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Involvement of Covalent Interactions in the Mode of Action of PPARβ/δ Antagonists

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Åsmund Kaupang,*[a] Siri Hildonen,[a] Trine G. Halvorsen,[a] Magnus Mortén,[b] Anders Vik[a] and Trond Vidar Hansen*[a]

A broad range of chemical structures modulate the inductive and repressive transcriptional regulation of the peroxisome proliferator-activated receptor β/δ (PPARβ/δ). In order to shed light on mechanistic differences in the modes of action of three classes of the reported PPARβ/δ antagonists, an investigation into their *in vitro* biological and chemical reactivities, with particular focus on covalent reactivity, was undertaken. The results reported here, substantiate the covalent modification of Cys249 as a part of the mode of action of the 5-trifluoromethyl-2-sulfonylpyridine class of antagonists. In contrast, GSK0660 does not appear to be a covalently binding antagonistic ligand. Additionally, we demonstrate the electrophilic nature of the recently published antagonist DG172 towards thiolates, although a covalent adduct with PPARβ/δ is not detected in our experiments.

Introduction

The peroxisome proliferator-activated receptor β/δ is one of three known members of the PPAR subfamily of nuclear receptors (α, β/δ and γ, NR1C1 – 3). PPARβ/δ is widely distributed in the human body and its transcriptional regulation affects the production of a proteome involved in processes such as energy uncoupling,[1] lipid metabolism,[2] inflammation,[3] proliferative regulation[4–6] and the pathophysiologies of cancer[7,8] and psoriatic lesions.[4,9]

Molecules that exert classical PPAR agonism have received attention from the medicinal chemistry community as candidates in the development of drugs to treat metabolic disorders, such as atherosclerosis, dyslipidemia and type II diabetes mellitus. So far, only compounds targeting PPARα and PPARγ have seen clinical applications.[10,11] On the other hand, reports from the last decade on the multi-faceted transcriptional regulation of the PPARs,[12–22] have stimulated an interest in partial- and antagonistic modulators, that may display improved toxicity profiles compared to synthetic, classical agonists and their derivatives, as well as expand the horizon of the therapeutic applications of PPAR modulation.[23,24]

Canonically, ligand-dependent transcriptional induction by a PPAR receptor bound to an agonist occurs through the release of bound corepressor proteins and subsequent recruitment of coactivator proteins to the liganded PPAR/RXR heterodimer. This, in turn, leads to chromatin remodelling and the engagement of the transcriptional machinery.[25,26] In the absence of agonists, PPARβ/δ interacts with corepressor proteins such as silencing mediator for retinoid and thyroid hormone receptors (SMRT), SMRT and histone deacetylase-associated protein (SHARP) or nuclear receptor corepressor (NCoR).[27–30] These interactions have been linked to the active role of PPARβ/δ in the repression of PPARα- and PPARγ target gene transcription induced by agonists against these receptor subtypes.[28] During the last decade, *in vitro* assays that aim to describe the molecular events occurring prior to transcriptional induction or repression, such as those based on time-resolved fluorescence resonance energy transfer (TR-FRET),[31] have become more frequently employed in the characterization of PPAR ligands. The various formats of these assays have yielded data on the affinity of a ligand for a given receptor, but also on the affinity profile of the resulting ligand-receptor complex for fluorescently labelled peptides derived from various coregulator proteins. In the context of PPARβ/δ antagonists, results from the latter format of these assays indicate that some, but not all, of the reported antagonistic ligands positively affect the affinity of the ligand-receptor complex for corepressor proteins.[32,33] These findings encouraged us to study events involved in the binding of a selection of the PPARβ/δ antagonists reported to date, in order to provide grounds for further classification of these ligands.

The PPARs share a conserved cysteine residue, located centrally in the ligand binding pocket (LBP). The nucleophilicity of its thiol moiety towards electrophiles of both endogenous and synthetic origins, has been demonstrated in all three PPAR subtypes and the functional outcomes of covalent ligation of the central cysteine have ranged from agonism[34–41] to

[a] Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, PO Box 1068, Blindern, 0316 Oslo, Norway.
[b] Department of Chemistry, University of Oslo, PO Box 1033, Blindern, 0315 Oslo, Norway.

