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Defining metabolic and non-metabolic regulation of histone acetylation by a NSAID chemotype

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are well known for their effects on inflammatory gene expression. Although NSAIDs are known to impact multiple cellular signaling mechanisms, a recent finding is that the NSAID salicylate can disrupt histone acetylation, in part through direct inhibition of the lysine acetyltransferase (KAT) p300/CBP. While salicylate is a relatively weak KAT inhibitor, its CoA-linked metabolite is more potent; however, the ability of NSAID metabolites to inhibit KAT enzymes biochemically and in cells remains relatively unexplored. Here we define the role of metabolic and non-metabolic mechanisms in inhibition of KAT activity by NSAID chemotypes. First, we screen a small panel of NSAIDs for biochemical inhibition of the prototypical KAT p300, leading to the finding that many carboxylate-containing NSAIDs, including ibuprofen, are able to function as weak inhibitors. Assessing the inhibition of p300 by ibuprofen-CoA, a known NSAID metabolite, reveals that linkage of ibuprofen to CoA increases its biochemical potency towards p300 and other KAT enzymes. In cellular studies, we find that carboxylate-containing NSAIDs inhibit histone acetylation. Finally, we exploit the stereoselective metabolism of ibuprofen to assess the role of its acyl-CoA metabolite in regulation of histone acetylation. This unique strategy reveals that formation of ibuprofen-CoA and histone acetylation are poorly correlated, suggesting metabolism may not be required for ibuprofen to inhibit histone acetylation. Overall, these studies provide new insights into the ability of NSAIDs to alter histone acetylation, and illustrate how selective metabolism may be leveraged as a tool to explore the influence of metabolic acyl-CoAs on cellular enzyme activity.

Keywords

epigenetics; acetylation; inflammation; NSAIDs; ibuprofen

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Supporting Information. Supplementary figures, full gel data, and characterization data for all new compounds. This material is available free of charge via the internet at <http://pubs.acs.org>.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most prevalently prescribed pharmaceuticals in the world. These molecules, which include aspirin, ibuprofen, and salicylate, are utilized to treat a range of conditions ranging from mild aches and pains, to arthritis, to cancer. To date, the most well-characterized mechanism of action of NSAIDs is inhibition of cyclooxygenase (COX) enzymes, which play a key role in biosynthesis of prostaglandins.¹ However, substantial evidence suggests many NSAIDs may engage additional cellular targets.² For example, doses higher than those necessary to inhibit COX are required to maximize the anti-inflammatory effects of some NSAIDs,^{2–3} and these drugs show activity even in COX-deficient cell and animal models.^{4–7} These observations have led to the characterization of additional NSAID targets including I κ B kinase,⁸ AMP-activated protein kinase,⁹ and caspases.¹⁰ In this vein, our groups recently characterized an interaction between the NSAID salicylate and the lysine acetyltransferase (KAT) enzyme p300 (Figure 1).¹¹ Salicylate and its analogues were found to inhibit p300 in biochemical assays, cause p300-dependent inhibition of histone acetylation in cells, and inhibit cell growth in a p300-dependent model of acute myeloid leukemia.¹¹ In addition, a brain penetrant pro-drug of salicylate was shown to inhibit p300-dependent acetylation of tau in cell and animal models of Alzheimer's disease, which resulted in increased tau clearance and rescue of tau-induced memory deficits.¹² It is important to note that salicylate is a pleiotropic drug, and no single target is likely to be wholly responsible for its phenotypic effects. Rather, the significance of these studies is that they i) expand our knowledge of NSAID polypharmacology, ii) specify for the first time the ability of these drugs to influence lysine acetylation, a posttranslational modification (PTM) associated with epigenetic regulation of gene expression and iii) leverage this observation to identify new therapeutic opportunities for these clinically-approved drugs.

