Deuterium exchange and mass spectrometry reveal the interaction differences of two synthetic modulators of RXR α LBD

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Abstract

Protein amide hydrogen/deuterium (H/D) exchange was used to compare the interactions of two antagonists, UVI 2112 and UVI 3003, with that of the agonist, 9-*cis*-retinoic acid, upon binding to the human retinoid X receptor alpha ligand-binding domain (hRXR α LBD) homodimer. Analysis of the H/D content by mass spectrometry showed that in comparison to 9-*cis*-retinoic acid, the antagonists provide much greater protection toward deuterium exchange-in throughout the protein, suggesting that the protein–antagonist complex adopts a more restricted conformation or ensemble of conformations in which solvent accesses to amide protons are reduced. A comparison between the two antagonists shows that UVI 3003 is more protective in the C-terminal region due to the extra hydrophobic interactions derived from the atoms in the benzene ring of the carboxylic acid chain. It was less protective within regions comprising peptides 271–278 and 326–330 due to differences in conformational orientation, and/or shorter carboxylic acid chain length. Decreased deuterium exchange-in in the segment 234–239 where the residues do not involve interactions with the ligand was observed with the two antagonists, but not with 9-*cis*-RA. The amide protons of helix 12 of the agonist- or antagonist-occupied protein in solution have the same deuterium exchange rates as the unliganded protein, supporting a suggestion made previously that helix 12 can cover the occupied binding cavity only with the cofactor present to adjust its location.

Keywords: hRXR α ; ligand-binding; conformational changes; antagonist and agonist; H/D exchange and mass spectrometry; UVI 2112; UVI 3003

The retinoid X receptor (RXR) is a member of the nuclear receptor (NR) superfamily which plays important roles as a modulator affecting growth, differentiation, development, metabolism, cellular homeostasis, and even profoundly affects the immune response (Mangelsdorf and Evans 1995; Szanto et al. 2004; Grenningloh et al. 2006).

RXR has a similar structural motif and mechanism of signal transduction as other members of the NR superfamily, i.e., retinoid ligands bind and promote protein conformational changes. If the ligand is an agonist, the conformational change drives the dissociation of corepressors and the association of coactivators with helix

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Abbreviations: RXR, retinoid X receptor; NR, nuclear receptor; LBD, ligand binding domain; 9-*cis*-RA, 9-*cis*-retinoic acid; H/D,

hydrogen/deuterium; ESI, electrospray ionization; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; CHAPS, 3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate.

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12 in an active position. If the ligand is an antagonist, H12 does not adopt this conformation. This further enhances corepressor association and coactivator dissociation. Therefore, agonists and antagonists initiate two distinct patterns of gene expression resulting in the modulation of diverse signaling pathways (Xu et al. 2002; Togashi et al. 2005). The important role of NRs in regulating transcription has led to an increased interest in the use of retinoid drugs for controlling specific gene expression in the treatment of diseases such as cancer, coronary heart disease, diabetes, obesity, and asthma (Mukherjee et al. 1997; McKenna and O'Malley 2002; Xu et al. 2002; Hede 2004; Villarroya et al. 2004; Altucci et al. 2005; Grenningloh et al. 2006).

RXR α is the predominant NR type I and II heterodimerization partner that plays a nonredundant essential role in the NR superfamily (Rastinejad 2001; Ahuja et al. 2003; Hede 2004; Vivat-Hannah and Zusi 2005; Haraguchi et al. 2006; Taschner et al. 2006). The molecular mechanism includes the key steps of ligand binding, dimerization, and interaction with transcriptional coregulators that occur in the ligand-binding domain (LBD) of NRs. Thus, the RXR α LBD is one of the most important targets for pharmaceuticals. Different agonists show different gene expression profiles, allowing for the possibility of reducing adverse effects in the treatment of diseases (e.g., RA syndrome) (Szanto et al. 2004; Vivat-Hannah and Zusi 2005; Haraguchi et al. 2006). These findings suggest that the ligand-binding pocket in the RXR LBD can adapt to different ligands to form multiple active conformations, and the individual interaction specificity of ligands plays a key role promoting the diversity of biological responses of NRs. Understanding the differences in the molecular mechanism by which agonists and antagonists interact with the RXR LBD and the development of technologies to analyze the effects of these drugs on the structure of the RXR LBD is important in the design and screening of therapeutic molecules.

