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Remodelin Is a Cryptic Assay Interference Chemotype That Does Not Inhibit NAT10-Dependent Cytidine Acetylation

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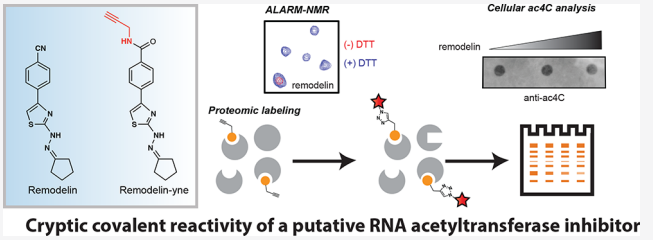
ABSTRACT: Remodelin is a putative small molecule inhibitor of the RNA acetyltransferase NAT10 which has shown preclinical efficacy in models of the premature aging disease Hutchinson–Gilford Progeria Syndrome (HGPS). Here we evaluate remodelin's assay interference characteristics and effects on NAT10-catalyzed RNA cytidine acetylation. We find the remodelin chemotype constitutes a cryptic assay interference compound, which does not react with small molecule thiols but demonstrates protein reactivity in ALARM NMR and proteome-wide affinity profiling assays. Biophysical analyses find no direct evidence for interaction of remodelin with the NAT10 acetyltransferase active site. Cellular studies verify that N4-acetylcytidine (ac4C) is a nonredundant target of NAT10 activity in human cell lines and find that this RNA modification is not affected by remodelin treatment in several orthogonal assays. These studies display the potential for remodelin's chemotype to interact with multiple protein targets in cells and indicate remodelin should not be applied as a specific chemical inhibitor of NAT10-catalyzed RNA acetylation.



Cryptic covalent reactivity of a putative RNA acetyltransferase inhibitor

KEYWORDS: *Acetyltransferase, pan-assay interference, chemical probes, RNA modification, NAT10, Hutchinson–Gilford Progeria Syndrome*

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The identification of remodelin, a putative small molecule inhibitor of *N*-acetyltransferase 10 (NAT10), represents a unique story in modern inhibitor discovery.[1] Inspired by reports of altered chromatin in HGPS,[2,3] in 2013 Larrieu and co-workers tested four histone deacetylase inhibitors and five putative lysine acetyltransferase (KAT) inhibitors for their ability to correct nuclear shape defects in an immortalized human osteosarcoma cell line in which Lamin A/C had been depleted. Of these nine molecules, a compound previously characterized as an inhibitor of yeast GCN5 (CPTH2, **1**, Figure 1)[4] was found to induce nuclear circularity. The observation that **1**, but not other molecules reported to inhibit GCN5, impacted nuclear shape in this model led to the hypothesis that novel targets of **1** may underlie its effects. To test this, a biotinylated analogue of **1** was synthesized and incubated with lysates, which led to the capture and LC-MS/MS identification of >50 proteins. Twenty-nine of these proteins were known to exhibit nucleolar localization, including NAT10. A clickable analogue of **1** was found to colocalize with NAT10 and other nucleolar proteins. Probing the direct interaction of this small molecule with recombinant NAT10 (partially purified from HEK-293 cells) by circular

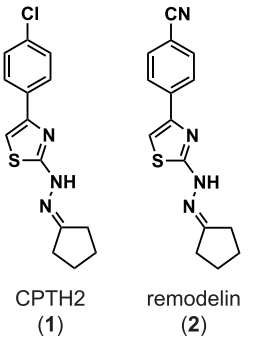

Figure 1. Structures of the putative NAT10 inhibitors CPTH2 (**1**) and remodelin (**2**).