Structurally, salicylate and related NSAIDs are defined by the presence of a pendant aromatic carboxylic acid. Notably, this chemical feature is shared with anacardic acid and C646 (Figure S3), two of the most well-known small molecule KAT inhibitors.^{13–15} Two hypotheses have been proposed for the prevalence of this chemotype in KAT inhibitors (Figure 1). First, KATs are known to interact strongly with the negatively charged cofactor acetyl-CoA, and modeling studies suggest aromatic carboxylates may mimic these interactions.^{14–15} Thus, aromatic carboxylates themselves may represent a privileged chemotype for KAT binding. Second, aromatic carboxylates such as salicylate are known to form acyl-CoAs as a key step of their metabolic clearance via glycine conjugation.^{16–17} Our group has recently found that many metabolic acyl-CoAs, including NSAID-CoAs, can potentially interact with KATs in vitro.^{18–20} This suggests the ability of aromatic carboxylates to inhibit cellular histone acetylation may, in part, arise from metabolic formation of NSAID-CoAs. However, the ability of NSAID carboxylates as well as their CoA conjugates to inhibit KAT activity has not yet been systematically explored. Towards this goal, here we define the scope and metabolic dependence of KAT inhibition by NSAID chemotypes. By screening a small panel of NSAIDs for biochemical inhibition of the prototypical KAT p300 we have discovered that many carboxylate-containing NSAIDs, including phenylacetic acids such as ibuprofen, are able to function as modest KAT inhibitors. Further analysis reveals

that CoA conjugation increases the KAT inhibitor potency of ibuprofen, as previously observed with other NSAIDs. Cellular studies reveal that carboxylate-containing NSAIDs, in contrast to the non-carboxylate NSAID celecoxib, inhibit histone acetylation, and that this inhibition does not correlate with NSAID metabolism. Overall, these studies provide new insights into the ability of NSAIDs to alter histone acetylation, and illustrate how selective metabolism may be leveraged as a tool to explore the influence of metabolic acyl-CoAs on KAT activity.

Experimental Section

General materials and methods

Recombinant p300 (1195–1662) was from Enzo and pCAF (503–668) was obtained from Caymen Chemical. (R)- and (S)-ibuprofen and celecoxib were purchased from Santa Cruz. Haloxyfop was purchased from Fluka. Salicylic acid, racemic ibuprofen, naproxen, ketoprofen, diclofenac, 5-fluorosalicylic acid, phenylacetic acid, benzoic acid and sodium acetate were purchased from Sigma. All purchased chemicals were used without further purification unless otherwise noted. All synthesized CoA analogues were HPLC purified, with purity verified by LC-MS prior to use, and concentrations were measured by UV analysis on a Thermo-Fisher Nanodrop 2000 spectrophotometer in distilled and deionized water (ddH₂O) using the molar extinction coefficient (ϵ) for Coenzyme A of 15,000 M⁻¹cm⁻¹ at λ_{max} of 259 nm. Qubit Protein Assay kit (Life Technologies) was used to determine histone extract concentrations. Labchip EZ-Reader 12-sipper chip (#760404) and ProfilerPro Separation Buffer (#760367) were purchased from Perkin-Elmer. Antibodies¹¹ used are as follows: H3K9Ac (9649P), H3K14Ac (7627P), H3K27Ac (8173) antibodies were purchased from Cell Signaling Technologies. The H2B K12/K15Ac (ab1759), total H2B (ab1790), and H3K56Ac (ab76307) antibodies were purchased from abcam. The pan-H4Ac (06–866) and pan-H3Ac (07–690) antibodies were purchased from Millipore Sigma.

