An Innovative Method To Study Target Protein−Drug Interactions by Mass Spectrometry

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We report the combination of chemical cross-linking and high-resolution mass spectrometry for analyzing conformational changes in target proteins that are induced by drug binding. With this approach conformational changes in the peroxisome proliferator-activated receptor alpha (PPARα) upon binding of low-molecular weight compounds were readily detected, proving that the strategy provides a basis to efficiently characterize target protein−drug interactions.

Introduction

The rate of drug discovery is greatly dependent on the development and improvement of rapid and reliable analytical methods for screening target protein−drug interactions. In those cases where high-resolution methods for structural analysis are applicable, such as X-ray crystallography and NMR spectroscopy, the solved three-dimensional structure of a protein with its bound ligand gives insights into stable interactions within the complex. Theoretical modeling might reveal further interactions, using the known three-dimensional structure as a starting point.

The aim of this study was to develop an approach for analyzing protein−drug interactions based on building a set of structurally defined interactions by covalently connecting pairs of functional groups within a protein using chemical cross-linking. Recently, chemical cross-linking of proteins has been combined with a proteolytic digestion of the protein and a subsequent mass spectrometric analysis of the created cross-linked peptides.[1−5] Our innovative analytical strategy for analyzing target protein−drug interactions is presented in Scheme 1. Pinpointing the location of the created cross-links within the protein by high-resolution mass spectrometry allows drawing conclusions on the changed distance geometries and thus on conformational changes of a protein induced by drug binding.

Analysis of cross-linked peptides by high-resolution mass spectrometry makes use of several advantages: Large proteins, membrane proteins (e.g., G-protein-coupled receptors[6]), and proteins that exist as mixtures of different species (post-translational modifications, splice variants) are amenable to analysis, the mass of the protein or the protein complex under investigation is theoretically unlimited, and, in favorable circumstances, only femto- to attomole amounts of total protein are required. Moreover, the broad range of specificities available for cross-linking reagents toward certain functional groups, such as primary amines, sulfhydryls, or carboxylic acids, and the wide range of distances that different cross-linking reagents can bridge offer the possibility to perform a great variety of experiments.[7] Using lysines as cross-linking sites has the minor drawback that charges are removed; however, if only a few cross-links are incorporated into the protein molecule, there are no conformational changes in the protein caused by the cross-linking reaction itself.

However, despite the straightforwardness of the cross-linking approach, the identification of the cross-linked products can be quite cumbersome because of the complexity of the reaction mixtures. Several strategies have been employed to enrich cross-linker-containing species by affinity chromatography or to facilitate the identification of the cross-linked products, e.g., by using isotope-labeled cross-linkers or proteins,[8−10] fluorogenic cross-linkers,[11−14] cleavable cross-linkers,[15] or photoaffinity labeling.[16,17]

The peroxisome proliferator-activated receptors (PPARs[a]) are ligand-activated transcription factors that belong to the nuclear receptor protein family. Three subtypes of PPARs (α, δ (or β, Nuc-1), and γ) have been identified so far.[18] PPARs are activated by fatty acids and eicosanoids and are also targets for antidiabetic and antidyslipidemic drugs.[19−21] Activated PPARs form heterodimers with the retinoid X receptor α (RXR) and bind to specific DNA sequences.[22] In general, PPARα promotes fatty acid catabolism in the liver and skeletal muscle, while PPARγ regulates fatty acid storage in adipose tissues.[23] Fibrates have been used since the 1960s for lowering hypertriglyceridemia and were recently shown to be weak PPARα agonists.[21] Dual PPARα/γ agonists (glitazars) bind to both PPAR subtypes α and γ.[24]

In the present paper we report on analyzing the conformational changes in PPARα upon binding of the high-affinity antagonist **1** (GW6471, $IC_{50} = 0.24\ \mu M$ [25]) and the agonist **2** (YS81, $EC_{50} = 7\ \mu M$,[26] Figure 1a). For the PPARα/**1** complex an X-ray structure exists (PDB entry 1KKQ); therefore, our strategy was shown as a "proof-of-principle" to demonstrate

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[a] Abbreviations: PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; LBD, ligand binding domain; NHS, *N*-hydroxysuccinimide; BS^3, bis(sulfosuccinimidyl)suberate; BS^2G, bis(sulfosuccinimidyl)glutarate; TFA, trifluoroacetic acid; HCCA, α-cyano-4-hydroxycinnamic acid; TCEP, tris(2-carboxyethyl)phosphine; AF2, activation function helix 2; XL, cross-linker.