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antagonism.[42–45] In PPARβ/δ, the binding of the few ligands reported to covalently modify Cys249 has resulted in antagonism.[43–45]

The covalent ligation of the central cysteine residue may impact on several aspects of the structural dynamics of PPARβ/δ, and consequently on its transcriptional regulation. The conserved triad of residues, located between helices 5, 11 and 12 (His287/His413/Tyr434, Figure 1), are thought to play an important role in the stabilization of helix 12 in its active conformation, through the formation of a network of hydrogen bonds e.g. involving the carboxylate head group of fatty acid agonists.[26,46] The covalent attachment of a molecular fragment centrally in the LBP, may thus directly perturb the interactions of these residues or impede the access of agonists to this arm of the LBP. In parallel, a covalent modification of Cys249 may affect the structural dynamics of other residues in helix 3, such as the conserved residue Lys265, which together with Glu435 in helix 12 (Figure 1), constitute a pair of ionic contacts that are instrumental to the binding of coactivator proteins.[26,47,48] In summary, more research into the structural basis of PPARβ/δ antagonism is needed to elucidate these mechanisms.

Since the discovery of PPARβ/δ in *Xenopus* and in humans,[54,55] a structurally diverse collection of antagonistic ligands have been discovered, such as sulindac sulfide,[56,57] indomethacin,[56] GSK0660,[58] ST-247,[32] PT-S58[32] and their analogues,[32,59] the imidazoline-2,4-dione "9",[60] SR13904,[61] carboxylic acids "3a" and "3g – i",[62] FH535,[63] GSK3787,[44] CC618[45] and DG172.[64] We chose to study the *in vitro* biological and chemical reactivities of GSK3787 (**1**), CC618 (**2**), GSK0660 (**3**) and DG172 (**4**) (Figure 2) using Mass Spectrometry (MS) techniques and Nuclear Magnetic Resonance (NMR) spectroscopy, in order to shed light on the involvement of covalent interactions in their mode of action. These ligands

