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Synthetic triterpenoid inhibition of human ghrelin O-acyltransferase: Involvement of a functionally required cysteine provides mechanistic insight into ghrelin acylation

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Abstract

The peptide hormone ghrelin plays a key role in regulating hunger and energy balance within the body. Ghrelin signaling presents a promising and unexploited target for development of small-molecule therapeutics to treat obesity, diabetes, and other health conditions. Inhibition of ghrelin *O*-acyltransferase (GOAT), which catalyzes an essential octanoylation step in ghrelin maturation, offers a potential avenue for controlling ghrelin signaling. Through screening a small molecule library, we have identified a class of synthetic triterpenoids that efficiently inhibit ghrelin acylation by the human isoform of GOAT (hGOAT). These compounds function as covalent reversible inhibitors of hGOAT, providing the first evidence for involvement of a nucleophilic cysteine residue in substrate acylation by a MBOAT family acyltransferase. Surprisingly, the mouse form of GOAT does not exhibit susceptibility to cysteine modifying electrophiles revealing an important distinction in the activity and behavior between these closely related GOAT isoforms. This study establishes these compounds as potent small molecule inhibitors of ghrelin acylation and provides a foundation for the development of novel hGOAT inhibitors as therapeutics targeting diabetes and obesity.

Graphical Abstract

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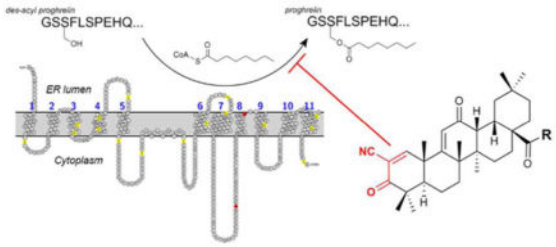
Competing Financial Interest Statement

J.L.H. and J.D.C. have patent interests in the use of compounds reported herein to target ghrelin signaling and associated health conditions.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications Website at: XX Synthetic methods and characterization of hGOAT inhibitor candidates; Activity versus inhibitor concentration plots for hGOAT inhibitors; ^{1}H and ^{13}C NMR for novel compounds



The increasing incidence of diabetes and obesity within the American population presents an urgent and growing threat to public health. The prevalence of type II diabetes has recently risen dramatically, with ~26 million adults and children suffering from diabetes and an estimated 79 million American adults classified as pre-diabetic as of 2011.[1] The global situation is also foreboding, with 382 million people suffering from diabetes in 2013, a figure expected to increase to 592 million by 2035.[2] These sobering statistics underscore the need for new therapeutic avenues to treat these diseases, particularly small-molecule based treatments to complement lifestyle modification and surgical approaches already being employed.

The peptide hormone ghrelin is a key component of a promising and unexploited target for development of small-molecule therapeutics to treat obesity, diabetes, and many related health conditions. Ghrelin is a 28-amino acid secreted peptide, discovered in 1999 by Kojima and coworkers,[3] which has been implicated in a wide array of physiological pathways ranging from energy regulation to neurological processes (for examples and reviews, see [4–8]). Ghrelin is perhaps most well-known for its ability to stimulate appetite.[5] Beyond hunger signaling, ghrelin has been linked to maintenance of body energy balance through regulation of fat mass and modulation of insulin signaling and glucose metabolism sensitivity, while des-acyl ghrelin and des-acyl ghrelin analogs block some of these effects.[4, 9–15] In general, an increase in the level of acyl ghrelin inhibits glucose-stimulated insulin secretion,[16–20] with studies suggesting the ratio of acyl to des-acyl ghrelin may be the controlling factor in modulating insulin sensitivity.[13, 21, 22] Such a multi-input signaling system could explain the conflicting results reported in studies of the effects of acyl ghrelin on insulin sensitivity, with differing effects reported in different cell lines, healthy and obese humans, and in cases of type I diabetes as described in recent reviews.[7, 11] Inhibition of GOAT-catalyzed acylation of ghrelin would not only decrease levels of acyl ghrelin, but increase levels of des-acyl ghrelin, and therefore caution must be taken when evaluating the potential benefits and hazards of such treatments in an organismal context.