KAT inhibition assays

Acetyltransferase activity of p300 (1195–1662) and pCAF (503–668) were assessed using a separation-based assay as reported previously.²⁷ For p300 and pCAF activity analyses, acetylation of a FITC-labeled p300 substrate peptide (histone H4 3–14; FITC-Ahx-RGKGGKGLGKGG) and FITC-labeled pCAF substrate peptide (histone H3 5–20; FITC-Ahx-TARKSTGGKAPRKQL) were separated based on its altered electrophoretic mobility relative to a non-acetylated peptide and quantified by fluorescence detection. Briefly, KAT assays consisting of reaction buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.05% Triton-X-100) with p300 [200 nM] and FITC-H4 [2 μ M] or pCAF [75 nM] and FITC-H3 [2 μ M] were plated in 384-well plates and allowed to equilibrate at room temperature for 10 min in the presence or absence of inhibitor. Reactions were initiated by addition of acetyl-CoA (final concentration = 1 μ M), bringing the final assay volume to 30 μ L. End-point experiments were quenched at appropriate time points (<15% product accumulation) by the addition of 5 μ L of 0.5 M neutral hydroxylamine. Reactions were then transferred to a PerkinElmer Lab-Chip EZ-Reader instrument for analysis by microfluidic electrophoresis. Optimized separation conditions were as follows: downstream voltage of –500 V, upstream voltage of –2500 V, and a pressure of –1.5 psi for FITC-H4 (3–14) and

FITC-H3 (5–20). Percent conversion is calculated by ratiometric measurement of substrate/product peak heights. Percent activity represents the relative acetylation in inhibitor treated reactions relative to untreated control, measured in triplicate, and corrected for nonenzymatic acetylation. IC₅₀ values represent the concentration that inhibits 50% of KAT activity. All calculations were performed using Prism 7 (GraphPad) software.

Cell culture and Western blot analysis

HEK293T cells were cultured at 37°C under 5% CO₂ atmosphere in a growth medium of DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. For histone acetylation analyses of p300 rescue experiments, HEK-293T cells were plated in 6-well dishes (4×10^5 cells/well), and allowed to adhere overnight. At this point, transfections were performed using Lipofectamine 2000 (10 µL/well; Life Technologies) and plasmid DNA (EV and p300 at 5000 ng) according to manufacturer's protocol. Following 48 hours of transfection, cells were dosed with racemic ibuprofen by adding a 10x stock solution as a neutralized sodium salt directly to growth medium at the specified concentrations. Cells were incubated for 24 hours prior to histone extraction. For all other experiments that did not involve overexpression of p300, HEK-293T cells were dosed with (R)-ibuprofen, (S)-ibuprofen, or salicylate by adding a 10x stock solution as a neutralized sodium salt or dosed with celecoxib by adding a 1,000x stock solution in DMSO directly to growth medium at the specified concentrations. Following removal of media, cells were washed once with PBS and incubated with 300 µL of Triton Extraction Buffer (TEB: PBS with 0.5% Triton-X 100, 2 mM PMSF, and 0.02% NaN₃), harvested by gentle lifting, and transferred to microcentrifuge tubes. Samples were incubated in TEB for 30 min on ice and pelleted by centrifugation (6500g x 10 min, 4 °C). Supernatant was removed and saved for cytosolic protein analysis. Nuclei were resuspended, washed again with TEB (150 µL), and pelleted by centrifugation (6500g x 10 min, 4 °C). For acid extraction of histones, each pellet was treated with 0.4N H₂SO₄ (75 µL) and rotated overnight at 4 °C. Samples were centrifuged (11000g x 10 min, 4 °C), and histones were precipitated from the supernatant by addition of 20% TCA (750 µL). After at least 1 hour, samples were centrifuged (16000g x 10 min, 4 °C), and the pellets were washed with acetone/0.1% HCl (750 µL) and neat acetone (750 µL), with centrifugations (16000g x 10 min at 4 °C) following each wash. Samples were air-dried at room temperature, dissolved in ddH₂O, and quantified by Qubit protein assay kit (Life Technologies). Samples were prepared for western blot analysis using Bis-Tris NuPAGE gels (12%) and MES running buffer in Xcell SureLock MiniCells (Invitrogen) according to the manufacturer's instructions. Following primary antibody incubation, washing, secondary antibody incubation and washing according to the manufacturer's instructions (Cell Signaling Technology), Lumiglo (CST #7003) was added and the chemiluminescent signal was visualized using an ImageQuant Las4010 Digital Imaging System (GE Healthcare). Immunoblot signals were quantified using ImageQuant TL software (GE Healthcare).