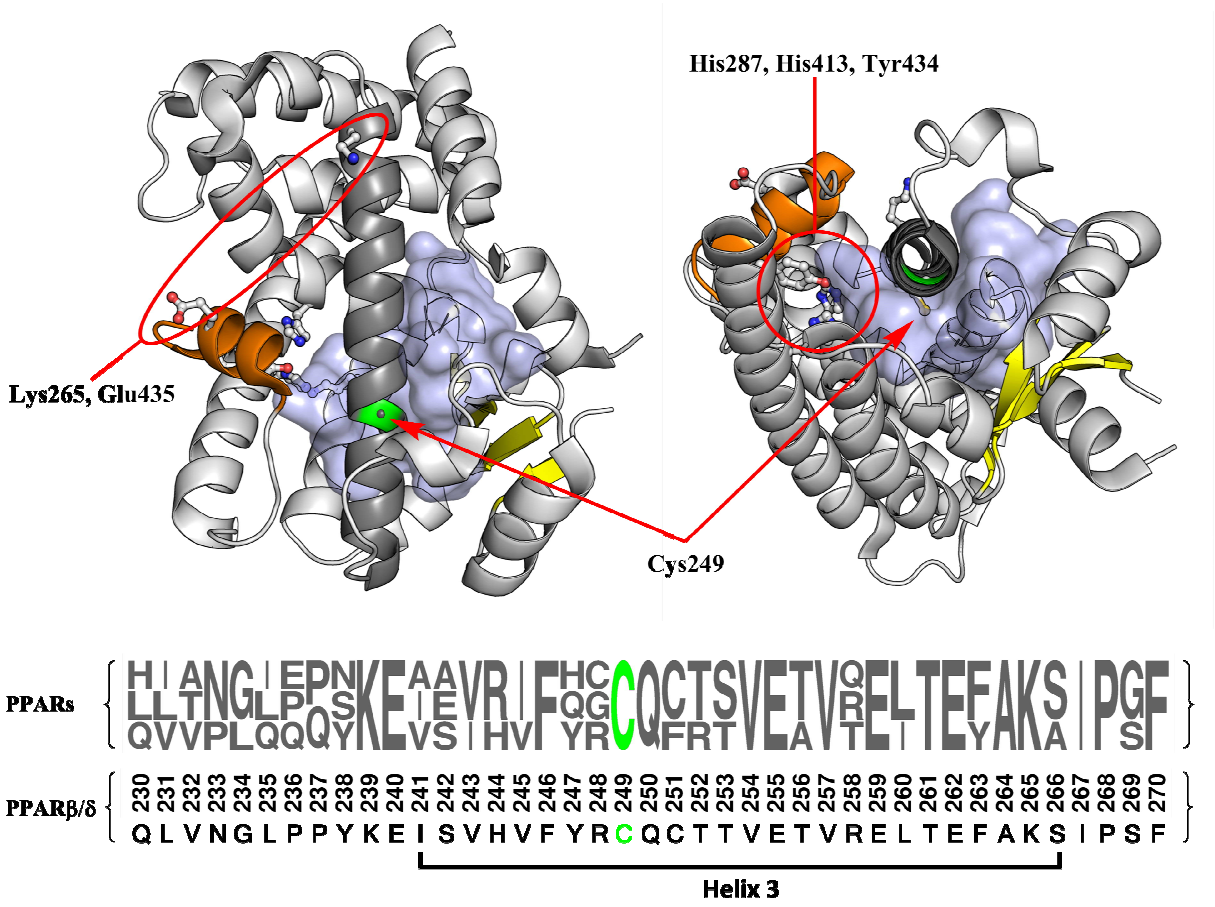


Figure 1. *Top*: The structure of the PPARβ/δ protein in the active conformation (PDB Code: 2XYX).[49] The conserved cysteine, Cys249 (UniProt hPPARβ/δ numbering[50]) is shown in green, helix 3 in dark grey and helix 12 in orange. A surface of the ligand binding pocket (LBP) is shown in light blue (generated with HOLLOW[51]/PyMOL[52]). *Bottom*: A WebLogo[53] representation of residues 230 – 270 (in PPARβ/δ), generated from a sequence alignment of the three known PPARs, with the corresponding PPARβ/δ sequence shown below the residue numbers. The conserved cysteine residue is shown in green, inside the boundaries of helix 3, denoted by the bracket.

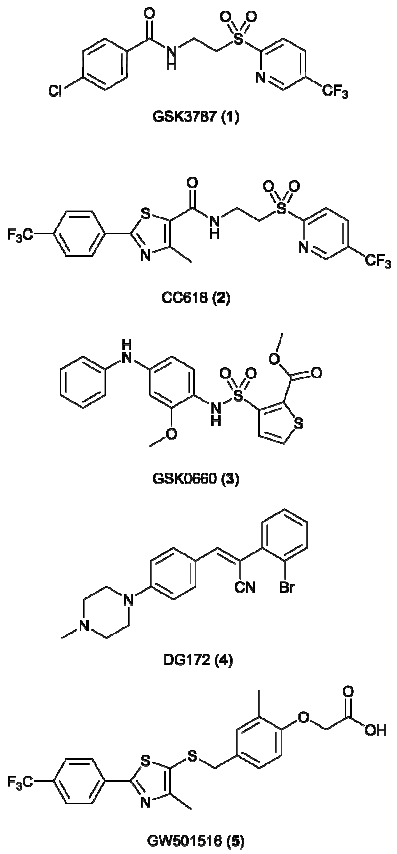
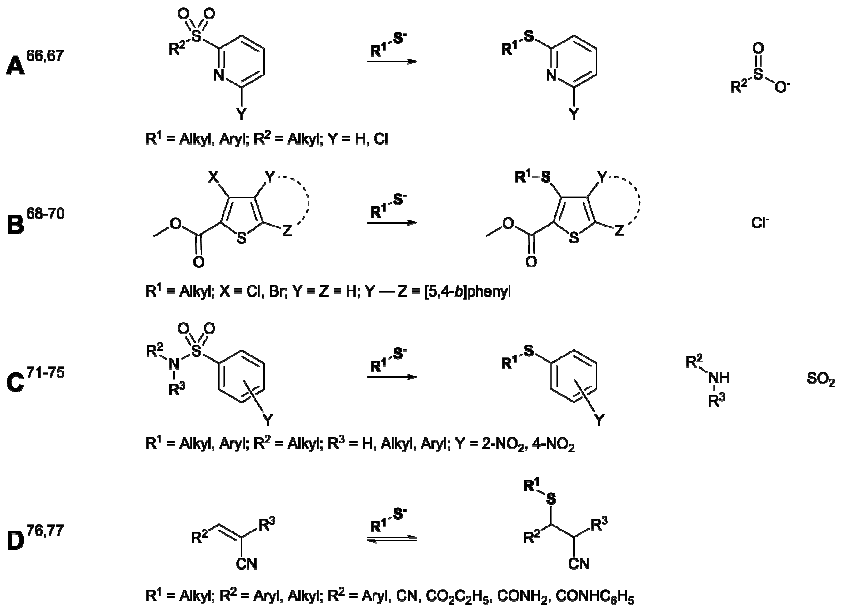