Recent studies also suggest ghrelin signaling can play a developmental role in defining metabolic and body weight “set points” within the hypothalamus, as both elevated and depressed ghrelin levels in neonatal mice have been linked to metabolic dysregulation later in life.[23] This suggests the potential for prophylactic inhibition of ghrelin signaling in infants with elevated ghrelin levels to reduce obesity and metabolic disturbances such as those observed in patients with Prader-Willi syndrome exhibiting hyperghrelinemia.[24, 25] However, studies such as that by Sominsky and coworkers demonstrate that disruptions of ghrelin signaling due to overeating early in life resolve in adult rats, indicating that the

connection between ghrelin signaling and metabolic regulation in adulthood remains to be fully defined.[26] Such studies will be aided by the development of potent small-molecule agents for effectively modulating ghrelin signaling within organisms.[27]

Ghrelin-dependent pathways present attractive targets for drug development, as ghrelin requires multiple covalent modifications for biological activity.[5] Ghrelin maturation involves a unique posttranslational modification of the third serine from the N-terminus of the 94-amino acid ghrelin precursor des-acyl proghrelin, wherein this serine is acylated by an octanoyl (C8) fatty acid group (Figure 1a). Ghrelin *O*-acyltransferase (GOAT), the integral membrane enzyme shown to be responsible for acylation of both des-acyl proghrelin and the unacylated form of mature 28-amino acid ghrelin, was identified in 2008.[28–30] While both acylated ghrelin and des-acyl ghrelin are present in blood serum, only the acylated form of ghrelin (hereafter referred to as “ghrelin”) can bind and activate signaling through its cognate GHSR-1a receptor.[5] Ghrelin is the only substrate predicted for GOAT within the human proteome,[28, 31] which reduces the potential for off-target effects due to the loss of GOAT-catalyzed acylation of other proteins upon inhibition of GOAT activity. The unique and essential nature of ghrelin octanoylation makes this modification an excellent target for inhibiting ghrelin activity.

While the potential of ghrelin signaling as a therapeutic target has been discussed in the literature,[5, 7, 9–11, 32] the lack of small molecule inhibitors with demonstrated potency in targeting this pathway in a cellular or organismal context has hampered evaluation of this approach. Our group and others have reported examples of GOAT inhibitors based on either mimics of ghrelin or screening of small molecule libraries (Figure 1b).[30, 32–36] However, other than the GO-CoA-Tat inhibitor discussed below, none of these GOAT inhibitors has been reported to block ghrelin octanoylation in cell- or animal-based studies.[33, 35, 36]

The strongest evidence supporting the potential for GOAT inhibitors to modulate serum levels of acylated ghrelin comes from the work of Barnett and coworkers with their peptide-based bisubstrate mimetic GO-CoA-Tat inhibitor.[32] This inhibitor effectively inhibited GOAT in both cultured mammalian cells and mice, with treated mice demonstrating increased glucose tolerance and reduced weight gain.[32] However, the pharmaceutical utility of GO-CoA-Tat is limited by its susceptibility to proteolytic degradation and its large size (MW ~3600 Da). The absence of readily available potent small molecule GOAT inhibitors remains the principal obstacle in validating ghrelin and ghrelin-related signaling pathways as treatment avenues.

To address this challenge in exploiting ghrelin signaling for therapeutic development and catalyze the creation of potent inhibitors of GOAT, we sought to identify “drug-like” small molecules with inhibitory activity against GOAT. Our screen of a library of small molecules with diverse structures revealed a new small molecule human GOAT (hGOAT) inhibitor, CDDO-Im, with subsequent structure-activity analysis revealing this compound and related molecules function as reversible covalent inhibitors of hGOAT. This class of compounds exhibits robust inhibition of ghrelin octanoylation by hGOAT, and effects reported in previous animal and clinical studies employing CDDO-family compounds support the potential for these compounds to impact ghrelin signaling.[37–44] Our study establishes these

synthetic triterpenoids as effective small molecule inhibitors targeting ghrelin signaling, provides the first evidence implicating a cysteine thiol group in the catalytic mechanism of a MBOAT family acyltransferase, and offers a foundation for continued development of novel hGOAT inhibitors as therapeutics targeting diabetes and obesity.

Experimental Procedures

General

Data plotting and curve fitting were performed with Kaleidagraph (Synergy Software, Reading, PA, USA). Methyl arachidonyl fluorophosphonate (MAFP) was purchased from Cayman Chemical (Ann Arbor, MI) as a stock in methyl acetate and diluted into DMSO prior to use. Octanoyl coenzyme A (octanoyl-CoA) was solubilized to 5 mM in 10 mM Tris–HCl (pH 7.0), aliquoted into low-adhesion microcentrifuge tubes, and stored at −80 ¼C. Acrylodan (Anaspec) was solubilized in acetonitrile, with the stock concentration determined by absorbance at 393 nm on dilution into methanol (ε = 18,483 M^{-1} cm^{-1} per manufacturer's data sheet). Compounds were obtained as follows: compounds **1–5**, gift from Gordon Gribble and Michael Sporn (Dartmouth University);[45–50] compounds **6–8** and **15** were provided by the Developmental Therapeutics Program (DTP/NIH); compounds **9–14** and **16–17** were synthesized as described in the Supporting Information; estrone (**15**) was purchased from Cayman Chemical (Ann Arbor, MI); cyclohexenone (**18**) was purchased from Alfa Aesar (Ward Hill, MA). The $GSSFLC_{NH2}$ peptide for fluorescent labeling with acrylodan was synthesized by Sigma–Genosys (The Woodlands, TX, USA) in the Pepscreen format. The $GSSFLC_{NH2}$ peptide was solubilized in 1:1 acetonitrile : H_2O and stored at −80 ¼C. Peptide concentration was determined spectrophotometrically at 412 nm by reaction of the cysteine thiol with 5,5'-dithiobis(2-nitrobenzoic acid) using ε_{412} = 14,150 M^{-1} cm^{-1}.[51]