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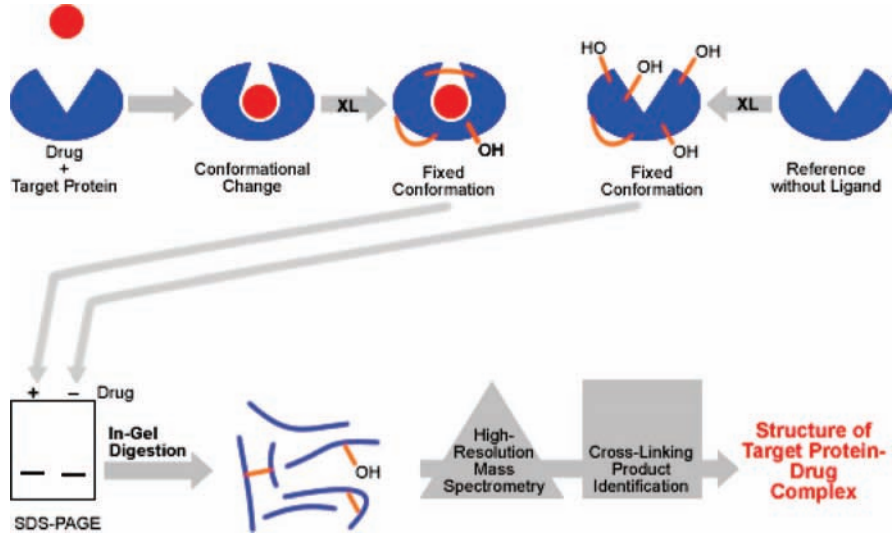
Scheme 1. Analytical Strategy for Analyzing Conformational Changes in Proteins upon Ligand Binding by Chemical Cross-Linking and High-Resolution Mass Spectrometry



the validity of our approach. In the next step, the complex between PPARα and the agonist **2** was investigated by our strategy in order to gain insight into the binding mode of agonist **2**.

Results and Discussion

The crystal structure of the PPARα ligand binding domain (LBD) has been determined in complex with the antagonist **1**.[25] We expressed the 25k Da LBD of PPARα as C-terminally His-tagged construct[27] and employed this construct for cross-linking experiments. In the following, PPARα-LBD is referred to as PPARα. PPARα was incubated with the ligand, either the antagonist **1** or the agonist **2** (a pirinixic acid derivative[26,28]), for 30 min (Figure 1).

The cross-linking reactions were conducted using the amine-reactive *N*-hydroxysuccinimide esters **3a** (BS^2G, bis(sulfosuccinimidyl)glutarate) and **3b** (BS^3, bis(sulfosuccinimidyl)suberate) bridging distances of ∼7.7 and 11.4 Å, which were employed as 1:1 mixture of nondeuterated (D_0) and four times deuterated (D_4) derivatives in order to facilitate a subsequent MS identification of cross-linking products (Figure 1).[29,30]

After the cross-linking reaction, the protein−ligand complexes and PPARα were separated by one-dimensional gel electrophoresis (SDS−PAGE), the bands of ligand **1**- or **2**-bound proteins and that of the free protein were excised from the gel and in situ digested. For enzymatic digestion, a number of endoproteinases were employed yielding different specificities, such as trypsin (cleaving C-terminal of Lys (K) and Arg (R)), AspN (cleaving N-terminal of Glu (E) and Asp (D)), and chymotrypsin (cleaving C-terminal of Leu (L), Val (V), Met (M), Phe (F)). The resulting highly complex peptides mixtures were subsequently separated by nano-high-performance liquid chromatography (nano-HPLC), which was either offline coupled to matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) mass spectrometry or online coupled to nanoelectrospray ionization (ESI) linear ion trap (LTQ) Orbitrap mass spectrometry. Both mass spectrometric techniques are capable of yielding data with excellent mass accuracies and resolution, which is a prerequisite for correctly assigning cross-linking products.