Analysis of cellular acyl-CoAs

Ibuprofen-CoA was quantified by modification of a previous method for acyl-CoAs.²¹ Briefly, cell pellets were quenched with 10% trichloroacetic acid, spiked with [¹³C₃¹⁵N₁]-acyl-CoA internal standards generated as previously described,^{22–23} and extracted by solid phase extraction using an Oasis HLB column (Waters, Milford, MA). Eluent was evaporated

to dryness under nitrogen, resuspended in 50 μ L 5% sulfosalicylic acid in water, and 5 μ L was injected onto a Waters XBridge C18 column (2.1 $\times$ 100 mm, 3.5 μ m) in an Ultimate 3000 UHPLC coupled to a Q Exactive Plus MS. Ibuprofen-CoA was quantified by its M+H ion in full scan (m/z 956.2426), with confirmation of molecular identity by the specific CoA derived fragment at M-507 (M-506.9957 with accurate mass, m/z 956.2->449.2469) at the same retention time. Acyl-CoAs were quantified by their matching stable isotope labeled internal standard, or in the case of Ibuprofen-CoA, by [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-acetyl-CoA.

Results

Structurally diverse NSAIDs can inhibit p300 in biochemical assays

To better understand the potential of carboxylate-containing NSAIDs to modulate protein acetylation, we first assessed a small panel of NSAIDs and related compounds for inhibition of p300 activity in biochemical assays. In designing our panel, we focused on analogues of benzoic acid (salicylic acid, 5-fluorosalicic acid, benzoic acid), phenylacetic acid (diclofenac and phenylacetic acid), and methylphenylacetic acid (ibuprofen, ketoprofen, naproxen, haloxyfop; Figure 2a). Our reasoning was that each of these chemotypes incorporate a putative KAT-binding carboxylate, and have further been shown to undergo metabolism to acyl-CoA thioesters in cells and/or organisms.¹⁷ Since salicylic acid is a relatively weak p300 inhibitor in biochemical assays, we assayed each compound at 1.25 mM, analogous to concentrations applied in fragment screens. In addition, we included the organic carboxylate acetic acid as a control. Consistent with the literature, we saw modest inhibition of p300 by salicylic acid (Figure 2b). The most potent compound was diclofenac (>50% inhibition at 1.25 mM), followed by the methylphenylacetic acid compounds haloxyfop and (S)- and (R)-ibuprofen. Of note, acetic acid itself did not cause significant inhibition of p300, indicating additional binding determinants besides a carboxylate moiety are necessary for KAT inhibition. These studies suggested inhibition of KATs by NSAID chemotypes may extend beyond the salicylate-p300 pair, and inspired us to further characterize these interactions.

NSAID-CoA metabolites inhibit KATs with 100-fold greater potency than parent compounds

Next, we sought to more fully characterize the inhibition of p300 by NSAID chemotypes, focusing on ibuprofen. Our rationale for these studies stemmed from the fact that i) both (R)- and (S)-ibuprofen were relatively potent hits in our initial NSAID-p300 screen, ii) ibuprofen is one of the most utilized pharmaceuticals on earth, so any new insights into its mechanism may be useful, and iii) ibuprofen's metabolism to CoA analogues is well-characterized, making it an ideal test case to assess the relevance of these metabolites to KAT inhibition.²⁴ As an initial step, we performed biochemical studies to better understand the potency and selectivity of KATs by NSAID and NSAID-CoA metabolites in vitro. To facilitate these studies, (R)- and (S)-ibuprofen CoA were prepared from their parent carboxylic acids using a conventional two step synthesis (Figure 3a, Scheme S1).²⁵⁻²⁶ Next, a recently developed microfluidic mobility shift assay was used to assay these compounds in dose-response for inhibition of the prototypical KATs p300 and pCAF.²⁷ We found that both stereoisomers of ibuprofen were modest inhibitors of p300, with IC₅₀ values similar to those previously

