

# ExSAR<sup>TM</sup>

## **LIGAND BINDING SITE AND ACTIVITY CORRELATIONS BY HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY**

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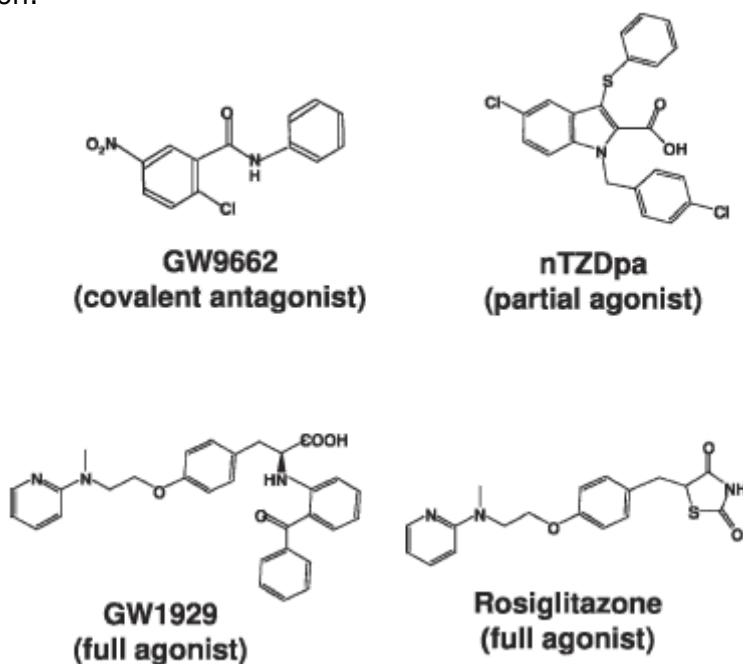
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## OVERVIEW

Amide hydrogen/deuterium exchange, coupled with proteolysis and liquid chromatography-mass spectrometry (H/D-Ex), is gaining widespread use for the analysis of protein structure dynamics<sup>1-3</sup>, protein-protein interactions<sup>4,5</sup>, and protein-ligand interactions<sup>1,6-8</sup>.

In this example the ligand binding domain of a nuclear receptor (NR), peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), was exposed to various ligands and analyzed by H/D-Ex. NRs are a superfamily of transcriptional regulators that modulate biological processes as diverse as cellular differentiation and metabolism. Their flexibility in regard to gene regulation has been attributed to their conformational flexibility. Many studies suggest that different types of ligands induce changes in conformation and dynamics to the ligand binding domain (LBD) of PPAR $\gamma$ . These changes consequently alter the expression profile of certain genes relating to lipid and glucose homeostasis. In fact, PPAR $\gamma$ 's role in lipid and glucose homeostasis has made it a common target of strategies seeking to restore insulin sensitivity in patients with type II diabetes.

Herein is described how H/D-Ex was used to detect differences in the protein dynamics of apo and drug-bound PPAR $\gamma$ -LBD. Three different classes of drugs were surveyed: agonists, partial agonists and antagonists (see Figure 1). The value of this technology is that H/D-Ex can be used to bin newly identified ligands (or drug-leads) into groups that elicit similar conformation or dynamic responses upon complex formation.



**Figure 1.** Structures of PPAR-gamma ligands (drugs).

## I. Dynamics/Conformational Analysis by H/D-Ex

The protein dynamics of free and ligand bound N-terminal HexaHIS tagged PPAR $\gamma$ -LBD was probed by ExSAR's H/D-Ex technology<sup>9</sup>. A schema of the technique is illustrated in section II.