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dichroism found that **1** caused a dose-dependent perturbation of NAT10s structure. Noting the instability of **1**, the authors synthesized an analogue of this compound (remodelin, **2**, Figure 1) which had similar effects on nuclear shape in Lamin A/C-depleted osteosarcoma cells. This molecule's ability to interact with NAT10 purified from HEK-293 cells was probed using a commercial KAT assay kit. Here incubation of NAT10 and tubulin was found to stimulate hydrolysis of acetyl-CoA, an effect consistent with KAT activity, which was limited by **2**. In subsequent follow-up experiments, both remodelin (**2**) as well as genetic knockdown of NAT10, were found to have significant effects on HGPS phenotypes.[1]

This study established that CPTH2 (**1**), remodelin (**2**), and siRNA knockdown of NAT10 have overlapping effects in phenotypic assays of HGPS. However, several recent observations led us to revisit whether **1** or **2** can be properly qualified as an on-target chemical inhibitor of NAT10s acetyltransferase activity. Specifically, a recent analysis of putative KAT inhibitors found **1** possesses several features of an assay interference chemotype.[5] In addition to its previously reported gross chemical instability, this analysis of KAT inhibitors found **1** to be thiol reactive. **1** was also found to inhibit the activity of the bacterial β-lactamase AmpC, indicative of its potential artifactual inhibitory effects in biochemical assays. Furthermore, inhibition of the KAT p300 by **1** was found to be detergent- and thiol-sensitive, suggesting its capacity to inhibit enzymes through nonselective aggregation-based and covalent mechanisms. While small molecules with these properties often exhibit effects in phenotypic assays, they are not generally considered to be optimizable by medicinal chemistry, or useful cellular probes of specific enzyme activities such as NAT10.[6,7] In addition, while a clickable analogue of **1** was found to enrich and colocalize with nucleolar proteins including NAT10 in Larrieu et al.'s initial study, no data for the cellular target occupancy or specificity of remodelin (**2**) was provided, and evidence for its effects on NAT10s acetyltransferase activity was limited to the aforementioned analysis using a commercial KAT assay kit in an $n = 1$ experiment.[1] Finally, a series of papers published since remodelin's discovery have provided substantial evidence that the nonredundant enzymatic activity of NAT10 is catalysis of cytidine acetylation in RNA.[8−10] However, the pan-assay interference characteristics and effects of remodelin on cytidine acetylation were not studied. Given this emerging context, as well as the proposed significance of remodelin as a therapeutic lead in HGPS, we set out to more comprehensively characterize this molecule and its effects on NAT10.

We began our studies by studying the protein reactivity of remodelin **2** by ALARM NMR (Figure 2a). This experiment monitors chemical shift perturbations in the La antigen caused by reaction of its two hyperreactive cysteine residues (C232 and C245) with electrophilic compounds.[11] Compounds are typically tested with and without excess dithiothreitol (DTT), to distinguish protein-reactivity from nonspecific protein perturbation. As previously reported for CPTH2 (**1**),[5] which causes similar effects as remodelin (**2**) in cell-based HGPS assays, analysis of remodelin by ALARM NMR resulted in observation of a DTT-dependent chemical shift perturbation indicative of thiol reactivity. Given the established utility of ALARM NMR in high-throughput screening triage, this experiment flags remodelin as an assay interference molecule potentially capable of nonspecific target engagement in biochemical assays.