Ligand binding to the hRXR α LBD results in α -helix rearrangement and major conformational changes in the structure. Since it is influenced by involvement in hydrogen bonds and/or hydrophobic interactions as well as the conformational dynamics of the protein that regulate solvent access to backbone amide protons, amide hydrogen exchange is a very sensitive probe for detecting such changes. Previous studies in our laboratory on the RXR α LBD by H/D (hydrogen/deuterium) exchange using mass spectrometric analysis yielded information on the protection of amide hydrogens from exchange in regions that are not directly in contact with bound 9-*cis*-retinoic acid (9-*cis*-RA), a result that was not obvious from the X-ray structures (Yan et al. 2004). The effects of different agonists on binding specificities can be explored quickly by mass spectral analysis of the global levels of exchanged

amide hydrogens on the intact protein. More detailed information on local binding specificities and the local dynamics of the exchange process can be obtained through an analysis of the time dependence of the deuterium exchange-in of certain small- or medium-sized peptide obtained by proteolysis (Yan et al. 2006).

In the present study, the interactions of the hRXR α LBD homodimer with two antagonists (Fig. 1) were explored using H/D exchange monitored by electrospray ionization (ESI) mass spectrometry. Unliganded protein was used as a control in studies to determine the differences in protein conformations that occurred between the RXR α LBD complexed with the two antagonists in comparison to the full agonist, 9-*cis*-RA. The results from these studies should contribute to an understanding of how antagonists interact with the RXR α LBD and provide a useful guide in the design of new selective agonists and antagonists with more desirable properties for improved treatment of associated diseases.

Results and Discussion

The rate of backbone amide H/D exchange depends on several factors. These include the stabilization of the proton by hydrogen bonds and/or dipolar interactions with charged amino acid side chains. Solvent accessibility must also be considered in that binding of a ligand may sterically hinder solvent access or reduce protein conformational fluctuations, decreasing solvent penetration into the protein interior. The potential interactions between the RXR LBD and ligands inferred from X-ray crystallographic data and the role that these interactions may play in either steric hindrance to solvent access or reduction in H/D exchange by modulation of protein conformational fluctuations must also be considered. Although the relative affinities of UVI 2112 (8 nM) (Koch et al. 1996) and UVI 3003 (30 nM) (E. Pérez, P. Germain, G. Lemaire, H. Gronemeyer, and A. Lera, unpubl.) are comparable to 9-*cis*-RA, and the experimental conditions were such that the RXR LBD was likely to be completely occupied by all of the ligands, it turned out that both antagonists provided greater protection against deuterium uptake than did 9-*cis*-RA. No difference was observed between the two antagonists on the H/D exchange rate of amide hydrogens for the intact protein, even though their structures are different (Fig. 2). The basis for the antagonist properties of these ligands is thought to arise in part from the nature of the 3-alkoxy side chain on the tetrahydronaphthyl group (Koch et al. 1996). A methoxy substituent results in an agonist, an ethoxy group a partial agonist, and the larger propyloxy and pentyloxy groups give rise to antagonist molecules.

X-ray crystallographic data show that the 9-*cis*-RA molecule adopts a conformation in the complex in which

Figure 1. Ligand structures and possible conformations within the ligand-binding site. Assumed ligand conformations (ball-stick model) for UVI 2112 and UVI 3003 based on crystal structures of complexes or similarity of structural motifs (see Materials and Methods). Atoms are numbered in 9-*cis*-RA according to the assignment in the crystal structure of the complex.

its atoms are spread mainly within two planes: the cyclohexene ring in one plane and the aliphatic chain in another (Fig. 1). The crystal structures for the hRXR α LBD antagonist complexes used in the present study are not available at this time. However, crystallographic data are available for the hRXR β -LG100268 complex (Love et al. 2002), where the beta subtype shares 89% of the sequence with the alpha subtype (Fig. 3A; Yan et al. 2006) and the agonist, LG100268, shares the same tetrahydronaphthalene motif with the two antagonists but with a different carboxylic acid chain. The crystallographic data have also been determined for the heterodimer hRXR α -L79 complex (Haffner et al. 2004). The structures showed that different ligands occupying the cavity contact exactly the same residues as does 9-*cis*-RA in the RXR α LBD ligand complex (Fig. 3B). Thus, it is assumed that these antagonists interact with the protein at the same sites as 9-*cis*-RA. It is further assumed that the tetrahydronaphthalene ring adopts a coplanar orientation, and the carboxylic acid group is in a plane orthogonal to it (Fig. 1). Therefore, a comparison of deuterium exchange data for these antagonists with the full agonist, 9-*cis*-RA may reveal additional insights into the interaction and binding in the ligand RXR α LBD complex.