### Experimental Results:

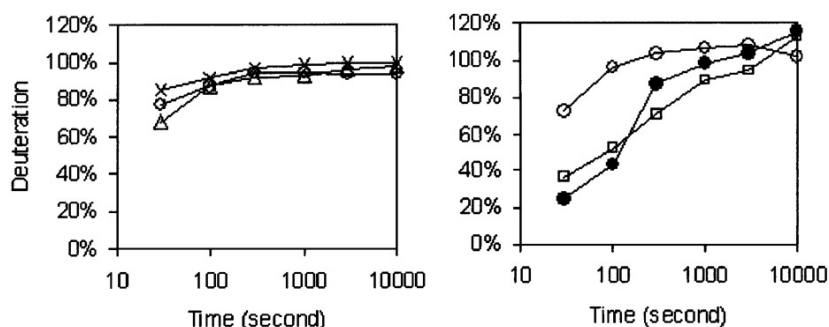
97% of the PPAR $\gamma$  amino acid sequence correlated to one of 23 pepsin derived peptides of an average length of 12 residues. Illustrated in Figure 2 are two graphs depicting deuterium buildup of one representative PPAR $\gamma$ -LBD pepsin derived peptide (+/- ligands). The experimental time frame consisted of six time points spanning 30 to 10,000 seconds. Graphs were also generated for the remaining 22 peptides.

Deuterium content was determined by employing the following equation,

$$\text{Deuteration level(\%)} = \frac{m(\text{P}) - m(\text{N})}{m(\text{F}) - m(\text{N})} \times 100$$

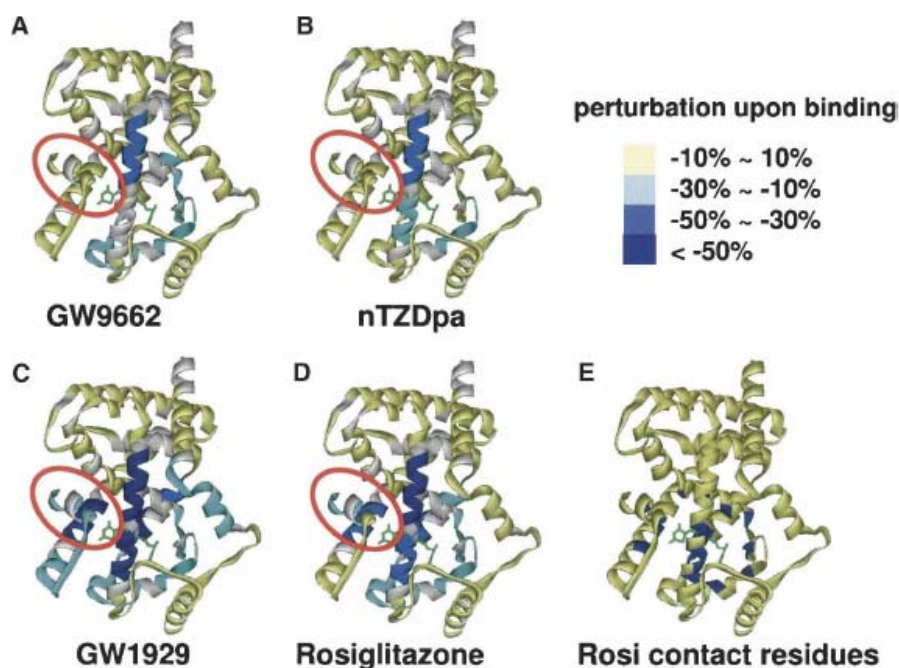
where m(P), m(N) and m(F) correspond to the LC/MS-ESI detected centroid mass values of the partially deuterated, non-deuterated, and fully deuterated peptides respectively.

The average difference in deuteration of apo versus ligand bound PPAR $\gamma$ -LBD was calculated for each peptide. For example in the presence of GW1929, PPAR $\gamma$ -LBD sequence segment 472-477 was 22% less deuterated than apo PPAR $\gamma$ -LBD. This difference in deuteration is referred to as a perturbation. Negative perturbations are indicative of stabilization, positive perturbations of greater mobility or motion.



**Figure 2.** Deuterium content over time of PPAR $\gamma$ -LBD amino acid segment 472-477 in the absence or presence of various ligands: ligand-free (o), GW9662 (x), nTZDpa ( $\Delta$ ), GW1929 ( $\square$ ), and rosiglitazone ( $\bullet$ ).