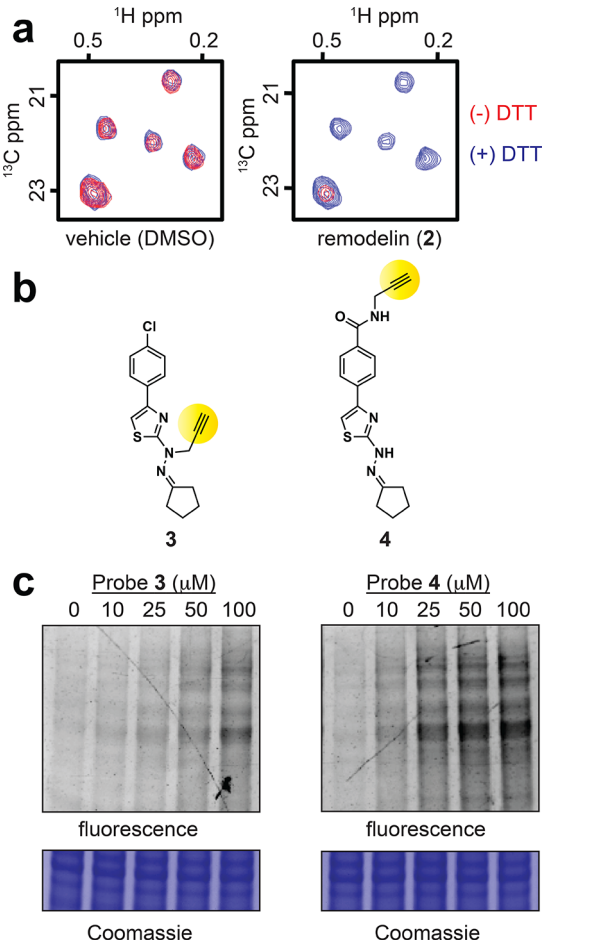

Figure 2. Remodelin is a thiol reactive chemotype. (a) ALARM NMR analysis of DMSO-treated and remodelin-treated La-antigen. Attenuation of chemical shift peaks in DTT-negative samples can be caused by small molecule reactivity. (b) Structure of clickable chemotype probes **3** and **4**. (c) Protein reactivity of clickable chemotype probes **3** and **4** with HeLa cell lysates.

Next we studied the chemical stability of remodelin in solution. In the initial study characterizing the effects of the remodelin chemotype in HGPS, **1** was observed to be unstable to exposure to air and light but has been applied in biological assays over periods of 3−10 days.[1,12] Such chemical instability raises the possibility (in the absence of purity determinations) that degradation products, rather than the parent compound, may be responsible for phenotypic effects in biological assays. To extend our understanding of this phenomenon to remodelin, we analyzed solutions of **2** for stability over time in a number of buffers. Protected from light, we found remodelin to be relatively stable over a period of 7 days in aqueous buffer (data not shown). However, in solutions of DMSO we observed solutions of remodelin to turn a dark brown color, as well as a second set of resonances in the aromatic region to arise (Figure S1). We were unable to identify a differentially eluting product by UHPLC-MS, suggesting this degradation product may be a molecule structurally related to remodelin, such as an isomerization product. Although we were unable to determine the identity of this side product (see Supplementary Discussion in the Supporting Information), our observation suggests it is important to make solutions of remodelin fresh before

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biological analyses, to ensure dosing is being performed with the intended compound.

Previous studies have found that reaction of electrophilic compounds with the CoA thiol can cause false-positives when screening for inhibitors using fluorogenic KAT activity assays.[13] However, remodelin (**2**) did not significantly interfere with this assay reagent, indicating it does not possess gross chemical reactivity with small molecule thiols (Figure S2). Furthermore, and in contrast to the previously characterized **1**, remodelin (**2**) was not found to be an aggregator by the AmpC aggregation counter-screen or possess detectable redox activity (Figure S3).[5] Remodelin did show evidence of colloidal aggregate formation by dynamic light scattering (Figure S3C). This suggests remodelin may possess more subtle pan-assay interference characteristics than its parent compound. To better understand the protein reactivity of this chemotype, as well as its potential proteome-wide consequences, we synthesized two clickable probes (Figure 2b). Probe **3** is identical to the clickable analogue employed by Larrieu and co-workers in their initial identification of NAT10 as a target of CPTH2 (**1**),[1] with a latent alkyne affinity handle installed at the hydrazone NH group. Since the hydrazone conjugated core of remodelin constitutes a potential site of reactivity, we also synthesized analogue **4**, in which this portion of the molecule is unmodified and the alkyne is installed as a propargyl carboxamide at the *para*-position of the phenyl ring. This design preserves the electron-drawing nature of the *p*-substituent of remodelin (**2**). Incubation of **3** and **4** with K562 cancer cell lysates, followed by click chemistry with a rhodamine azide,[14] led to the observation that both probes show dose-dependent labeling. However, probe **4**, which contains the unmodified hydrazone, was found to be more reactive, displaying promiscuous proteomic reactivity at concentrations as low as 10 μM (Figure 2c). This reactivity was competed by remodelin (**2**), albeit weakly, and clickable probe **4** also exhibited less discoloration than remodelin when exposed to ambient light (Figure S4). The observation that *p*-Cl compound **1** is more reactive than **2** in thiol reactivity assays, but that *p*-Cl probe **3** is less reactive than **4** in chemoproteomic labeling experiments, suggests that an unmodified hydrazine is critical for covalent protein labeling by the remodelin chemotype. Interestingly, previous research has found remodelin (**2**) is nontoxic to HGPS cells over prolonged time periods,[1,12] implying that the observed protein reactivity may exert polypharmacological or benign effects, rather than highly cytotoxic consequences. These studies characterize *p*-substituted cyclopentylidene-[4-(phenyl)thiazol-2-yl]-hydrazones as a cryptic assay interference chemotype whose covalent labeling properties are sensitive to the installation of latent affinity handles.