Previous work showed that the peptides comprising the RXR α LBD pocket with residues in direct contact with the full agonist, 9-*cis*-RA (Fig. 3B) exhibited significantly higher protection than the rest of the LBD (Yan et al. 2004). A different extent of protection against deuterium exchange induced by other agonists was

mainly observed in these areas (Yan et al. 2006). In the present study, the intact protein was fragmented by on-line pepsin digestion and analyzed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), and the location and extent of deuterium exchange-in protection on this protein due to the binding of different antagonists were revealed using the same approach. First, the patterns of deuterium exchange-in protection along the protein backbone by binding of the two full antagonists, UVI 2112 and UVI 3003, are very similar and broad, though the extent of protection varies

Figure 2. Deuterium levels observed at amide bonds in the subunit of the liganded and unliganded homodimer hRXR α LBD as a function of deuterium exchange-in time, 0.25–60 min. Open circle, unliganded protein; open triangle, 9-*cis*-RA liganded protein; open diamond, UVI 2112 liganded protein; filled diamond, UVI 3003 liganded protein.

Figure 3. Location of ligand (9-*cis*-RA) contact points (labeled by solid black circles) in the sequence. (A) Peptic peptides used in the present study are indicated by the double-headed arrows. (B) Schematic plot representing hydrophobic contacts (dashed lines within 4.5 Å) between the protein and ligand 9-*cis*-RA. The diagram was generated with the program LIGPLOT (Wallace et al. 1995).

in some regions (Fig. 4). Both ligands reduced the deuterium level in segments 255–270 (H2–H3 loop and the N terminus half of H3), 271–278 (helix 3), 301–309 (helix 4 and 5), 310–319 (helix 5 and 3_{10} helix), 320–325 (loop and following $\beta 1$), 326–330 (loop between $\beta 1$ and $\beta 2$), 331–346, 347–353 ($\beta 2$, helix 6, helix 7, and the loop between them), 353–362 (helix 7 and the following loop), 363–370 (helix 8), 419–425, 426–432 (helix 11), 431–438

and 439–449 (helix 11 and 12, and the loop between them). In many respects, this protection pattern is similar to that observed upon binding of the full agonist, 9-*cis*-RA, although the extent of protection may differ (Yan et al. 2004). Second, the protected regions are mainly located at the 9-*cis*-RA binding pocket or nearby segments, namely helix 3, the two β -sheets and the loop between them, helix 5, helix 7, helix 11, helix 12, and the loop

Figure 4. Antagonist binding induced deuterium exchange-in relative and absolute level changes along the hRXR α LBD backbone (*upper panel*: antagonist UVI 2112; *lower panel*: antagonist UVI 3003). Deuterium level difference is given for each peptide fragment at eight time points (0.25, 1, 2, 3, 5, 10, 30, and 60 min, *left to right*) as a series of vertical bars. Negative value means lower deuterium content compared to the segment in the unliganded protein and thus increased protection toward deuterium exchange-in within the fragment. The maximum absolute number of deuteriums exchanged-in is shown for each fragment. The total exchangeable amide hydrogens for each fragment are listed in the *upper panel* in parentheses.

between helix 11 and 12. These results are consistent with the available crystal complex structures and with previous experimental results on RXR–agonist interactions in that these two full antagonists also bind to the same site as does 9-*cis*-RA and interact with those residues that trigger protein conformational changes (Yan et al. 2004, 2006), that affect the dynamics of the protein–antagonist complex. In the deuterium exchange reaction period (0.25–60 min), the location and relative extent of maximum protection toward exchange resulting from the two antagonists show some minor differences in H3, H7, H11, H12, and a larger difference for H1 where 50% exchange was recorded for UVI 2112 and 30% for UVI 3003 (Figs. 4, 5). If exchange-in is allowed to continue long enough in each case, then the exchange will be essentially the same for the liganded and nonliganded protein. This trend is clearly revealed (Fig. 4) over the period of 60 min.