The identified cross-linking products within the PPARα were different with and without a bound ligand, depending on the conformational state of the protein (Table 1). We found a number of cross-linking products for antagonist **1**-bound PPARα, whereas the number of cross-linking products was much lower for the free PPARα. This finding might indicate a high flexibility of the free protein and could give a hint that ligand binding leads to a more rigid structure, which in the case of PPARα resulted in a higher number of cross-links. Indeed, NMR studies on the liganded and apo-PPARα showed an overall stabilization of the LBD upon ligand binding, resulting in a more compact and rigid structure.[31,32] On the other hand, recently conducted studies support a model of PPARα in which the AF2 helix transiently adopts a relatively stable active conformation even in the absence of agonists, in agreement with the existence of a basal activity in the absence of ligands.[33] Our data confirm that PPARα does not adopt a well-defined structure in the absencene of the ligand but rather exists in an equilibrium of conformations.

In detail, the connection between lysine-349 and lysine-449 was found in the free PPARα as well as in both PPARα/ligand complexes (Table 1).

The occurrence of a cross-link with **3b** between lysine-222 and lysine-449 in the PPARα/**1** complex (Figure 2) reveals a large conformational change within in the protein upon ligand binding: Lysine-449 gets positioned close to the activation function helix AF2 (amino acids 458−467) in PPARα, which flips over upon **1** binding in the direction toward lysine-222 and subsequently closes the binding pocket ("mouse trap principle")[31] (Figure 3). The fact that this cross-link is just observed for the longer cross-linker **3b** bridging ∼11.4 Å between both lysines gives insight into the distance between the two residues.

Additionally, the cross-link with **3a** and **3b** between lysine-252 and lysine-449 indicates that a huge conformational change is induced in the highly flexible Ω loop in PPARα (amino acids 252−267) and the AF2 helix upon binding of the antagonist **1**. Both domains come close to each other and jointly close the binding pocket (Figure 3). The respective cross-links are neither observed for free PPARα nor for the PPARα/**2** complex (Table 1).

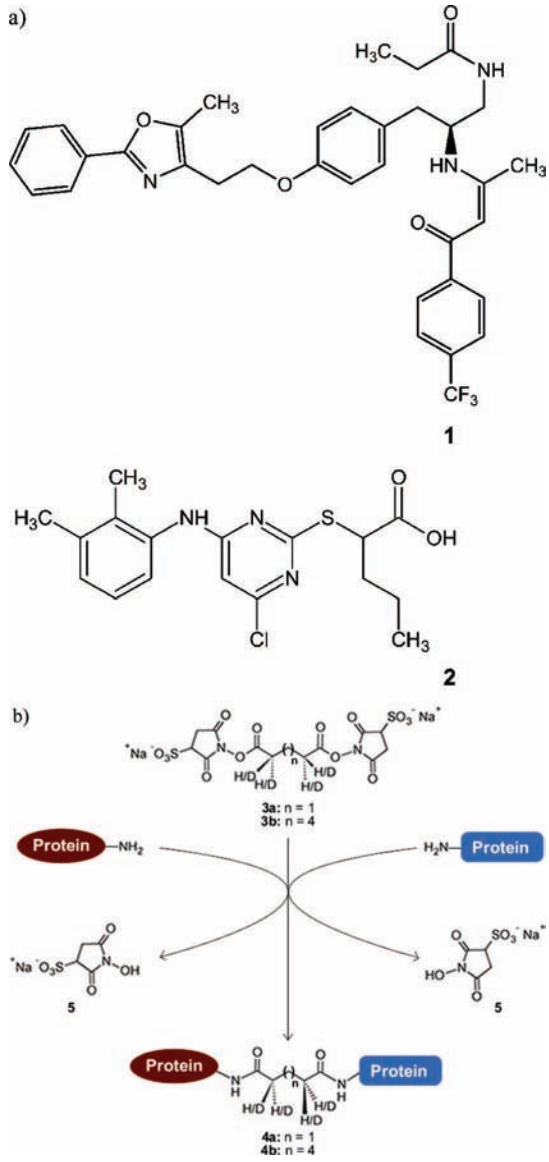


Figure 1. PPARα ligands: (a) **1** antagonist GW6471, **2** agonist YS81; (b) reaction mechanism of homobifunctional cross-linking with NHS esters.