Biochemical assays of NAT10 have proven challenging, with only one study to date reporting reconstitution of the protein's RNA acetyltransferase activity.[8] As an alternative measure of inhibitory potential, we evaluated NAT10-remodelin interactions using a suite of biophysical and chemoproteomic assays (Figure 3a). Analysis of full-length NAT10 by differential static light scattering (DSLS)[15] found the enzyme was stabilized by the endogenous cofactors ATP and acetyl-CoA but not remodelin (Figure 3a). Since full-length NAT10 is unstable and prone to precipitation, we next analyzed the more well-behaved recombinant NAT10 acetyltransferase domain (residues 494−753) by isothermal titration calorimetry (Figure 3b).[16] Here again, the excised domain was found to bind to

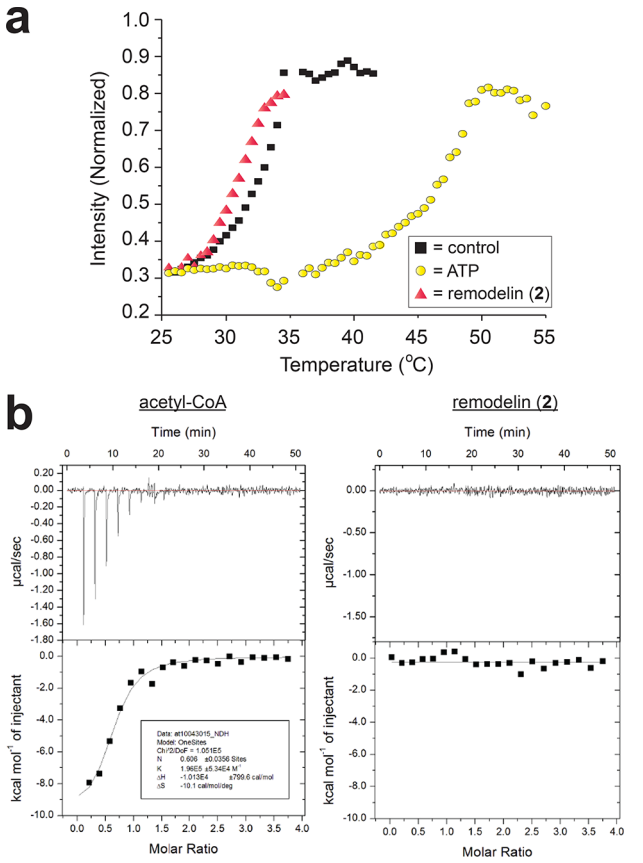

Figure 3. (a) Full length recombinant NAT10 is stabilized by ATP but not remodelin. (b) Recombinant NAT10 acetyltransferase domain binds acetyl-CoA but not remodelin.

acetyl-CoA but not remodelin. Importantly, these experiments do not rule out that remodelin may interact with NAT10 in a manner that is undetectable by our biophysical analysis methods or via a nonactive site-directed mechanism. However, our studies provide no confirmatory evidence for a direct remodelin−NAT10 interaction.