observed for salicylate (~6 mM; Figure 3b-c). However, in contrast to salicylate which selectively inhibits p300 over pCAF, ibuprofen was found to inhibit pCAF with slightly greater potency. Exploring the metabolites of these compounds, we found that conjugation to CoA greatly increased the inhibitory effects of these compounds. (R)- and (S)-ibuprofen-CoA were found to be ~200-fold more potent than their parent carboxylates, and ~10-fold more potent than salicyl-CoA towards both p300 and pCAF. NSAID-CoAs show overall similar, but modestly reduced, inhibitory potency relative to reference compounds that have been previously studied using mobility shift assays, including C646 (p300 IC₅₀ ~7 μ M)¹⁵ and CoA (Gcn5 [pCAF homologue] IC₅₀ ~1–5 μ M).²⁸ Interestingly, we found that testing salicylate as its cognate salicyl-CoA reverses its selectivity and makes it a more potent inhibitor of pCAF than p300, which had not been previously investigated.¹¹ In the case of ibuprofen-CoAs, nearly identical inhibitory potency was observed for both the (R)- or (S)-stereoisomer (Figure 3c). This is consistent with the biochemical effects of the parent carboxylates, as well as previous studies of KAT bisubstrate inhibitors which have shown the CoA binding tunnel of p300 can accommodate methylene groups near the thiol substituent.²⁹ These studies benchmark ibuprofen as a p300 inhibitor, and confirm that CoA-linkage of NSAIDs potentiates KAT inhibition in biochemical assays. These findings prompted us to further examine the effects of these molecules on acetylation in biological settings.

The commonly utilized NSAID ibuprofen inhibits cellular histone acetylation

Previous studies have found that carboxylate NSAIDs, specifically salicylate and diflunisal (Figure S3), can inhibit histone acetylation in the human embryonic kidney cell line HEK-293.¹¹ Therefore, we used this model to assess the effects of ibuprofen on cellular acetylation. Cells were subjected to treatment with (R)-ibuprofen (0.5–2.5 mM) or vehicle control for 24 hr, and assessed for changes in histone acetylation by Western blot (Figure 4a). This analysis revealed dose-dependent inhibition of several different p300-regulated acetylations by ibuprofen. The most potent inhibition was observed for H2BK12/K15Ac and H3K56Ac, consistent with previous studies of salicylate.¹¹ Ectopic overexpression of p300 rescues ibuprofen's inhibition of histone acetylation at high concentrations (2 mM) (Figure S1). While this rescue effect is consistent with a cellular ibuprofen-p300 interaction, it is important to note that it does not conclusively rule out other mechanisms of ibuprofen, including inhibition of alternative KATs or HDAC activation, that may be also rescued by p300 inhibition. One caveat to these observations is the possibility that histone acetylation may lie downstream of other pathways disrupted by NSAIDs, such as COX activity. To better understand whether inhibition of histone acetylation may be a general consequence of COX inhibition, we compared the effects of carboxylate NSAIDs ((R)-ibuprofen and salicylate) to a non-carboxylate COX inhibitor (celecoxib; Figure 4b-c). To address the known selectivity of celecoxib towards the COX2 isoform, we used a relatively high dose (50 μ M) that has been shown in the literature to be sufficient to also inhibit COX1.³⁰ Analyzing the NSAID-sensitive acetylation of histone H2B, we found that in contrast to (R)-ibuprofen and salicylate, celecoxib treatment causes an increase in cellular acetylation. This suggests the observed changes in acetylation are not a consequence of COX inhibition. Of note, although these findings are consistent with the biochemical inhibition of KATs by NSAIDs and NSAID-CoAs, they do not rule out the possibility that modulation of an alternative target, such as I κ B kinase or AMPK, may drive or contribute to ibuprofen's

effects on acetylation. To our knowledge, these studies provide the first evidence that the commonly utilized NSAID ibuprofen can alter histone acetylation in cells.