Table 1. Interpeptide Cross-Linking Products in PPARα/Antagonist **1** and PPARα/Agonist **2** Complexes with the Amine-Reactive Cross-Linkers **3a** and **3b**[a]

m/z				
$[M + H]^+_{exp}$	$[M + H]^+_{theo}$	ligand	XL[b]	sequence[c]
1152.5601	1152.5605		BS^3	$\mathbf{K}_{349}$PFC + $\mathbf{K}_{449}$TES
1152.5600	1152.5605	GW6471	BS^3	$\mathbf{K}_{349}$PFC + $\mathbf{K}_{449}$TES
1152.5600	1152.5605	YS81	BS^3	$\mathbf{K}_{349}$PFC + $\mathbf{K}_{449}$TES
1379.7401	1379.7352	GW6471	BS^3	NFNMN$\mathbf{K}_{222}$VK + $\mathbf{K}_{449}$T
1389.7843	1389.7835	GW6471	BS^3	E$\mathbf{K}_{252}$TLVAK + $\mathbf{K}_{449}$TES
1110.5127	1110.5136		BS^2G	$\mathbf{K}_{349}$PFC + $\mathbf{K}_{449}$TES
1110.5123	1110.5136	GW6471	BS^2G	$\mathbf{K}_{349}$PFC + $\mathbf{K}_{449}$TES
1110.5129	1110.5136	YS81	BS^2G	$\mathbf{K}_{349}$PFC + $\mathbf{K}_{449}$TES
1347.7360	1347.7366	GW6471	BS^2G	E$\mathbf{K}_{252}$TLVAK + $\mathbf{K}_{449}$TES

[a] Masses are given for D_0 cross-linkers. All sequences were confirmed by MS/MS data. [b] XL: cross-linker. [c] All cysteines (C) are carbamidomethylated.

Conclusively, upon binding of the antagonist **1**, PPARα reveals a large conformational change in the AF2 helix and in the flexible Ω loop, which is not resolved in the X-ray structure (PDB entry 1KKQ).

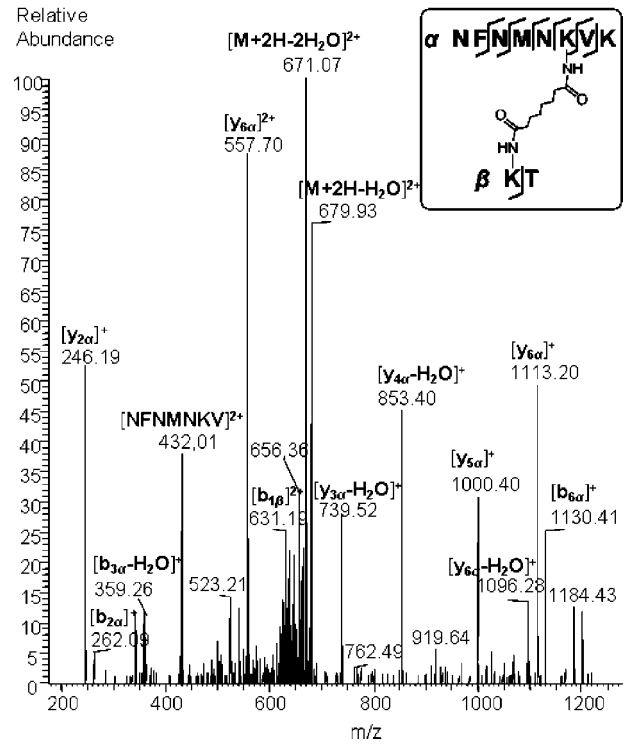


Figure 2. Tandem mass spectrum of the doubly charged precursor (*m/z* 688.36) of the intramolecular cross-linked product lysine-222 connected with lysine-449 of PPARα with **3b** in the presence of the antagonist **1** (Table 1).

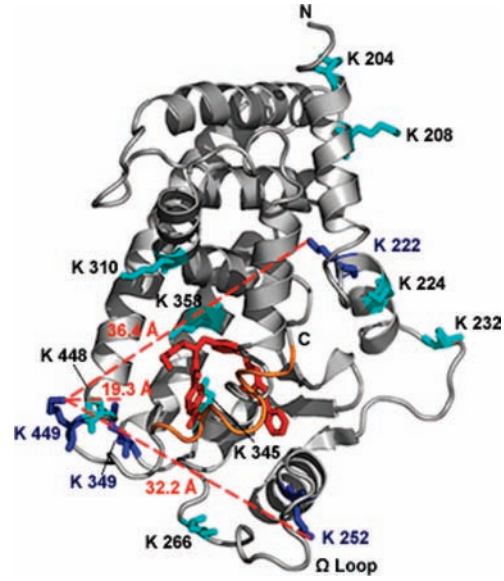


Figure 3. Crystal structure of PPARα/GW6471 complex (PDB entry 1KKQ). Lysines cross-linked with **3b** are shown blue. Lysines modified with hydrolyzed **3b** are shown cyan. The antagonist **1** is shown red, the activation function helix AF2 orange. Identified cross-links are represented by dashed red lines. The distances between lysine-N atoms are indicated; distances between C_α atoms are 15.2 Å (Lys-349 to Lys-449), 31.0 Å (Lys-222 to Lys-449), and 26.0 Å (Lys-252 to Lys-449).