Figure 2. The structures of the PPARβ/δ antagonists GSK3787 (**1**), CC618 (**2**), GSK0660 (**3**) and DG172 (**4**), and that of the agonist GW501516 (**5**).

were chosen based on their respective reports, as well as on the chemical literature describing the reactivity of electrophilic motifs similar to those found in **1** - **4**. These chemical reactivities, summarized in Scheme 1, are detailed below together with a brief introduction to each ligand.

GSK3787 (**1**, Figure 2) was introduced in 2010 as an antagonistic ligand that was found to covalently modify PPARβ/δ.[44] CC618 (**2**, Figure 2) was disclosed more recently, as a structural hybrid between the potent PPARβ/δ agonist GW501516[65] (**5**, Figure 2) and GSK3787 (**1**) that displayed PPARβ/δ-selective antagonism, with similar *in vitro* effects to those of GSK3787.[45] Notably, both ligands contain a 5-trifluoromethylpyridin-2-yl sulfone moiety. Similar electron-poor pyridin-2-yl sulfones have been demonstrated to be reactive towards thiolates in nucleophilic aromatic substitution (S_NAr) reactions (Scheme 1, A).[66,67] Based on these findings, we included **1** and **2** in our study, in order to examine their reactivity towards PPARβ/δ from a chemical perspective.

The PPARβ/δ antagonist GSK0660 (**3**, Figure 2) was identified in a high-throughput screen in 2008, in which it displayed high affinity and selectivity for PPARβ/δ in ligand displacement assays, but did not induce transcription in a GAL4 chimera reporter gene assay.[58] In a chemical context, it has been shown that 3-halothiophene-2-carboxylates may undergo nucleophilic aromatic substitutions with thiolates,[68] although these reactions proceed in higher yields with added copper metal or with the corresponding benzothiophenes as substrates (Scheme 1, B).[69,70] Furthermore, the reactivity of electron-poor arylsulfonamides, such as nitrobenzenesulfonamides, towards thiolates, has been studied and exploited in the synthetic preparation of the



Scheme 1. Selected examples of the chemical reactivity of electron-poor aromatic and heteroaromatic structures, towards thiolates, in nucleophilic aromatic substitution (S_NAr) reactions (A – C), as well as examples of 1,4-conjugate additions to acrylonitriles (D).

corresponding amines - a reaction that also produces 2- or 4-nitrophenylsulfides through a nucleophilic aromatic substitution mechanism (Scheme 1, C).[71–75] In summary, we found it prudent to consider the possibility that the methyl 3-sulfamoylthiophene-2-carboxylate head group of GSK0660 (**3**) could be reactive towards a nucleophilic cysteine residue.

The cyanostilbene DG172 (**4**, Figure 2) is a potent antagonist of PPARβ/δ, introduced by Lieber *et al*. During the SAR that led to its discovery, the authors also evaluated a series of compounds incorporating stilbene skeletons lacking the electron-withdrawing nitrile group on the central alkene moiety, and found that these were significantly less potent binders of PPARβ/δ.[64] These results provided support for the importance of the acrylonitrile moiety of DG172 (**4**) in its affinity for PPARβ/δ. The high potency of DG172 (**4**) as a PPARβ/δ ligand, combined with the demonstrated propensity of similar acrylonitriles to undergo 1,4-conjugate addition of thiolates (Scheme 1, D),[76,77] rendered the inclusion of DG172 (**4**) in our study of significant interest**.**

Results and discussion

LC-MS/MS and ESI-MS analyses

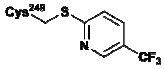
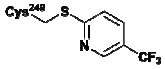
Previously, we have reproduced the LC-MS/MS findings of Shearer *et al.*[44] with GSK3787 (**1**), as well as with our analogue CC618 (**2**) (Entries 1 and 2, Table 1).[45] Thus, we initiated this investigation, by subjecting purified PPARβ/δ ligand binding domain (LBD) to each of the remaining antagonists GSK0660 (**3**, Figure 2) and DG172 (**4**, Figure 2), and analyzed the peptides obtained by tryptic digestion of the protein-ligand mixtures (see experimental details and mass spectra of the Cys249-containing peptides in the ESI). In parallel, we treated methanol solutions of each of the four antagonists (**1** – **4**) with 2-mercaptoethanol (2-ME) in potassium phosphate buffer (PB) and analyzed the resulting mixtures with electrospray ionization mass spectrometry (ESI-MS) (Table 1). In summary, a covalent modification of PPARβ/δ, was not detected upon treatment with GSK0660 (**3**) or DG172 (**4**) (Entries 3 and 4, Table 1), although GSK3787 (**1**), CC618 (**2**) and DG172 (**4**) were reactive towards 2-ME in buffered aqueous methanol.