Expression and enrichment of hGOAT and mGOAT

hGOAT and mGOAT were expressed and enriched in insect (Sf9) cell membrane fractions using a previously published procedure.[15, 31, 52]

Peptide substrate fluorescent labeling

Peptide substrates were labeled with acrylodan on a cysteine thiol and HPLC purified as previously reported.[31, 52]

hGOAT and mGOAT activity assays and analysis

hGOAT and mGOAT activity assays were performed using a modification of previously reported protocols.[15, 31, 52] For each assay, membrane fraction from Sf9 cells expressing hGOAT or mGOAT was thawed on ice and passed through an 18-gauge needle 10 times to homogenize. Assays were performed with ~100 μg of membrane protein, as determined by Bradford assay. Membrane fraction was preincubated with 1 μM methyl arachidonyl fluorophosphonate (MAFP) and inhibitor or vehicle as indicated in 50 mM HEPES pH 7.0 for 30 minutes at room temperature.[53] Reactions were initiated with the addition of 500 μM octanoyl CoA and 1.5 μM fluorescently-labeled ghrelin mimetic, $GSSFLC_{Acrylodan}$, for a total volume of 50 μL, and were incubated for 3 hours at room temperature in the dark. Reactions were stopped with the addition of 50 μL of 20% acetic acid in isopropanol, and

solutions were clarified by protein precipitation with 16.7 μL of 20% trichloroacetic acid, followed by centrifugation (1,000x*g*, 1 minute). The supernatant was then analyzed by reverse phase HPLC, as previously described.[52] Data reported are the average of three independent determinations, with error bars representing one standard deviation.

Library screening

For screening from the Diversity Set IV library of small molecules, hGOAT octanoylation reactions were performed as described above with the addition of library compounds at concentrations of 10 and 100 μM, achieved by dilution of 10 mM compound stocks in DMSO received from the Developmental Therapeutics Program. Compounds that met criteria for inhibition (dose-dependent decrease in activity, <50% activity at 100 μM) were confirmed with a secondary screen using the same protocol.

Determination of IC_{50} values in in vitro hGOAT and mGOAT activity assays

For inhibition experiments, reactions were performed and analyzed as described in the presence of either inhibitor or vehicle (DMSO or ethanol) as appropriate.[31] The percent activity at each inhibitor concentration was calculated from HPLC integration data using equations 1 and 2:

$$\%\,\text{activity} = \frac{\%\,\text{peptide octanoylation in presence of inhibitor}}{\%\,\text{peptide octanoylation in absence of inhibitor}} \quad (1)$$

$$\%\,\text{peptide octanoylation} = \frac{\text{Fluorescence of octanoylated peptide}}{\text{Total peptide fluorescence (octanoylated and non- octanoylated)}} \quad (2)$$

To determine the IC_{50} value, the plot of % activity versus [inhibitor] was fit to equation 3, with % activity_0 denoting hGOAT activity in the presence of the vehicle alone:

$$\%\,\text{activity} = \%\,\text{activity}_0 * \left(1 - \frac{[\text{inhibitor}]}{[\text{inhibitor}] + IC_{50}}\right) \quad (3)$$

Determination of inhibitor time dependence with hGOAT

To determine the time dependence of inhibition of hGOAT activity by CDDO-EA, assays were performed and analyzed as described above with the following two modifications: i) Membrane fraction was preincubated with 1 μM methyl arachidonyl fluorophosphonate (MAFP) in 50 mM HEPES pH 7.0 for 30 minutes at room temperature, followed by incubation with inhibitor or vehicle as appropriate for varying times (5, 10, 30, 60 minutes) prior to reaction initiation. ii) Reactions were incubated for 10 minutes at room temperature

in the dark, followed by addition of stop solution and reaction workup and analysis as described above.