Both antagonists exhibited more protection against deuterium exchange-in along the protein backbone than 9-*cis*-RA (Fig. 6). In the segment 255–270 of the RXR α LBD the X-ray structure shows that Ile 268 and Cys 269 hydrophobically interact with 9-*cis*-RA in the complex (Fig. 3B). These interactions caused the rate of deuterium exchange-in to decrease in the fragment 255–270 in the 9-*cis*-RA liganded protein compared to the unliganded protein as observed previously (Yan et al. 2004). In the two antagonists, a planar benzene ring, part of which corresponds to C6–C8 in 9-*cis*-RA, attached to the twisted cyclohexene plane results in an expanded area of coplanarity and an increased number of contacting atoms that should enhance the hydrophobic interaction with the corresponding segment of the protein. The crystal structure of the complex hRXR β LBD–LG100268 indicates that the residue which corresponds to Ile 268 in the hRXR α

Figure 5. (A) Ribbon structures of apo RXR α LBD (1LBD), with different colors indicating the secondary structure and binding 9-*cis*-RA sites (in gray and ball-stick model). The color coding in A does not apply to H/D exchange. The maximum deuterium exchange protection caused by antagonists, (B) UVI 2112 and (C) UVI 3003, in the exchange reaction period (0.25–60 min) relative to the unliganded protein is shown by color coding.

LBD is closer to the carboxylic acid chain, which causes it to interact mainly with the benzene ring. In addition, the two extra methyl groups in the cyclohexane ring of the two antagonists increase the local volume and may strengthen the hydrophobic interactions. Time course studies (Figs. 4, 6) showed that, in comparison to 9-*cis*-RA, protection by the antagonists was greatest at early time points and that the degree of protection for both antagonists and 9-*cis*-RA decayed over time. These results may indicate the dampening of conformational fluctuations in the unliganded protein by both ligands with the additional interactions of the antagonists further restricting the protein dynamics and decreasing the rate but not the extent of solvent accessibility at longer time points. This explanation is consistent with the similar degree of protection observed in segment 255–270 by the two antagonists UVI 2112 and UVI 3003 because of the similarity of conformation of the tetrahydronaphthalene moiety.

In the segment 271–278, Ala 272 and Gln 275 make hydrophobic contacts with 9-*cis*-RA on the long carboxylic acid chain at C10–14, C20, C14–15, and O1. The carboxylic acid chain moiety of UVI 2112 is identical to that of 9-*cis*-RA and therefore should adopt a similar conformation in the protein pocket. But the spatial adjustment of Ile 268 to the carboxylic acid chain and the increased volume from the two extra methyl and propoxyl groups should fill the protein pocket more completely, resulting in stronger hydrophobic interactions. The greatest deuterium exchange-in protection relative to 9-*cis*-RA observed in this region when binding UVI 2112 supports this conclusion. The antagonist UVI 3003 showed enhanced protection toward deuterium exchange-in compared to 9-*cis*-RA due to the

benzene ring that makes the molecule as a whole more spatially demanding within the protein pocket and with more restricted conformational options to the protein by the carboxylic acid chain. Thus, it exhibited less deuterium exchange-in protection in the segment 271–278 relative to that of UVI 2112. Again, the time course studies indicated that both antagonists showed greatest protection at the earliest time points compared to 9-*cis*-RA suggesting a mechanism similar to that described above for the adjacent peptide 255–270.

The crystal structure revealed that residues Trp 305 and Asn 306 in the protein interact hydrophobically with C19 only in 9-*cis*-RA, while Leu 309 interacts with C10 and C12 (Fig. 3B). Assuming C19 does not change position upon binding of the antagonists UVI 2112 or UVI 3003 (Fig. 1), and that the conformation and orientation of the carboxylic acid moiety in UVI 2112 is similar to 9-*cis*-RA, the minor protective effect toward deuterium exchange-in in segment 301–309 probably arises from the increased molecular volume. Similarly, the benzene ring in the chain containing the carboxylic acid group in UVI 3003 decreased deuterium exchange-in in segment 301–309 in comparison to that resulting from 9-*cis*-RA binding. The time course study on the exchange process does not easily lend itself to interpretation since the difference in exchangeable protons is small.