These results poised us to further investigate the chemical reactivity of these antagonists towards thiolates using NMR spectroscopy, in order to monitor the formation of the products observed with ESI-MS. Thus, inspired by recent reports involving the use of thiols in aqueous organic solvents at pH 7.0 – 8.0, in the study of the reactivity of their derived thiolates towards conjugate acceptors,[77–80] we subjected DMSO-d_6 solutions of each of the four antagonists listed in Table 1 to 2-ME in potassium phosphate buffer (0.050 M, pH 7.2) and monitored the mixtures by ^{1}H NMR (*vide infra*).

NMR Analyses

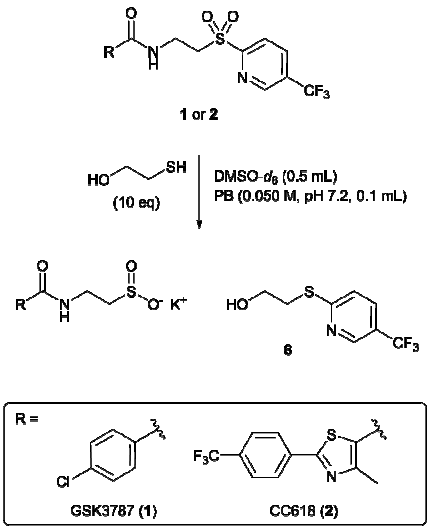
In light of their demonstrated reactivity towards PPARβ/δ,[44,45] we first subjected GSK3787 (**1**) or CC618 (**2**), to the above described conditions (Scheme 2). In this system we observe clean conversions of both the 5-trifluoromethyl-2-sulfonylpyridine antagonists, to give 2-(2-hydroxyethyl)sulfenyl-5-trifluoromethylpyridine **6**, in addition to their respective aroylamidoalkyl sulfinates (Figures S5, S6 and S8 in the ESI). The identities of these products were supported by the observation of their corresponding *m/z* values in the ESI-MS and by comparison of the obtained ^{1}H NMR data with that of the independently synthesized sulfide **6** (see ESI for NMR spectra, synthetic procedures and characterization). These results are thus examples of the known reactivity of pyridin-2-yl sulfones towards thiolates in nucleophilic aromatic substitution (S_NAr) reactions (Scheme 1, A). These reactions, also termed *ipso*-substitutions, have been studied in the context of organic synthesis, as preparative routes to both sulfides and sulfinic acids, the latter of which is produced when the sulfone acts as a leaving group.[66,67] The

Table 1. Summary of mass spectrometrical findings.

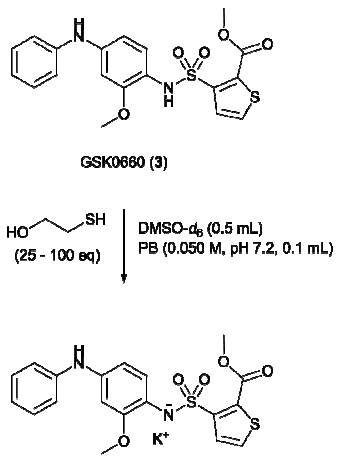
#	Compound	Covalent modification of PPARβ/δ (LC-MS/MS)	Reaction with 2-ME (ESI-MS)	pH[a]
1	GSK3787 (**1**)	Ref. 44,45 	Nucleophilic aromatic substitution	7.2
2	CC618 (**2**)	Ref. 45 	Nucleophilic aromatic substitution	7.2
3	GSK0660 (**3**)	None detected	None detected[b]	7.2 - 7.8
4	DG172 (**4**)	None detected	1,4-conjugate addition	7.2

[a]The initial pH of the buffer.

[b]Decarboxymethylated products were detected (also in the absence of 2-ME).



