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Antagonizing the Androgen Receptor with a Biomimetic Acyltransferase

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Abstract

The Androgen Receptor (AR) remains the leading target of advanced prostate cancer therapies. Thiosalicylamide analogs have previously been shown to act in cells as acyltransfer catalysts that are capable of transferring cellular acetate, presumably from acetyl-CoA, to HIV NCp7. Here we explore if the cellular acetyl-transfer activity of thiosalicylamides can be redirected to other cellular targets guided by ligands for AR. We constructed conjugates of thiosalicylamides and the AR-binding small molecule tolfenamic acid, which binds the BF-3 site of AR, proximal to the coactivator “FXXLF” binding surface. The thiosalicylamide-tolfenamic acid conjugate, YZ03, but not the separate thiosalicylamide plus tolfenamic acid, significantly enhanced acetylation of endogenous AR in CWR22Rv1 cells. Further analysis confirms that Lys720, a residue critical to FXXLF coactivator peptide binding, is a site of acyl-YZ03 acetylation. Under acyl-transfer conditions, YZ03 significantly enhances the ability of BF-3 site binding ligands to inhibit AR-coactivator peptide association. These data suggest that biomimetic acyltransferases can enhance protein-protein interaction inhibitors through covalent modification of critical interfacial residues.

Graphical abstract

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Author Contributions

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Notes

The authors declare no competing financial interest.

Supporting Information

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Complete protocols for cell-based and in vitro assays and analysis methods, supplemental figures, protein MS spectra, complete synthetic protocols and characterization including ^1H -NMR and ^{13}C -NMR spectra.

Protein acetylation is a ubiquitous mechanism for modulating protein function including altering protein-protein interactions in ways that traditional small molecules (inhibitors, agonists and antagonists) cannot. The prevalence of protein acetylation rivals that of protein phosphorylation.^{1,2} Here, we describe a general method to affect targeted acetylation of proteins in cells using small molecules that are charged as acetylthioesters by endogenous metabolites and can subsequently acetylate a protein of interest through ligand-guided proximity. We further demonstrate that a biomimetic acyltransferase can inhibit androgen receptor coactivator peptide association through proximity directed acetylation.

Proximity-directed reactions have been shown to be very useful for labeling proteins both inside and on the surface of cells.³ In addition to using covalent modifying enzyme substrates,⁴⁻⁶ several elegant strategies have been developed that rely on the proximity enhanced reactivity of ligand tethered reagents to selectively modify proteins of interest. Often these proteins need to be in the form of chimeras with high-affinity ligand-binding domains.⁷⁻⁹ Whereas most proximity-directed protein modifications are stoichiometric reactions, Hamachi *et al.* have recently described ligand-directed acyl transfer catalysts based on the common nucleophilic catalyst dimethylaminopyridine (DMAP). In the presence of excess added labeling reagent, these ligand-tethered catalysts can label selectively cell surface proteins by directing the transfer of reactive thioesters.¹⁰ They have also used ligand-DMAP conjugates to target intracellular protein targets but with only partial selectivity.¹¹

Previously, the Appella labs reported that thiosalicylic acid amide (thiosalicylamide) derivatives such as MT-1 (and SAMT-247) are able to catalyze the acetylation and inactivation of the HIV Gag polyprotein (Figure 1a). MT-1 also acts as a nucleophilic acyl transfer catalyst. Rather than requiring the addition of exogenous reagents, MT-1 obtains acetate equivalents from endogenous cellular metabolites, presumably acetyl-CoA, and transfers them to Cys39 and adjacent lysines of the NCp7 domain of gag.^{12,13} Herein we investigate the ability of ligand conjugates of thiosalicylamides to target other proteins of interest such as the androgen receptor (AR).

It was initially unclear if ligand conjugates of MT-1 would similarly acquire acetate in cells, as MT-1 analogs with even modest sized N-alkyl modifications had poor anti-HIV activity.¹³ We first determined if conjugates of MT-1 were acetylated in cells by synthesizing the biotin conjugate YZ01 and attempting to isolate the thioester intermediate from treated cells (Figure 1b). As the proposed thioester intermediate is expected to be labile and the acyl group could be lost to nonspecific nucleophiles within the cellular milieu, we also synthesized probe analog YZ02 that contains a primary amine that can serve potentially as an intramolecular acyl trap (Figure 1b).