To better conceptualize their significance, perturbations were color coded by magnitude and mapped to the crystallographic structure of PPAR $\gamma$ -LBD illustrated in Figures 3.



**Figure 3.** Average difference in deuteration levels (perturbations) of PPAR $\gamma$ -LBD with or without ligands overlaid onto a three-dimensional crystallographic structure of PPAR $\gamma$ -LBD with rosiglitazone (PDB identifier 2PRG). Blue indicates that the segment exchanges slower in the presence of ligand. Yellow indicates that the segment exchanges at about the same rate with or without ligand. Rosiglitazone is illustrated in light green. Gray regions are not covered in the current experimental set. Within the circled region are two helices, the shorter of the two corresponding to helix 12 and the other helix 11.

The rate of amide hydrogen exchange is dependent upon local fluctuations in protein structure<sup>10</sup>. For this reason the rate of H/D-Ex is a good indicator of regional flexibility within a protein. As illustrated in Figure 3, ligand binding in all cases reduced the exchange rate hence stabilized structural regions closely corresponding to the crystallographically determined rosiglitazone binding site (Figure 3E). Since ligand binding typically involves the formation of an intermolecular network involving hydrogen bonds and/or electrostatic interactions with the protein, complex formation is accompanied by stabilization. Protein stabilization is readily detectable by H/D-Ex.

Of particular interest to this study is the variability in the location and magnitude of stabilization. Note that these experiments were carried out with a 20-fold molar excess of high-affinity ligand. We can therefore assume that PPAR $\gamma$ -LBD was in a nearly 100% ligand bound state. Perturbation differences accordingly do not stem from differences in binding equilibrium.

In general, full agonists GW1929 and rosiglitazone (Figures 3C-D) stabilized PPAR $\gamma$ -LBD to a greater extent than antagonist GW9662, and partial agonist nTZDpa (Figures 3A-B). The exchange of amide hydrogens in Helix 11 and 12 was largely impeded upon binding either agonist; a negative perturbation in excess of 50% was observed in helix 11 and ~20% negative perturbation in helix 12. Both of these helices are highly dynamic in apo PPAR $\gamma$ -LBD. Upon binding antagonist or partial agonist helices 11 and 12 appear to be as dynamic as in the unbound form. The stabilization of helix 12 in the presence of agonist supports earlier studies showing that the maintenance of the helical conformation of this helix is critical for gene activation<sup>11</sup>. **These results accordingly demonstrate the ability to predict by H/D-Ex, the activities of various drugs by probing changes in conformation and dynamics upon drug binding.**

## II. Hydrogen/Deuterium Exchange Mass Spectrometry (H/D-Ex)

ExSAR's platform technology, Hydrogen/Deuterium Exchange Mass Spectrometry (H/D-Ex), can be used to probe the conformational dynamics of a protein's 3D structure in solution (Figure 7).

In brief, protein is mixed with deuterated buffer and incubated for a predetermined duration during which backbone amide hydrogens gradually exchange with bulk solvent deuterons. The exchange rate of each backbone amide hydrogen is unique to its environment; disordered regions and/or surface exposed regions exchange fast, ordered and/or buried regions exchange slow. Following the incubation period, the exchange reaction is essentially quenched by shifting the pH to around 2 while lowering the temperature to near 0°C. The exchanged protein is then proteolyzed with acid stable proteases. The peptic fragments are then chromatographically separated and their masses determined by mass spectrometry. The experiment is repeated in the absence of deuterium and the molecular weight difference of identical fragments attributed to deuteration.