Scheme 2. The two members of the 5-trifluoromethyl-2-sulfonylpyridine class of PPARβ/δ antagonists (**2** and **3**) react with 2-ME, in phosphate-buffered aqueous DMSO-d_6.



Scheme 3. GSK0660 (**1**) is slowly deprotonated in potassium phosphate-buffered aqueous DMSO-d_6, but does not appear to react with 2-ME.

rate-limiting step in these reactions is likely the formation of a Meisenheimer-type intermediate.[67] The energy barrier of formation of this negatively charged intermediate could, in theory, be lowered by the introduction of electron-withdrawing substituents on the pyridine ring. Correspondingly, an increase in reactivity upon the introduction of an electron-withdrawing substituent, is apparent from the lower temperature (50 °C vs. refluxing ethanol) needed to obtain high conversions of 2-chloro-6-methylsulfonylpyridine, compared to its 2-unsubstituted analogue, in reactions with ethoxide.[66] Bearing in mind this reactivity pattern, we subjected our previously described analogue of CC618 (**2**), 5-H-CC618 (**7**, Figure 3),[45] to the above described NMR-conditions. In the course of 24 hours, we did not observe conversion of 5-H-CC618 (Figures S7 and S8 in the ESI). The observed lack of reactivity of the 5-H-2-(alkylsulfonyl)pyridine moiety of **7**, towards thiols in buffered aqueous DMSO-d_6, is coherent with the observation that 5-H-CC618 does not covalently modify Cys249 in PPARβ/δ (Figure S2 in the ESI) and contrasts the observed electrophilic reactivity of the comparable 5-H-2-(methylsulfonyl)pyridine moiety towards thiolates in dry ethanol.[66]

We then turned our attention to GSK0660 (**3**). By ^{1}H-NMR, we did not observe conversion of **3** in the presence of up to 100 equivalents of 2-ME (Figures S9 and S10 in the ESI). Nevertheless, a limited formation of the GSK0660 sulfonamide anion is observed (Scheme 3 and Figure S9), confirmed by the disappearance of these signals upon acidification of the solution. This result prompted us to run ESI-MS analyses of mixtures with increased buffer capacity (1.0 M), in order to limit the effect the acidic sulfonamide moiety of **3** has on the pH of the mixtures, and thus on the available thiolate concentration. In these mixtures, we did not observe adducts that correspond to reactions between GSK0660 and 2-ME. However, both in the presence and in the absence of 2-ME, we observed some degree of decarboxymethylation of **3** (see mass spectrometrical details in the ESI). Although more frequently observed at higher temperatures, this type of reactivity has been demonstrated for thiophene-2-carboxylates.[81,82] In summary, the relative stability of GSK0660 (**3**) towards thiolates is in line with our observation of unmodified Cys249-containing tryptic peptides in the LC-MS/MS analysis of PPARβ/δ treated with **3** (Figure S3 in the ESI). These results suggest that the binding mode of GSK0660 to PPARβ/δ is non-covalent and reversible.

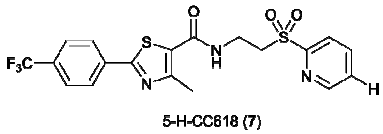
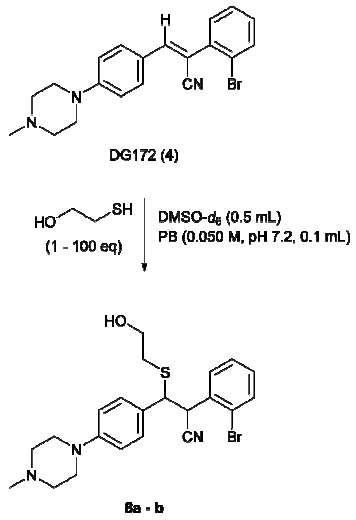


Figure 3. The structure of 5-H-CC618 (**7**).