HEK293t cells treated with YZ01 or YZ02 in the presence of ¹⁴C-labeled pyruvate or acetate were lysed and the biotin conjugates were isolated using streptavidin beads that were extensively washed before radiolabel incorporation was measured by scintillation counting. Non-specific binding was determined by treating identical aliquots with a large excess of “unlabeled” biotin prior to isolation. We found that despite the addition of the large tethered

ligand, both biotin probes were readily labeled in cells indicating that the thiophenol core was acetylated in a manner similar to that proposed for MT-1 (Figure 1c).

The amount of ^{14}C -pyruvate derived radiolabel incorporation increased with increasing concentrations of compound. YZ02 had roughly twice the amount of acetate label as YZ01 (Figure S1a). Under similar conditions, cells treated with pre-acetylated AcYZ01 in the presence of ^{14}C -labeled acetate incorporated similar levels of radiolabeled acetate demonstrating that thiosalicylamide can be reacylated in cells after acyl transfer and are in principle capable of turnover (Figure S1b).

We then synthesized the AR-targeting conjugate YZ03, which replaces the biotin handle of YZ01 with the AR-binding ligand tolfenamic acid, **3** (Figure 2a).¹⁴ Whereas many selective, high-affinity ligands are known that target the ligand-binding pocket of AR, tolfenamic acid binds to an allosteric site on the surface of the AR ligand-binding domain (termed BF-3), that is flanked by several lysine residues on the receptor surface.¹⁴ Tolfenamic acid is a poor AR antagonist in cells ($\text{IC}_{50} > 30 \mu\text{M}$), however, we have found the propargyl amide **4** to be a submicromolar potent AR partial antagonist ($\text{IC}_{50} = 0.67 \pm 0.10 \mu\text{M}$) in cellular reporter gene assays (Figure 2a and S2). Thus it was envisioned that the tolfenamic amide conjugate YZ03 would be metabolically charged in cells and subsequently transfer its *S*-acetyl group to proximal lysines on the ligand-binding domain (LBD) of AR.

HEK293t cells transiently expressing full-length AR were treated with $20 \mu\text{M}$ (unacetylated) YZ03 or the combination of equal concentrations of unlinked fragments, tolfenamic amide **4** plus thiosalicylamide **5** for 16h. Acetylation of the low-abundance AR cannot be directly identified from the background of numerous endogenously acetylated proteins of the cell but can be readily detected by immunoprecipitation (IP) of AR. Immunoprecipitated AR was then analyzed by western blot using anti-acetyllysine (anti-AcK) antibodies (Figure 2b). Treatment with YZ03 (lane 3), but not vehicle (lane 1) or the combination of **4** plus **5** (lane 2), resulted in a substantial increase in AR acetylation. Significantly, the intensity of YZ03 induced AR acetylation can be attenuated by the addition of the competing ligand tolfenamic amide **4** (Figure 2b, lanes 4 and 5) consistent with a ligand-directed process. We were unable to completely block all AR acetylation with **4** in cells as high concentrations ($>50 \mu\text{M}$) of **4** showed signs of cellular toxicity. Using purified AR ligand-binding domain, AR(LBD), we confirmed *in vitro* that AR(LBD) can be similarly acetylated using pre-acetylated YZ03, AcYZ03 (Figure 2c, lane 1). *In vitro* acetylation by AcYZ03 can be attenuated by $20 \mu\text{M}$ **4** and fully blocked by $50 \mu\text{M}$ **4** (Figure 2c, lanes 2 and 3).