Instead of reacting with two ε-amine groups in lysines (Figure 1), NHS esters can be hydrolyzed at one NHS ester function in case no protein amine group is available. These peptides, which are modified by a partially hydrolyzed cross-linker, do not give direct structural information on the 3D structure of a protein, however, they yield valuable information on the surface

Table 2. Different Hydrolyzed Cross-Linking Products in Ligand-Free PPARα, PPARα/Antagonist **1**, and PPARα/Agonist **2** Complexes with NHS Esters **3a** and **3b**[a]

m/z				
$[M + H]^+_{exp}$	$[M + H]^+_{theo}$	ligand	XL[b]	sequence[c]
1454.7778	1454.7778		BS^3	DQVTLL**K$_{310}$**YGVY
1454.7774	1454.7778	GW6471	BS^3	DQVTLL**K$_{310}$**YGVY
1904.1223	1904.1225	GW6471	BS^3	QLVTEHAQLVQII **K$_{448}$**K
1777.8477	1777.8465		BS^2G	IYEAYL**K$_{216}$**NFNM$_{ox}$NK
2303.3093	2303.3092		BS^2G	V**K$_{224}$**ARVILSGKASNNPPFVIH
1449.7191	1449.7155	YS81	BS^2G	NFNMN**K$_{222}$**V **K$_{224}$**AR
902.5190	902.5193	GW6471	BS^2G	E**K$_{252}$**TLVAK
902.5191	902.5193	YS81	BS^2G	E**K$_{252}$**TLVAK
634.3082	634.3083		BS^2G	EP**K$_{358}$**F
634.3082	634.3083	YS81	BS^2G	EP**K$_{358}$**F
1820.9068	1820.9069	GW6471	BS^2G	DRPGLLNVGHIE **K$_{399}$**MQ
1590.8781	1590.8783	GW6471	BS^2G	DDIFLFP**K$_{425}$**LLQK

[a] Masses are given for D_0 cross-linkers. All sequences were confirmed by MS/MS data. [b] XL: cross-linker. [c] M_{ox}: oxidized methionine.

accessibility of a protein. These lysines that were found to be modified by a partially hydrolyzed cross-linker (Table 2 and Supporting Information Table 1) also differ depending on the state of the protein (free vs ligand-bound). As such, lysine-425 and lysine-399 were found to be modified by a partially hydrolyzed cross-linker **3a** only in the PPARα/**1** complex. In addition, lysine-222 was exclusively found to be modified by a partially hydrolyzed cross-linker **3a** when **2** was bound (Table 2). MS/MS data of a PPARα peptide that was found to be modified by a partially hydrolyzed cross-linker are presented in Supporting Information Figure 1.

In summary, the cross-links found in the complex between PPARα and the novel agonist **2** did not show a conformational change in the flexible Ω loop and the AF2 helix as was observed for antagonist **1**. This indicates that the agonist **2** does not bind in the binding pocket of PPARα in the same manner as the antagonist **1** does but seems to exhibit a different binding behavior. Whereas the antagonist **1** is able to fill the T-shaped binding pocket and therefore yields a number of cross-linking products by stabilizing the protein conformation, the novel agonist **2** does not fill the binding pocket and therefore yields less cross-linking products. In fact, the cross-linking data of free vs **2**-bound PPARα are very similar, indicating that **2** does not induce a conformational change in PPARα as does **1**. There are indications that the binding pocket of PPARα adopts a similar stable conformation in the free and in the agonist-bound form.[34] We cannot, however, exclude the possibility that for **2** we were not able to detect specific cross-links, which would point to a different conformation of PPARα upon ligand binding.

Conclusions

We demonstrate that with our approach we are able to readily detect conformational changes in PPARα upon low-molecular weight compound binding, proving that our strategy can serve as basis to efficiently characterize target protein−drug interactions. This innovative method can be expected to be of particular importance for these proteins, which are not amenable to other structural analysis methods, such as NMR spectroscopy or X-ray crystallography. It possesses a great potential for the pharmaceutical industry as femtomole amounts of target protein−drug complexes might be analyzed.