Selective metabolism of ibuprofen enantiomers suggests metabolism-independent effects

(R)-Ibuprofen's effects on histone acetylation in cells are observed at concentrations 2–5x lower than its biochemical IC₅₀ (Figures 3 and 4). A similar observation has been made for inhibition of lysine acetylation by salicylate.¹¹ This could stem from accumulation of (R)-ibuprofen in cells, engagement of a non-KAT target, or metabolism to a more potent KAT inhibitor. Inspired by our biochemical studies, we sought to explore the role of membrane impermeable ibuprofen-CoA in histone acetylation using a selective metabolism approach. Our strategy makes use of the wealth of literature regarding ibuprofen metabolism, particularly studies of its chiral inversion.^{31–32} Specifically, classic pharmacology studies have shown that (R)-ibuprofen is preferentially activated by acyl-CoA synthetase (ACS) enzymes to (R)-ibuprofen-CoA (Figure 5a). This intermediate is epimerized by alpha-methylacyl-CoA racemase (AMACR) to form (S)-ibuprofen-CoA, which in turn is hydrolyzed by an acyl-CoA thioesterase (ACOT) to release (S)-ibuprofen. Literature evidence suggests that (S)-ibuprofen is a poor substrate for ACS enzymes, which prevents a futile cycle by blocking the reverse pathway. (Figure 5a).³³ We hypothesized that we could exploit this stereoselective metabolism to better define the contribution of ibuprofen-CoA on histone acetylation, since (R)- and (S)-enantiomers should form different levels of this metabolite. To establish this approach, we first treated HEK-293 cells with either (R)- or (S)-ibuprofen and extracted acyl-CoA metabolites for LC-MS/MS analysis. Using a Stable Isotope Labeling by Essential nutrients in cell Culture (SILEC) protocol²³ we normalized acyl-CoA levels in each biological sample, and assessed total ibuprofen-CoA levels in cells treated with 1 mM (R)- and (S)-ibuprofen. At early time points (2 hr), we observed substantial formation of ibuprofen-CoA from the (R)-stereoisomer but negligible from the (S)-stereoisomer (Figure 5b), consistent with the literature. This preferential formation of ibuprofen-CoA from (R)-ibuprofen was still observed at later time points (24 hr), although the difference was less pronounced (~5-fold difference between two samples). This validates the selective metabolism of (R)-ibuprofen to ibuprofen-CoA. Next, we sought to examine the effects of these two stereoisomers on cellular histone acetylation. Cells were treated (R)- or (S)-ibuprofen for 24 hrs at concentrations that were not limiting for proliferation (Figure S2), acid-extracted to isolate histones, and analyzed for effects on H2B acetylation. In contrast to their selective metabolism, we observed that both ibuprofen stereoisomers similarly inhibit histone acetylation (Figure 5c). While we cannot absolutely rule out a role for ibuprofen-CoA (due to its formation from each stereoisomer), these studies indicate that formation of ibuprofen-CoA and inhibition of histone acetylation are poorly correlated, and therefore imply that this metabolite may not be a major driver of ibuprofen's effects on histone acetylation in cells. Furthermore, they establish selective metabolism as an approach for defining the cellular activities of NSAIDs and NSAID-CoA metabolites.