We also confirmed that YZ03 is able to acetylate AR expressed at endogenous levels in the prostate cancer cell line CWR22Rv1. CWR22Rv1 cells were treated with $20 \mu\text{M}$ YZ03 or equal concentrations of the two unlinked fragments, tolfenamic amide **4** plus probe **5** (Figure 2d, lanes 1–3). Western analysis of the immunoprecipitated AR again shows that only the intact YZ03 promotes AR acetylation. A similar pattern is observed at $40 \mu\text{M}$ though the intensity is lower (Figure 2d, lanes 4 and 5). AR acetylation by YZ03 is substantially greater than endogenous acetylation levels which can be detected at longer exposures (Figure 2d middle). Based on comparison to acetylated protein standards, approximately 5% of AR is acetylated (Figure S3).

As further evidence that acetylated YZ03 can serve as a proximity directed reagent, we synthesized the S-acetylated analog of the control **5** (**Ac5**) and compared its ability to acetylate purified AR(LBD) in vitro to acetylation by AcYZ03 (Figure 3a). Again, only AcYZ03, and not the combination of **4** plus **Ac5**, caused detectable levels of AR(LBD) acetylation in vitro. As an additional control, we confirmed in HEK293t cells that the off-targeted biotin ligand conjugate (AcYZ01) did not significantly acetylate AR under conditions where AcYZ03 caused substantial AR acetylation (Figure 3b). We also analyzed the AR content of the immunoprecipitated acetylome (i.e. IP with anti-AcK) of HEK293t cells treated with AcYZ03 (Figure 3c, lanes 1 and 2). Conversely, we selected HSP70 as prototypical off-target protein that contains a similar number of lysines (50 lysines) as AR (40 lysines). No HSP70 could be detected in the vehicle treated or AcYZ03 treated cells (Figure 3c). Finally, we confirmed that neither YZ03 nor the biotin-conjugate YZ01 significantly affected the overall acetylation pattern of cellular proteins, suggesting that YZ01 and YZ03 are not grossly modifying endogenous protein acetylation or deacetylation (Figure S4). Taken together, these studies provide additional support for YZ03 acting through a proximity direct acyl transfer mechanism that uses an endogenous source of acetate to selectively target AR.

The sites of acetylation mediated by YZ03, were evaluated using pre-acetylated YZ03 (AcYZ03) and recombinant AR(LBD) in vitro. Recombinant AR(LBD) was incubated with AcYZ03 at 30°C for 3h and tryptic fragments were analyzed by mass spectrometry (ESI-Orbitrap). The peptide sequence analysis provided 30% coverage of AR(LBD). The peptide ⁷¹⁸WAK(Ac)LPGFR⁷²⁵, with acetylation at Lys720, was the only acetylated tryptic peptide identified with statistical confidence ($q < 0.05$) processed with either C-18 or cation-exchange micro column (Figure 4b and S5). Steered molecular dynamics simulations suggests that in addition to Lys720, the thioacetate of AcYZ03 can readily access several other lysines (Lys717, Lys822, Lys825 and Lys836) when bound to the BF-3 binding pocket (Figure S6). Therefore, other acetylations may occur and these tryptic peptides may not appear as significant by our analysis or perhaps Lys720 is more reactive. Lys720 (18.5Å) is the second closest lysine to BF-3 bound tolfenamic acid (Lys836 is 15.8Å) and is uniquely flanked by both Lys717 and Arg726 which could account for an increased reactivity by effectively lowering its pKa (Figure 4a).

Lys720 is a critical residue for coactivator binding and is part of the “charge-clamp” motif used to bind the conserved amphipathic FXXLF helix of coactivators (Figure 5a).¹⁵ Mutations to Lys720 have been shown to block association of coactivator peptides and suggest that acetylation of Lys720 may similarly affect coactivator association.¹⁶ This presents the intriguing possibility that YZ03 may be able to enhance the antagonist activity of BF-3 site ligands through proximity directed acyl transfers.