In the case of DG172 (**4**), we observed conversion of the starting material upon treatment with 1 equivalent of 2-ME, under the above described conditions (Scheme 4). The addition of 2-ME to DG172 (**4**) proceeded with significant conversion of **4** upon increasing the thiol concentration (Figure S12 in the ESI). The products were identified as the expected, diastereomeric 1,4-conjugate adducts, by the appearance of two pairs of doublets around 4.90 and 4.55 ppm in the ^{1}H NMR spectrum, corresponding to the central spin systems of the *syn*- and the *anti* adducts (Figure S11 in the ESI). The origin of the diastereomeric products lies in the stepwise nature of the 1,4-conjugate addition reaction, in which the protonation of the intermediate α-carbanion, resulting from the attack of the thiolate on the β-carbon of the alkene, may occur from either face.[76,77,83,84] The identities of the DG172-2-ME adducts (**8a - b**) were further corroborated by the finding of their



Scheme 4. DG172 (**4**) is reactive towards 2-ME in phosphate-buffered aqueous DMSO-d_6.

corresponding *m/z* value in the ESI-MS analysis, as well as by their independent chemical synthesis and characterization (see experimental details and characterization in the ESI).

The structure of DG172 (**4**) includes a tertiary amine, which under physiological conditions would be partially protonated (calc. pK_a 7.89[85]). At the phosphate concentration present in our NMR assay, the presence of DG172 (7.5 µmol) would significantly influence the pH and, in turn, the available thiolate concentration. Thus, in order to determine in which pH interval the addition of 2-ME to DG172 takes place, we investigated the reaction of DG172 (5 µmol) with 2-ME (10 eq) in 1.0 M phosphate buffers at pH 7.2, 7.5, 7.8 and 8.1 (Figure S11 and Table S3 in the ESI). After 24 hours under these conditions, the 1,4-conjugate addition had proceeded with conversions > 50% at pH 7.8 and 8.1, while at pH 7.2 and 7.5, the conversions were around 10%. Thus, in phosphate buffered aqueous DMSO-d_6, in the presence of 10 equivalents of 2-ME, a thiolate concentration sufficient for significant addition to DG172 (**4**), appears to become available between pH 7.5 and 7.8.

In contrast to the above demonstrated electrophilic reactivity of DG172 (**4**) towards thiolates, we did not observe Cys249-containing peptides with an increased mass, corresponding to a conjugate-type addition of PPARβ/δ to **4**, in the protein LC-MS/MS analysis (Table 1 and Figure S4 in the ESI). Thus, taking cues from previous reports on the reversible covalent modification of a nucleophilic cysteine residue, by acrylonitrile-containing kinase inhibitors,[78] we investigated the reversibility of the 1,4-conjugate addition of 2-ME to **4**, by monitoring the addition reaction and the subsequent dilution of this reaction mixture in phosphate buffer (0.050 M, pH 7.2), with UV-spectroscopy. Our results indicate that, under the investigated conditions, the reaction is slowly reversible (Figure S13 and Table S4 in the ESI). The viability of the reverse reaction did not, however, preclude the isolation of the DG172-2-ME adducts (**8a** - **b**), even though the work-up of the preparative reaction involved washing the organic phase with dilute aqueous sodium hydrogen carbonate (see experimental details in the ESI). On the other hand, the propensity towards reversal of an eventual addition of Cys249 to **4** would be affected by the chemical microenvironment of the PPARβ/δ LBP. We thus employed a time-resolved fluorescence resonance energy transfer (TR-FRET)-based assay to compare the rate of displacement of DG172 (**4**) from the LBP of PPARβ/δ, by a high-affinity fluorescent tracer ligand, to those of the agonist GW501516 (**5**) and the antagonist GSK3787 (see Table S5 and experimental details in the ESI). The TR-FRET assay demonstrated a marked difference between the observed rates of displacement of the ligands and DG172 (**4**) and GW501516 (**5**), which are both readily and similarly displaced, and that of the covalent irreversible antagonist GSK3787 (**1**) ($k_{off} \approx 0$). Furthermore, DG172 (**4**) was displaced at apparently similar rates in both the low- and the high concentration experiments (Figure S14 in the ESI). In summary, while DG172 (**4**) is demonstrably reactive towards thiolates, a covalent modification of Cys249 in PPARβ/δ is not detected in our experiments.

Conclusions

We have described a mechanistically oriented investigation into the modes of action of three classes of PPARβ/δ antagonists, in which their *in vitro* biological and chemical reactivities towards thiolates were evaluated. Our results indicate significant differences in the behaviour of these ligands in the PPARβ/δ LBP, in particular towards the conserved nucleophilic cysteine residue, Cys249. By building on these results, as well as on those of reported investigations into the differential transcriptional regulation by PPARβ/δ in complex with antagonists/inverse agonists,[18,33] future studies may seek to shed light on how the observed mechanistic and structural differences in the binding modes of these antagonistic ligands, affect the transcriptional outcome.

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