The residue Phe 313 has multiple hydrophobic interactions with the 9-*cis*-RA from C10 to C15, C18, C20 and hydrogen-bonding interaction with O1. Arg 316 contacts C15 and the two carboxyl O atoms in 9-*cis*-RA. The introduction of the benzene ring in the antagonists increases steric constraints for the interaction of C18

Figure 6. Deuterium level changes through antagonist binding relative to 9-*cis*-RA binding as shown for the peptic peptides. *Upper* panel: antagonist UVI 2112 relative to 9-*cis*-RA; *middle* panel: antagonist UVI 3003 relative to 9-*cis*-RA. The maximum absolute difference of deuterium exchanged-in is shown for each peptide, and the total number of exchangeable amide hydrogens for each peptide is listed (*upper* panel) in parentheses. The deuterium level difference caused by binding of the antagonist UVI 2112 relative to the antagonist UVI 3003 in the peptic peptides is presented in the *lower* panel.

with its residues, and the propoxyl or pentyloxyl groups in the antagonist may hinder solvent access to the residues that are normally exposed to the carboxylic acid chain in the 9-*cis*-RA complex. The alkyl chain may strengthen the interactions between the atoms on the carboxylic acid chain and the nearby amino acid residues. Thus much greater deuterium exchange-in protection was observed for segment 310–319 upon binding of the two antagonists compared to 9-*cis*-RA. The time course study on the protective effects differs from that observed for peptides 255–270 and 271–278 in that it is greatest at longer times, which suggests that slower fluctuations of protein structure in this region are more strongly affected by the bound antagonists than by 9-*cis*-RA. Leu 325 is involved in hydrophobic and hydrogen-bonding interac-

tions with O2 in 9-*cis*-RA. Ala 327 also interacts with O1 and C15. Thus, it is reasonable to have observed significant deuterium exchange-in protection for segments 320–325 and 326–330 when the protein binds 9-*cis*-RA. The two antagonists showed similar protection (but greater than 9-*cis*-RA) toward deuterium exchange-in in segment 320–325, indicating that the different carboxylic acid chains interact similarly at the carboxyl terminus. For segment 326–330, however, UVI 2112 exhibited greater deuterium exchange-in protection than UVI 3003. UVI 3003 has a shorter carboxylic acid chain, which probably reduces its interaction with neighboring residues. In addition, the benzene ring in the carboxylic acid chain may make the carboxylic acid group adopt a slightly different orientation. The combination of these effects may

be responsible for the decreased deuterium exchange-in in segment 326–330 by UVI 3003 compared to UVI 2112. Both antagonists show different time-dependent effects on the adjacent peptides. Protection against exchange increases with time for 320–325 but decreases for 326–330, suggesting different conformational dynamics for different peptides at the ligand-binding site depending on the structure of the particular ligand bound and whether the ligand is an agonist or antagonist.

For the segment 331–346, two residues, Val 342 and Ile 345, interact with C3 and C3–4 atoms of 9-*cis*-RA, respectively, as observed in the crystal structure of the complex hRXR α LBD–9-*cis*-RA. In the present study, the cyclohexene ring conformation of the two antagonists is proposed to be the same, as indicated by the nearly identical deuterium exchange-in results in this segment at most time points. But, at the earliest time point, this segment exhibited detectable deuterium exchange-in differences among these two antagonists and the full agonist, 9-*cis*-RA, indicating that the extra methyl groups on the cyclohexene ring of 9-*cis*-RA had a minor influence on H/D exchange in this segment and the importance of experimental timing for such an approach to explore conformational subtleties.

Segment 347–353 showed significant protection against deuterium exchange-in in the case of 9-*cis*-RA binding, as C18 interacts with residue Val 349. The two antagonists showed greater protection than 9-*cis*-RA did in this segment which was probably due to the increased molecular volume and the presence of the alkoxy groups. Interactions from these substituents appeared to affect slower conformational transitions to the open state since protection increased over time. There is no interaction between 9-*cis*-RA and the residues from 353 to 370 in the protein backbone so the increased protection at longer times probably derives from a conformational change affecting slower structural transitions when the antagonist binds (Yan et al. 2004). The same time dependence is also observed for 353–363 and 363–370 (Fig. 6), suggesting that 347–370 may be affected by the same dynamics in the protein.