Evaluation of YZ03 as an antagonist of AR-dependent transcription in cells proved challenging as the parent ligand (tolfenamic acid-amide) is already an effective AR antagonist. We synthesized control compound, BrYZ03, a nonreactive isostere of YZ03 that has the thiol of YZ03 replaced by an isosteric bromine atom (Figure 2a). However, in reporter gene assays in HEK293t cells, the isostere was a slightly more efficacious antagonist than AcYZ03 (Figure S7). As it is unlikely that the distal bromine significantly

alters ligand binding, we reasoned that the cellular bioavailability of YZ03 might be affected by the propensity of the thiol to oxidize over the course of the 18-hour cellular assay. However, the thiol is an intrinsic part of the YZ03's acyl transfer activity, therefore, we turned to *in vitro* studies to evaluate YZ03's antagonist activity.

Using a modified version of the standard *in vitro* TR-FRET coactivator association assay (Lanthascreen™, Figure 5b and S8) we found that incubation of AR(LBD) with just 10 μ M AcYZ03 reduces dihydrotestosterone (DHT)-induced coactivator association by 36% ($p < 0.0001$) or 5-times greater antagonism than the same concentration of unacetylated YZ03. Just 10 μ M AcYZ03 is also significantly more effective than treatment with acetic anhydride at 100-times the concentration (1000 μ M) (Figure 5c). Additionally, inhibition by unacetylated YZ03 can be enhanced 3-fold by the addition of 100 μ M of the acetyl-CoA mimic, S-acetyl-N-acetyl cysteamine (AcSNAC) (Figure 5c). Together, these studies show that proximity directed acyl transfer reactions can be used as a novel approach to enhance the activity of weak antagonists that bind proximal to the coactivator binding interface.

TR-FRET was also used to estimate the binding constant of AR for YZ03 by synthesizing a fluorescein labeled derivative, FIYZ03 (Figure 2a). Although limited solubility prevented us from obtaining a fully saturated binding curve, we estimated the dissociation constant through direct binding and in competition with AcYZ03 to be approximately 59 μ M (Figure S9). YZ03 is therefore considerably less potent than amide **4**. While one can obviously improve the efficiency of our proximity-directed catalyst by increasing its AR binding affinity, we chose to explore how thioester reactivity might change acyl-transfer efficiency.

We designed acyl transfer catalyst YZ06 (Figure 2a). Compared to YZ03, YZ06 has an intervening methylene group that breaks conjugation of the electron withdrawing amide with the thiophenol and is expected to form a less reactive thioester. *In vitro* acyl transfer assays at 30°C, AcYZ06 is 4.6-times less effective than AcYZ03 as an acyltransfer reagent for AR(LBD) (Figure 3d). In the presence of the competing nucleophiles of cell lysates, structurally similar probes also show the same pattern of reactivity (Figure S10). However, in acyl-transfer reactions performed in HEK293t cells, preacetylated YZ06 (AcYZ06) is 46% more effective at acylating AR without detectable background acylation of control protein HSP70 (Figure 3c, lane 3). In CWR22Rv1 cells, (unacetylated) YZ06 is 2.2-times more efficient at catalyzing the overall acetyl group relay from cellular metabolites to AR (Figure S11). This suggests that tuning the reactivity of the thiol can further improve acyl-relay efficiency, which does not simply parallel the reactivity of the intermediate thioester.

Discussion

Proximity directed reactions commonly rely on stoichiometric reagents tethered to high affinity ligands. Recently, Hamachi et al. developed ligand-tether DMAP catalysts that can work in conjunction with excess thioester labeling reagents to label proteins of interest.¹⁰ Although proximity directed DMAP catalysis was quite selective for cell surface proteins where competing nucleophiles are limited, intracellular protein targets showed only partial selectivity even after optimization of reagent reactivity.¹¹ These DMAP based nucleophilic catalysts are reminiscent of the prior work by Appella et al. who previously demonstrated

that the thiosalicylamide MT-1 can serve as a nucleophilic acyl transfer catalyst capable of inactivating HIV Gag by endogenous cellular metabolites as a source of reactive acetate.¹³ Of course, endogenous acetate is not very useful as a “label”, but it is capable significantly altering protein function when appropriately placed.

