from unbound with a fast size exclusion chromatography (SEC). The eluate is injected directly into a reversed phase HPLC column and the amount of marker is determined in a linear ion trap MS.

IC_{50} 's are calculated from the binding curve based on the bound marker and can be converted into K_i values reliably.



In the case study of the orphan NR the setup is to detect the amount of co-regulator and reporter-agonist with MS. 3 types of "hits" can occur: **Type-1 hits** (non-productive: no agonist and co-regulator found); **Type-2 hits** (co-regulator competitive: agonist found, co-regulator not); **Type-3 hits** (agonist competitive: agonist not found, some co-regulator present).

| Antagonist (15µM) | TR-FRET IC_{50} HTS (µM) | MS Area co-regulator peptide (%) | MS Area Agonist (%) | ratio peptide / agonist |
|-------------------|----------------------------|----------------------------------|---------------------|-------------------------|
| "Active Ctrl" | none (no Protein) | 0.3 | 0.7 | 1.0 |
| "Neutral Ctrl" | none | 100.0 | 100.0 | 1.0 |
| Compound 1 | 0.1 | 43.7 | 9.6 | 4.6 |
| Compound 2 | 0.2 | 76.8 | 86.4 | 0.9 |
| Compound 3 | 1.0 | 51.3 | 33.7 | 1.5 |
| Compound 4 | 2.1 | 65.2 | 17.5 | 3.7 |
| Compound 5 | 2.2 | 73.6 | 35.9 | 2.1 |

The table above shows first results obtained with this mechanistic MS approach. Up to now more than 70 compounds have been measured but we plan to characterize the whole hit list.

Results flowchart summary



Conclusion

Key biophysics findings with this difficult orphan NR target:

- SPR can not be easily applied universally with all proteins especially not with this NR
- The protein stability assay and the competitive MS assay were established recently and first data are encouraging. Measurement of more compounds is ongoing
- Through this target approach a X-Ray structure of this orphan NR could be obtained with one of the compound discovered in this process
- X-ray structure of agonist/NR complexes could be established already

References

1. Mangelsdorf DJ, & al. (1995) The nuclear receptor superfamily: the second decade. Cell; 83: 835-839.
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 3. Posier at SBS Dresden 2008: [Esser O & al. (2008)] Microtiter-plate based screening for direct binding of small molecules on target proteins. A comparison of the EPIC technology with the ProteOn XPR36 SPR technology for fragment based screening and hit characterization. NIBR.