Discussion

Small molecule inhibitors are often segmented into two classes: highly specific chemical probes and pan-assay interference compounds (PAINs).³⁴ Lying between these two

classifications are drugs such as ibuprofen, clinically-useful compounds that likely engage multiple cellular targets, of which any single one is likely insufficient to explain their clinical effects. While inherently partial, inquiry into the mechanism of these compounds has the potential to highlight new clinical applications, as well as biomarkers for assessing therapeutic efficacy. Towards this goal, here we have characterized the effects of commonly-utilized NSAID chemotypes on KAT activity. Inspired by recent studies of salicylate,^{11–12} we find that several carboxylate-containing NSAIDs are able to inhibit p300 in biochemical assays. Further analysis of the methylphenylacetic acid NSAID ibuprofen revealed this molecule can act as a modest KAT inhibitor itself ($IC_{50} \sim 6$ mM). In contrast to salicylate, ibuprofen is not highly selective for p300, and also inhibits the KAT pCAF ($IC_{50} \sim 6$ mM). These concentrations may be physiologically-relevant, as previous studies have observed ibuprofen to reach millimolar levels in plasma, and higher concentrations are believed to accumulate in cells and tissues.³⁵ The KAT inhibitory potency of ibuprofen in biochemical assays is increased >200-fold by conjugation to CoA, as occurs during metabolism of this compound in vivo. This observation supports previous studies from our lab, which have found that acyl-CoA metabolites can interact with KATs as endogenous bisubstrate inhibitors, and that these metabolites may contribute to the signaling function of fatty acids in vitro and in vivo.

To build on our biochemical findings, we examined ibuprofen's effects on cellular histone acetylation in cells. (R)-Ibuprofen was found to cause dose-dependent inhibition of several histone acetylations, including H2B K12/K15Ac and H3K56Ac. Of note, the specificity of the H3K56Ac results should be interpreted with caution, as a recent study reported the antibody used in these studies may possess cross-reactivity towards H3K9Ac and H3K27Ac.³⁶ In the future, the specificity of these effects may be better assessed via quantitative mass spectrometry assays.³⁷ Apart from specificity, the inhibitory effect of carboxylate NSAIDs on acetylation is particularly notable given that many structurally-related compounds function as HDAC inhibitors. For example, butyrate and propionate are two well-known HDAC inhibitors derived from the diet and gut microbiota,^{38–39} and aromatic-containing carboxylates (phenylbutyrate, trans-cinnamic acid) have also been shown to stimulate acetylation in cells.^{40–41} These contrasting observations provide evidence that structurally simple, pleiotropic carboxylate drugs can engage different subsets of targets to manifest their cellular effects. Only nascent studies, including this report, have begun to examine the potential of the plethora of carboxylic acid containing compounds that are known to form acyl-CoAs to alter protein acylation.^{18, 42}

Finally, to probe the inhibition of acetylation by ibuprofen and ibuprofen-CoA, we devised a selective metabolism strategy to distinguish the effects of these species. LC-MS/MS studies found (R)-ibuprofen is preferentially processed to ibuprofen-CoA relative to the (S)-stereoisomer, consistent with the literature.³³ In contrast, other prominent acyl-CoAs including acetyl-, propionyl-, and succinyl-CoA were not markedly affected by either stereoisomer (data not shown). However, assessment via Western blot found that both stereoisomers inhibit histone acetylation to a similar extent. This suggests that metabolism of ibuprofen to ibuprofen-CoA may not form steady state concentrations of ibuprofen-CoA sufficient to inhibit KAT activity, or, alternatively, formation of ibuprofen-CoA may be spatially distinct from KATs. Of note, many acyl-CoA synthetases as well as glycine-N-