Experimental Section

Expression and purification of the ligand-binding domain (LBD) of PPARα was carried out as described recently.[27]

Reagents. Nano-HPLC solvents were spectroscopic grade (Uvasol, VWR, Darmstadt, Germany). Water was purified with a Direct-Q5 water purification system (Millipore, Eschborn, Germany). The endoproteases trypsin (cleaves C-terminal side of lysine and arginine except when either is followed by proline), chymotrypsin (cleaves C-terminal side of phenylalanine, valine, methionine, and leucine), and AspN (cleaves N-terminal side of aspartic acid and glutamic acid, all sequencing grade) were obtained from Roche Diagnostics (Mannheim, Germany). MALDI matrixes and proteins for MALDI-TOF-MS calibration were obtained from Bruker Daltonik (Bremen, Germany). All other chemicals were purchased from Sigma (Taufkirchen, Germany).

For chemical cross-linking, an equimolar mixture of PPARα and ligand (10 μM each, final concentration) containing 20 mM HEPES buffer, 150 mM NaCl, 1 mM TCEP, 10% glycerol, pH 7.4, was incubated for 30 min at room temperature. Cross-linkers **3a** (BS^2G) and **3b** (BS^3, 200 mM stock solutions in DMSO) were prepared as 1:1 mixtures of nondeuterated (D_0) and four times deuterated (D_4) reagent, added in 50 and 100 M excess over the protein/ligand complex, and the reactions were allowed to proceed for 30 and 60 min. After quenching with ammonium bicarbonate (20 mM final concentration), reaction mixtures (200 pmol of PPARα) were separated by one-dimensional gel electrophoresis (SDS−PAGE). Gel bands of interest were excised and in-gel-digested with trypsin and endoproteinase AspN.

Nano-HPLC/MALDI-TOF/TOF-MS. Proteolytic peptide mixtures were separated by nano-HPLC (Ultimate 3000, Dionex Corporation, Idstein, Germany) on a C18 column (PepMap, 75 μm × 150 mm, 3 μm, 100 Å, Dionex, Idstein, Germany) using a 30 min gradient of 5−50% B (B, 80% acetonitrile, 0.04% TFA) at flow rates of 300 nL/min. The eluates were fractionated postcolumn (15 s per spot) onto a 384 MTP 600 μm AnchorChip MALDI target (Bruker Daltonik, Bremen, Germany) using an LC/MALDI fraction collector (Proteineer fc, Bruker Daltonik, Bremen, Germany) controlled via HyStar 3.2. MALDI-TOF/TOF-MS was performed in positive ionization mode on an Ultraflex III instrument (Bruker Daltonik, Bremen, Germany) equipped with a Smart beam laser[35] using α-cyano-4-hydroxycinnamic acid (HCCA) as matrix.

Nano-HPLC/Nano-ESI-LTQ-Orbitrap-MS. Fractionation of the peptide mixture was carried out on an Ultimate 3000 β Nano-HPLC system (Dionex Corporation, Idstein, Germany) using a C18 column (Acclaim, 75 μm × 150 mm, 300 Å, Dionex) using a 120 min gradient of 0−100% B (B, 30% ACN, 0.1% FA). The nano-HPLC system was directly coupled to the nano-ESI source (Proxeon, Odense, Denmark) of an LTQ-Orbitrap hybrid mass spectrometer (ThermoFisher Scientific, Bremen). The mass spectrometer was operated in positive ionization mode with data dependent MS/MS acquisition of the three most abundant signals of the corresponding survey scan.

Analysis of Cross-Linked Products. Cross-linked products were identified by analyzing the MS data with the CoolToolBox software program, which is a major upgrade of the VirtualMassSpectrometryLab (VMSL) software.[36] MS/MS data of cross-linked products were identified using General Protein Mass Analysis for Windows (GPMAW[37]), version 8.00 (Lighthouse Data, Odense, Denmark,

http://www.gpmaw.com), and BioTools 3.1.1.36 (Bruker Daltonik, Bremen, Germany).

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Supporting Information Available: The tandem mass spectrum of a hydrolyzed cross-linked product of PPARα (amino acids 251−257) with **3a** in the presence of the antagonist **1**; a complete list of hydrolyzed cross-linking products in ligand-free PPARα, PPARα/**1**, and PPARα/**2** complexes with NHS esters **3a** and **3b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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