Whereas the compact MT-1 structure provides both Gag-NCp7 protein binding and acyl-transfer activity, in this work we have separated ligand binding and acyl transfer functions to direct acetylation to a new protein of interest. In contrast to reactions performed in solution or on the cell surfaces, acyl transfer reactions performed in the cytoplasm are complicated by the myriad of competing nucleophiles within the cellular milieu.^{17,18} We were able to modify the thiol reactivity independent of ligand binding function and confirm that although AcYZ06 is a less effective acylating agent than AcYZ03 in vitro, YZ06 is a more effective acyltransfer catalyst in cells. This suggests that catalyst efficiency is a balance between thiol nucleophilicity and acyl-reactivity, which must function selectively in the presence of a large number of competing nucleophiles within the cell.

While the primary target of MT-1 acetylation is a cysteine that is part of the Gag-NCp7 zinc-finger, YZ03 appears to target cell surface lysines on AR. Lys720 was the only significant AR acetylation site observed by tryptic analysis. Although no cysteine acetylation was observed, one cannot rule out the possibility that cysteine acetylations were lost during processing. In addition to being proximal to the BF-3 binding site, Lys720 is expected to be more reactive by virtue of having a lower effective pKa induced by the adjacent Lys717 and Arg726. Lys720 plays a critical role in coactivator association.¹⁹

The androgen receptor is the principle target of antiandrogens used for the treatment of advanced prostate cancer.²⁰ Mutations acquired under the selective pressure of antiandrogen treatment represent a common pathway that leads to antiandrogen resistance.²¹ While the majority of antiandrogens that target the AR bind to the hormone-binding pocket, new compounds that target the coactivator-binding interface offer an alternate strategy to circumvent mutations associated with resistance to traditional antiandrogens.^{22–24}

Protein-protein interaction inhibitors have long been considered difficult drug targets. Tolfenamic acid and related BF-3 site binders are partial antagonists that are thought to allosterically modulate the AR-coactivator interface.¹⁴ Lys720 is a significant acetylation target of the BF-3 site binding compound YZ03. However, in vitro coactivator binding assays, the acyltransfer reactivity of AcYZ03 greatly enhanced YZ03's ability to block coactivator binding. This study suggests that acyltransfer catalysis can effectively expand or amplify the effects of weak protein-protein interaction inhibitors by chemically modifying adjacent residues important to the binding interface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Cellular labeling of biotin probes. a. Structures of HIV inhibitors SAMT-247 and MT-1 developed by Appella *et al.*; b. biotin conjugate probes; c. labeling of biotin probes (20 μ M) by 14 C-acetate in HEK293t cells.

Figure 2.

Cellular and *in vitro* acetylation of AR by AR-targeting thiosalicylamides. a. Structures of tolfenamic acid analogs and thiosalicylamide conjugates; b. AR acetylation by YZ03 in AR-expressing HEK293t cells. Immunoprecipitated (IP) AR analyzed by western immunoblot (IB) using anti-acetyllysine (AcK) and anti-AR; c. *In vitro* acylation of AR(LBD) by preacetylated AcYZ03; d. Acetylation of endogenous AR in CWR22Rv1 cells.

Figure 3. Selectivity and reactivity of AR-targeting thiosalicylamide conjugates. a. *In vitro* acetylation of purified AR(LBD); b. Acetylation of AR by AR-targeting (AcYZ03) and off-targeting (AcYZ01) in AR-expressing HEK293t cells; c. Analysis of AR and HSP70 content of cellular acetylome of AR-expressing HEK293t cells isolated by Anti-AcK IP; d. *In vitro* acetylation of purified AR(LBD).

Figure 4. Lysines proximal to the AR BF-3 binding site. a. Structure of AcYZ03 directed towards Lys720 after steered molecular dynamics simulation (based on PDB: 2PIX). b. MS/MS fragmentation map of K(Ac)720 containing tryptic peptide.

Figure 5. Coactivator peptide association assay. a. Lys720 forms critical hydrogen-bonding interactions with coactivator peptide (PDB: 1XOW); b. Coactivator association assay by TR-FRET; c. Inhibition of DHT-induced coactivator association determined by TR-FRET.