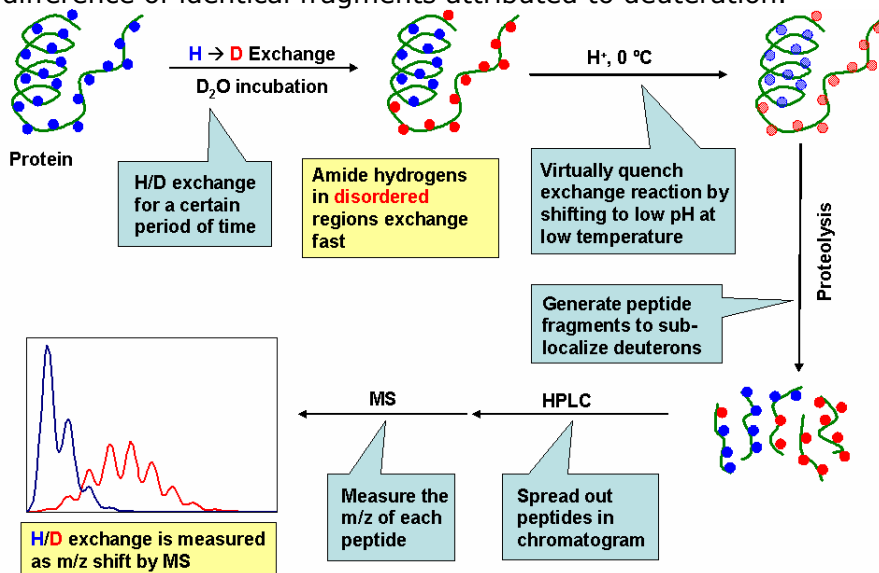


Figure 4. H/D-Ex Experiment Overview

## **EXSAR Competencies/Deliverables**

ExSAR has offered H/D-Ex services since 2002 for major pharmaceutical and biopharmaceutical clients. It offers a collaborative and consultative approach, with verbal and written presentation of results. Its scientific leadership includes Dr. Charles Cantor, Dr. William DeGrado, and Dr. S. Walter Englander, each members of the National Academy of Science. Projects are conducted under the supervision of Dr. Yoshitomo Hamuro.

### **William F. DeGrado, Ph.D.-Chairman of the ExSAR Scientific Advisory Board**

Dr. DeGrado is the George W. Raiziss Professor of Biochemistry and Biophysics at the University of Pennsylvania and he is a member of the National Academy of Sciences. His published research includes contributions to the fields of protein design, synthesis of peptidomimetics, and characterization of membrane-active peptides.

### **S. Walter Englander, Ph.D. – Member, ExSAR Scientific Advisory Board**

Dr. Englander is the Jacob Gershon-Cohen Professor of Medical Science and Professor of Biochemistry and Biophysics at the University of Pennsylvania. He is a member of the National Academy of Sciences, Honorary Fellow of the Biophysical Society and Honorary Fellow of the American Association for the Advancement of Science. His work has focused on internal protein motions and correlations to amide hydrogen exchange rates.

### **Charles R. Cantor, Ph.D., Director, ExSAR Board of Directors**

Dr. Cantor is the Chief Scientific Officer and Chairman of Sequenom, and a member of the National Academy of Sciences. He was previously the chair and professor of the department of biomedical engineering and biophysics, and director of the Center for Advanced Biotechnology, at Boston University. Prior to this, Dr. Cantor held faculty positions at Columbia University. He was also director of the Human Genome Center Project of the Department of Energy at Lawrence Berkeley Laboratory. Dr. Cantor has published more than 325 peer reviewed articles and has been granted 26 U.S. patents.

### **Yoshitomo Hamuro, Ph.D.—Senior Director of Technology Development**

Dr. Hamuro has led the development of hydrogen/deuterium exchange mass spectrometry analysis of protein dynamics, protein-ligand interactions and protein-protein interactions at ExSAR since joining in 2002. Prior to joining ExSAR, he was instrumental in the development of modern H/D-Ex technology at the University of California, San Diego, in the laboratory of Professor Virgil Woods. Dr. Hamuro conducted postdoctoral research on combinatorial chemistry and solid-phase chemistry at DuPont and later on antibacterial  $\beta$ -peptides at the University of Pennsylvania under Professor William DeGrado. He obtained his Ph.D. in 1996 from the University of Pittsburgh on protein structure mimetics.

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