Following the identification of remodelin as a modulator of nuclear shape defects in HGPS, multiple groups reported that NAT10 is a cytidine aceyltransferase enzyme which mediates formation of the minor nucleobase N4-acetylcytidine (ac4C) in RNA.[8,9] NAT10 is the only human enzyme known to catalyze this modification, which has been implicated in regulation of ribosome biogenesis, tRNA half-life, and mRNA stability.[17] Given the utility a specific chemical inhibitor would have in studying these processes, we evaluated remodelin's effects on NAT10-catalyzed ac4C. Evaluation of a previously reported hypomorphic HeLa cell line in which the NAT10 locus had been targeted using CRISPR-Cas9 revealed that NAT10 knockdown (NAT10 KD) affects RNA acetylation far more than tubulin acetylation (Figure S5).[10] This is consistent with previous characterization of this cell line and suggests the nonredundant activity of NAT10 in human cells is catalysis of RNA cytidine acetylation. In contrast to CRISPR-Cas9 disruption, treatment of wild type cells with remodelin did not cause a decrease in ac4C levels by LC-MS (Figure 4a), nor was it observable by dot or Northern blot using an anti-ac4C antibody (Figure 4b, Figure S6).[18] As orthogonal validation measures, we applied both a UV-HPLC assay[9] as well as a recently reported site-specific ac4C sequencing assay to study

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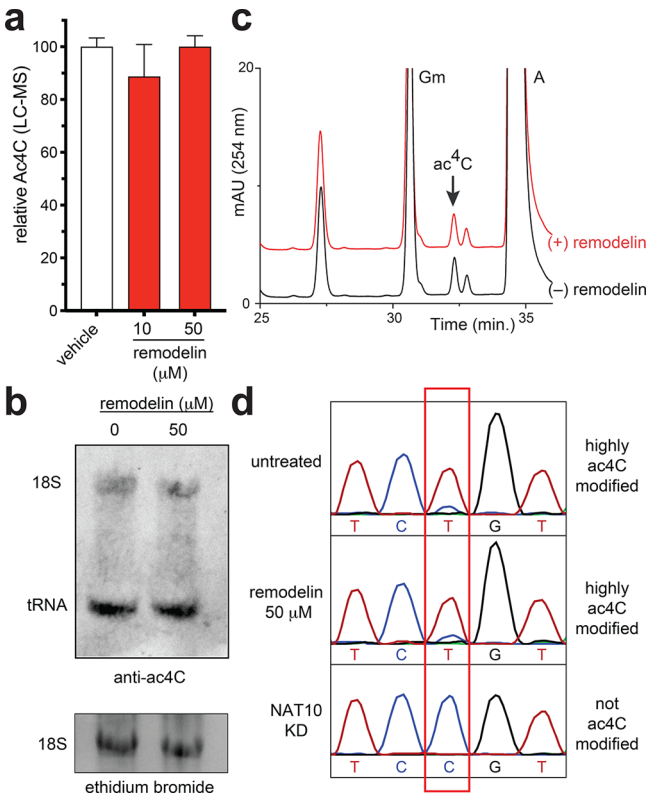

Figure 4. Remodelin does not modulate cellular ac4C levels. (a) LC-MS comparison of ac4C levels in response to remodelin treatment (48 h). (b) Northern blot analysis of ac4C levels in response to remodelin treatment (48 h). (c) Nucleotide resolution analysis ac4C in helix 34 and 45 of 18S rRNA by UV-HPLC after remodelin treatment (50 μM, 4 d). (d) Analysis of ac4C in helix 45 of 18S rRNA (C1842) by nucleotide resolution sequencing assay after remodelin treatment (50 μM, 48 h). C → T transversion indicates ac4C is present at the highlighted nucleotide, while a lack of mutation ("C", blue, exemplified by NAT10 KD) indicates ac4C disruption.

how site-specific acetylation of rRNA was affected by remodelin.[19,20] Again, we observed potent loss of ac4C in the NAT10 KD cell line, but not in response to remodelin treatment (Figures 4c–d and S6). We also did not observe large effects of remodelin on bulk tubulin acetylation in cellular models (Figure S7), although we note that the ability of remodelin to affect tubulin acetylation has not been previously reported in this cell line.[21,22] Overall, these studies indicate that remodelin is not a potent inhibitor of NAT10-catalyzed ac4C in HeLa cells and raise the possibility that its observed phenotypic effects may be unrelated to the RNA modification installed by this acetyltransferase.