Segment 234–248 corresponds to helix 1 and the helix 1–2 loop. Although positioned away from the binding cavity, it underwent a major rearrangement upon binding 9-*cis*-RA (Yan et al. 2004). Unlike 9-*cis*-RA, the two antagonists caused increased protection for helix 1 (segment 234–239) which slowly developed and then declined, suggesting that the exchange process is the result of multiple processes having different time domains. This is also suggested by the increased exchange in loop 241–248 for the antagonists compared to 9-*cis*-RA and the time dependence of the process, i.e., a slow increase followed by a decrease. Residues 371–415 comprise the carboxyl-terminal of H8, H9, and the amino terminus of H10. That this region experienced almost no effect upon H/D

exchange irrespective of ligand, suggests the absence of conformational changes compared to the apo-protein.

Segment 426–432 exhibited significant protection upon binding of the two antagonists. This is consistent with the interaction of residue Cys 432 with 9-*cis*-RA and the potential influence of the two methyl and the alkoxy groups. It also agrees with the decrease in the rate of deuterium exchange-in for the segment 431–438 when UVI 3003 binds to the protein as compared to binding of 9-*cis*-RA, due to the hydrophobic interactions between His 435, Leu 436, and carbons 16 and 17. UVI 2112 showed no difference in protection compared to 9-*cis*-RA, supporting the proposed conformational similarities between 9-*cis*-RA and UVI 2112. The extra atoms of the benzene ring located on the carboxylic acid chain of UVI 3003 may give rise to additional interactions with residues Cys 432 and Leu 436 similar to those found for the cyclohexene ring on the carboxylic acid chain in the crystal structure of the RXR α –L79 complex. These extra interactions resulted in lower deuterium exchange-in behavior in segments 431–438 and 426–432 of the protein. The additional time-dependent protection of the antagonists in comparison to 9-*cis*-RA appears biphasic, suggesting multiple, slower conformational processes mediate the H/D exchange in this region.

Crystallographic data suggest that a major reorientation of helix 12 covers the occupied ligand-binding site and creates a new surface that interacts with coactivators to initiate transcription (Freedman 1999). The C-terminal end of helix 12 and the following loop (segment 454–462) showed no difference in the rate of deuterium exchange-in after binding the agonist, 9-*cis*-RA, or either of the antagonists in this study. Moreover, segment 439–449, which includes most of helix 12, showed almost the same protection against deuterium exchange-in in the presence or absence of 9-*cis*-RA, UVI 3003, or UVI 2112. Thus, this segment is highly solvent exposed in solution. It has been suggested that the location of helix 12 is determined by the combination of both ligand and cofactor (copressor or coactivator) (Togashi et al. 2005; Folkertsma et al. 2006). The present results agree with this suggestion in that helix 12 in ligand-occupied protein (agonist or antagonist) has the same solvent accessibility of its amide protons as it does in the unliganded protein in solution. Helix 12, thus, has the ability to fold back to cover the occupied binding cavity and carry out its biological function, but the cofactor must be present to adjust its location.

Proteins manifest a unique three-dimensional structure with attendant surface crevices and interior cavities in order to carry out specific biological functions, such as ligand binding. Nevertheless, the flexible properties of proteins provide them with an ability to attain the best spatial fit and allow for interaction of individual ligands. These conformational transitions result in different gene expression profiles

for the RXR protein and can be detected by deuterium exchange-in monitored by a sensitive technique such as analytical mass spectrometry. In combination with detailed crystal structure data, the methodology used in the present study provides information for designing new ligands with properties for the effective treatment of diseases.

Conclusions

Binding specificities of two different antagonists compared to the agonist 9-*cis*-RA were explored in the recombinant human RXR α LBD homodimer by amide hydrogen/deuterium exchange and mass spectrometry. The affected regions in the protein were found to be the sites that involve protein–ligand interactions and regions in which changes in protein conformation and/or dynamics took place.

Global analysis of deuterium content from H/D exchange experiments showed that there is little or no difference in the antagonist/protein complexes with UVI 2112 and UVI 3003. However, binding specificities as observed by relative deuterium incorporation in different regions of the protein were often dramatically different. The proposed ligand conformations, which were based on available crystal complex structure or similar complex structures, were used for discussing possible interactions and influences on the protein segments. The method described here may be useful for analyzing the structural interactions between ligands and their receptors, and in drug design, screening, and development.