acyltransferase (GLYAT), which catalyzes transfer of xenobiotic-CoA analogues to glycine, are known to be located in the mitochondria.^{16–17} In the future, we envision that incorporation of chemical functionalities enabling selective metabolism could prove a generally useful approach for distinguishing the signaling functions of structurally simple carboxylic acid metabolites from their cognate acyl-CoAs. In addition, the spatial compartmentalization of acyl-CoA metabolism could provide an avenue into the development of new strategies for cellular KAT inhibition. For example, engineered cytosolic ACS enzymes could be used to activate ibuprofen, enabling potent inhibition of KATs by ibuprofen-CoA. Extending this approach, one could envision designing custom carboxylate precursors in order to form CoA conjugates capable of selectively targeting specific KATs or KAT subsets. Such a strategy would provide an approach to probe KAT activity complementary to selective small molecule inhibition of these enzymes, which has proven challenging to achieve.⁴³ Selective metabolism may also be a potentially useful approach for studying additional classes of drugs for whom the structural determinants of metabolism are well understood.^{44–45} Overall, these studies illuminate a novel interaction of the commonly used NSAID ibuprofen with the acetylation machinery, and provide new strategies for probing the metabolic regulation of protein acetylation in health and disease.

In conclusion, we have characterized the interaction of NSAIDs and NSAID-CoA metabolites with KAT enzymes. Our studies suggest that several carboxylate-containing NSAIDs, including ibuprofen, are able to function as weak-binding KAT inhibitor fragments. Ibuprofen-CoA, a commonly formed NSAID metabolite, inhibits the KAT enzymes p300 and pCAF ~200-fold more potently than its parent compound. Cellular studies indicate ibuprofen can inhibit cellular acetylation, but that this inhibition does not correlate with levels of metabolism to ibuprofen-CoA. These studies further highlight the emergent interaction of NSAIDs with acetylation biology, and provide new strategies for defining their metabolic and non-metabolic roles in vitro and in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Inhibition of KAT-catalyzed protein acetylation by NSAIDs and NSAID-CoA metabolites.

Figure 2.

Exploring the ability of NSAIDs to inhibit p300. (a) Structures of carboxylic acid NSAIDs and analogues tested in this study. (b) Single-point screen for p300 inhibition. Compounds were screened at 2.5 mM, with the exception of salicylic acid (5 mM), diclofenac (1.25 mM), and acetic acid (5 mM). For the single point screen all compounds were dosed in their protonated state and dissolved in DMSO.

Figure 3.

Inhibition of KAT activity by ibuprofen. (a) Synthesis of ibuprofen-CoA. Full characterization data is given in the Supporting Information. (b) IC₅₀ curve for inhibition of p300 by enantiomers of ibuprofen and ibuprofen-CoA. (c) Tabulated IC₅₀ values for inhibition of p300 and pCAF by NSAIDs and NSAID-CoAs. All compounds were dosed as their neutralized sodium salts. *IC₅₀ values were obtained from citation 11.

Figure 4.

Cellular inhibition of histone acetylation by (R)-ibuprofen. (a) (R)-Ibuprofen exhibits dose-dependent effects on histone acetylation. Numerical values indicate histone acetylation signal normalized to Ponceau. (b) Carboxylate ((R/S)-ibuprofen, salicylate) and non-carboxylate (celecoxib) NSAIDs. Asterisk indicates the stereocenter. Full (uncropped) gel images are provided in the Supporting Information. (c) NSAID inhibition of histone acetylation requires a carboxylate chemotype. Numerical values indicate histone acetylation signal normalized to Ponceau. Blue lines indicate where lanes have been re-ordered for clarity. Full (uncropped) gel images are provided in the Supporting Information. (R)-ibuprofen and salicylate were dosed as their neutralized sodium salts.

Figure 5.

Using selective metabolism to probe the role of NSAID-CoAs in modulation of histone acetylation. (a) Schematic illustrating stereoselective acyl-CoA metabolism responsible for chiral inversion of (R)- to (S)-ibuprofen. (b) Time-dependent formation of total ibuprofen-CoA (both stereoisomers) monitored by LC-MS/MS. (c) Effect of ibuprofen stereochemistry on NSAID inhibition of cellular histone acetylation. Blue lines indicate where lanes have been re-ordered for clarity. Full (uncropped) gel images are provided in the Supporting Information. (R)- and (S)-ibuprofen were dosed as their neutralized sodium salts.