Thorough characterization of small molecule inhibitors is critical information for their application as probes of biological function.[23−25] Here we report the assay interference characteristics of the putative NAT10 inhibitor remodelin. Our studies reveal that remodelin can covalently react with proteins, is unstable in DMSO, does not apparently interact with the NAT10 acetyl-CoA binding site, and minimally inhibits ac4C levels as compared to NAT10 knockdown. It is critical to specify that our studies do not conclusively prove that remodelin does not bind to or inhibit NAT10. However, they do indicate that the remodelin chemotype can interact with multiple protein targets in cells. Together with its lack of effect on ac4C, these findings suggest it should not be applied as a specific chemical inhibitor of NAT10-catalyzed RNA acetylation. This has several significant implications. For example, remodelin is marketed as a "NAT10 inhibitor" by multiple companies including Sigma, Tocris, and Cayman. Studies that have used this compound without consideration of its assay interference characteristics may have mistakenly ascribed biological phenotypes arising from its administration specifically to NAT10. In addition, the limitations of remodelin as a NAT10 inhibitor specify the development of a drug-like, reversible inhibitor of this enzyme as a still unmet need in the field. More broadly, remodelin highlights a unique pitfall in small molecule target elucidation, which is the potential for essential genes (such as NAT10) to be mistaken as targets of polypharmacological compounds in phenotypic screens. This is because knockdown of such genes can manifest nonspecific toxicity, which may inadvertently phenocopy a pleiotropic small molecule's effects. Taken thusly, the initial designation of remodelin as a NAT10 inhibitor activity was a plausible conclusion arising from the intersection of a cryptic pan-assay interference compound, an essential gene, and an uncharacterized biochemical activity. However, in the absence of orthogonal validation—for example biochemical reconstitution, determination of target occupancy by cellular thermal shift assay,[26] or rescue of the molecule's effect with an inhibitor-resistant mutant[27]—the evidence that a small molecule such as remodelin targets an essential protein such as NAT10 may be properly qualified as ambiguous.

Our studies raise several areas for future investigation. First, what is the nature of the covalent reactivity in the remodelin chemotype? The thiazole hydrazone core of **1** and **2** is not flagged by conventional assay interference filters (see Supplementary Discussion) and highlights the utility of ALARM NMR in identifying "cryptic" PAIN chemotypes that display protein, but not small molecule, reactivity.[13] Second, can recombinant NAT10 acetylate tubulin, and can remodelin inhibit this activity?[21] Additional structural and biochemical reconstitution studies will be critical to clarifying these disparate activities. Third, do off-targets contribute to remodelin's phenotypic effects in HGPS models? Literature evidence indicates compounds structurally related to **1** and **2** act as monoamine oxidase inhibitors,[28−31] suggesting one possible target class that may warrant investigation. A final question that is irrespective of remodelin's cellular target(s) is how does genetic modulation of NAT10 alter HGPS phenotypes in vitro as well as in vivo?[1,12,32] Interestingly, fibroblasts from HGPS patients have been found to display nucleolar expansion and increased translation,[33] and it is tempting to speculate that NAT10s role in nucleolar RNA biology may mitigate these effects. Of note, upstream ribosome biogenesis has shown efficacy in preclinical models of HGPS.[34] Overall, these studies demonstrate how the thorough characterization of small molecule inhibitors can fuel new lines of chemical and biological inquiry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00193.

Figures S1−S7, supporting schemes, materials, methods, and discussion (PDF)

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Notes

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