Materials and Methods

Materials

The hRXR α LBD was expressed and purified as described previously and confirmed as a homodimer (54 kDa) by size-exclusion chromatography and mass spectrometry (Yan et al. 2004). A 45 μ M (or 90 μ M concentration of monomers) hRXR α LBD stock solution was prepared in aqueous buffer consisting

of 0.5 M KCl, 0.5 mM CHAPS, 1 mM TCEP, and 50 mM potassium phosphate buffer (pH 7.4). Deuterium oxide (D₂O; 99.9% deuterium), 9-*cis*-retinoic acid, porcine pepsin, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Chemical Co. The reductant, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Pierce. Poros 20 AL media was purchased from Applied Biosystems. All ligands were prepared as 10 mM stock solutions in ethanol. All other chemicals were reagent grade and obtained from standard commercial sources.

Ligand synthesis and preparations

Synthesis of UVI 3003

The preparation of this antagonist is described elsewhere (V. Nahoum, E. Pérez, P. Germain, F. Rodríguez-Barrios, F. Manzo, S. Kammerer, G. Lemaire, O. Hirsch, C. Royer, H. Gronemeyer, et al., in prep.).

Synthesis of UVI 2112 (LG 100,754)

The preparation of UVI 2112 (**9** in Scheme 1) is a modification of the reported improvement (Hamann 2000) to the original lengthy procedure (Koch et al. 1996). It intercepts the improved method at the level of the allyl alcohol **5**. From this intermediate onward, the synthesis is similar to that described and features the Dess-Martin oxidation reaction with periodinane in pyridine (100%). Highly *E* stereoselective HWE condensation of the unsaturated aldehyde **6** with the anion of phosphonate **7** (70%) affords trienoate **8** and saponification leads to **9** (100%). For the incorporation of the alkenyl side chain with *Z* geometry a Suzuki cross-coupling of arylboronic acid **3** and iodide **4** (Dakoji et al. 2001) was selected to afford allyl alcohol **5**. Boronic acid **3** was obtained in quantitative yield by trapping with triisopropylborate, the aryl lithium generated by bromine-lithium exchange starting from the *n*-propyl ether (**2**) derivative of bromotetrahydronaphthol **1**.

All reactions were carried out under argon atmosphere (C-50), and those not involving aqueous reagents were carried out in oven-dried glassware for at least 12 h at 140°C. Solvents were dried according to published methods and distilled before use (Perrin and Armarego 1988). ¹H NMR spectra and ¹³C NMR spectra (recorded on a Bruker AVANCE DPX400 and Bruker ARX400 spectrometers at 400 MHz and 100 MHz, respectively)

Scheme 1. Reagents and reaction conditions: (a) NaH, *n*-PrI, DMF, 25°C, 100%. (b) *n*-BuLi, B(O*i*Pr)₃, TMEDA, THF, −78°C, HCl (10%), 6 h, 100%. (c) Pd(PPh₃)₄, Na₂CO₃, DME, 80°C, 5 h, 55%. (d) Dess-Martin Periodinane, Py, CH₂Cl₂, 20°C, 5 min, 100%. (e) *n*-BuLi, THF, DMPU, −78°C, 2 h, 70%. (f) KOH/MeOH, 90°C, 30 min, 100%.

and high-resolution mass spectra of the final product were consistent with those reported previously (Hamann 2000).

H/D exchange experiments

Deuterium exchange-in was initiated by 11-fold dilution of the protein stock solution (in the aqueous buffer) with the deuterated aqueous buffer at 30°C. Prior to the H/D exchange reaction, protein–ligand interaction equilibration was achieved by adding 1 μ L of ligand stock solution (final concentration was 278 μ M) or 1 μ L ethanol as blank for unliganded protein sample to 35 μ L homodimer hRXR α LBD stock solution upon which the solution was incubated for 30 min. The D₂O buffer, in which the same concentration of ligand or ethanol was added, was equilibrated at 30°C. After 0.25, 1, 2, 3, 5, 10, 30, and 60 min reaction, aliquots (20 μ L) were taken and added to a pre-chilled tube containing 20 μ L of 0.42% phosphoric acid to quench the reaction (pH 2.5). The quenched sample was immediately divided into two tubes and frozen in liquid nitrogen for duplicate LC-ESI-MS analysis.

General procedure for H/D exchange, digestion, and analysis

Since the dissociation constants for ligands are in the nanomolar range and the final concentration of binding sites was nearly 10 μ M after dilution and ligand concentrations were about 280 μ M, it was assumed that all ligand binding sites were occupied.

The protein was eluted through a C4 column (0.32 $\times$ 50 mm, Vydac, 10 μ m, in-house packed) within 6 min with a steep gradient (mobile phase A: 0.04% TFA in H₂O, mobile phase B: 0.04% TFA in CH₃CN). For intact protein analysis the column was coupled directly to the mass spectrometer. On-line proteolytic digestion was carried out within 3 min under quench conditions (0°C and pH 2.5), using an in-house prepared immobilized pepsin column as detailed by Wang (Wang et al. 2002). The peptic peptides were captured on a C₁₈ trap (1 $\times$ 8 mm, Michrom Bioresources, Inc.), then further separated through a C₁₈ column (0.32 $\times$ 100 mm, Luna C₁₈(2), 5 μ m, in-house packed) before delivery into the mass spectrometer within 9 min, using the same elution solvent as described above. The entire apparatus (injector, enzyme column, C₁₈ trap, C₁₈ column, and part of the solvent transfer tubing for delivery into the mass spectrometer) was immersed in an ice bath. Data are the average of two experiments with a typical difference of 5%–11% between experiments. If the protein or peptide retention time was different, a third experiment was conducted.

Mass spectra of the intact protein were acquired with an LCT mass spectrometer (Waters Corp.) that was equipped with a standard ESI source. All mass spectral measurements were taken with the following settings and conditions: capillary voltage 3 kV, sample cone voltage 60 V, extraction cone voltage 5 V, source temperature 80°C, desolvation temperature 110°C, desolvation gas 500 L/h. The intact protein molecular weight (deuterated and nondeuterated sample) was obtained by deconvoluting the combined ESI spectra of the protein with MaxEnt1 of the MassLynx 4.0 program (Waters). Pepsin-generated peptides were analyzed with a Thermo Finnigan LCQ mass spectrometer (Thermo Electron Corp.) with capillary temperature at 150°C. Fragmented peptides were identified through the data-dependent MS/MS mode with dynamic exclusion and SEQUEST database search engine against the sequences of the protein and pepsin for assignments. All peptide ion assign-

ments were further verified manually. The centroided mass of peptic peptides (deuterated and nondeuterated samples) was calculated from the ESI spectra of the peptide with the Magtran program (Z. Zhang, Amgen). The deuterium levels for protein or peptic peptides were determined from the mass difference between the deuterated and nondeuterated samples. The absolute number of deuterium exchange-in in each fragment for a certain reaction time can be calculated by multiplication of the deuterium level difference (percentage) and its amide hydrogen number.

Protein structure and ligand-binding pocket analysis were carried out with the Web site program LPC/CSU (Sobolev et al. 1999) at <http://lgin.weizmann.ac.il/space/servers/> and LIGPLOT (Wallace et al. 1995). Virtual hydrogen-bonding sites were identified using the HBPlus 3.15 program (McDonald and Thornton 1994). Ligand conformations were drawn based on the complex crystal structure or available similar structural motifs. The 9-*cis*-RA conformation was obtained from 9-*cis*-RA liganded RXR α LBD complex (PDB entry: 1fby). Based on the available crystal structure for complex hRXR β LBD–LG100268 (PDB entry: 1h9u), for which the protein shares 89% sequence homology with the alpha subtype (Yan et al. 2006) used in the present study and the ligand, LG100268, shares the same tetrahydronaphthalene motif as the antagonists used here, the UVI 2112 conformation within the complex was assumed to be similar. Likewise, the complexed UVI 3003 conformation was also assumed similar to that in complex hRXR β LBD–LG100268 and/or in complex hRXR α LBD–L79 propenoic acid (PDB entry: 1rdt), which contains a tetrahydronaphthalene motif, too. Dashed lines are used (Fig. 1) for connections where prior structural information was unavailable.

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