Inhibitor reversibility assay

Undiluted homogenized membrane protein fraction containing hGOAT (protein concentration ~7 μg/μL) was incubated with 10 μM MAFP and 3 x IC_{50} of inhibitor or equal volume vehicle (DMSO or ethanol) for 30 minutes at room temperature. Following preincubation, the membrane fraction-inhibitor solution was diluted 10-fold into a reaction mixture containing 500 μM octanoyl CoA, 1.5 μM $GSSFLC_{AcDan}$, 50 mM HEPES pH 7.0, and either vehicle or inhibitor (final concentration 3 x [IC_{50}]) in a total reaction volume of 50 μL. Reactions were incubated for 3 hours at room temperature in the dark and then analyzed as described above.

Results

Library screening reveals a new small molecule GOAT inhibitor

As described in the Introduction, the majority of reported GOAT inhibitors are substrate- or product-mimetic compounds. While effective to varying degrees in *in vitro* GOAT assays, many of these compounds appear to lack sufficient cell permeability to permit effective inhibition of ghrelin octanoylation in cell- or organism-based systems. To explore a broader expanse of chemical space for potential GOAT inhibitors, we utilized our fluorescence-based *in vitro* hGOAT activity assay to screen compounds from the Diversity Set IV library (Developmental Therapeutics Program, NCI/NIH) for inhibition of hGOAT (Figure 2).[52] This library consists of ~1600 compounds chosen to represent the molecular diversity of "drug-like" molecules within the DTP repository. Compounds were initially screened at 10 and 100 μM, with those compounds exhibiting a dose-dependent decrease in activity and <50% activity at 100 μM verified by a secondary screen under the same conditions. For compounds passing both screens, we then obtained and assayed structurally related compounds from the DTP repository for inhibitory activity against hGOAT.

Following screening, we identified the most promising candidate molecule from the Diversity IV library as a synthetic oleanate triterpenoid, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im, **1**) (Figure 2c) which inhibits hGOAT activity with an IC_{50} of 38 ± 6 μM. A structurally related molecule methyl 2-cyano-3,12-dioxooleana-1,9(11)dien-28-oate (CDDO-Me, **2**) also exhibits inhibitory activity against hGOAT (Figure 3).

Verification of CDDO scaffold activity against hGOAT

CDDO-Im and CDDO-Me belong to a class of orally available semisynthetic triterpenoids based on oleanolic acid.[54] These compounds have demonstrated antiangiogenic and antitumor activities in animal cancer models by modulating multiple signaling pathways including the Nrf2 and NF-κB pathways.[54–57] Given the inhibition of hGOAT by CDDO-Im and CDDO-Me, we determined the inhibitory activity of three other CDDO compounds with various carboxyl substituents (compounds **3–5**, Figure 3) against hGOAT using the *in vitro* hGOAT activity assay.[52] Of these five CDDO compounds, all but the acid **5** served as

inhibitors of hGOAT-catalyzed ghrelin octanoylation with the methyl ester and ethyl amide derivatives (CDDO-Me **2** and CDDO-EA **3**) demonstrating the most potent inhibition of hGOAT (Figure 3). The lack of inhibition exhibited by the parent CDDO bearing a carboxylate may reflect a general intolerance for negatively charged groups within the hGOAT active site and substrate binding sites. Substrate selectivity studies have revealed hGOAT does not accept peptide substrates bearing negatively charged side chains or C-terminal acids. [30, 31, 52]

Structure-activity analysis of the CDDO scaffold

These CDDO-derived compounds contain several functional groups that could be responsible for activity against hGOAT (Figure 4): the triterpenoid scaffold; the α-cyanoenone (ring A); or the α,β-unsaturated ketone (ring C). Given the multiple potential pharmacophores within the CDDO family compounds and our lack of knowledge regarding the structure and chemical nature of the hGOAT active site and substrate binding sites, we sought to determine the structure-activity parameters defining CDDO-based inhibitor potency against hGOAT by evaluating structural analogues. The natural product triterpenoid compounds ursolic acid (**6**) and oleanic acid (**7**, from which CDDO is derived) exhibit negligible inhibition of hGOAT activity at concentrations up to 100 μM. These compounds lack the activated α-cyanoenone group shown to be essential for CDDO derivative activity in previous studies targeting receptor signaling.[54, 58] However, as both molecules also bear unsubstituted carboxylate groups their lack of hGOAT inhibition could reflect the inability of hGOAT to bind negatively charged molecules.[30, 31] To separate these factors, we determined the ability of the triterpenoid taraxerol (**8**) to inhibit hGOAT. Taraxerol shares the same scaffold and 3-hydroxyl group as ursolic and oleanic acid but lacks the carboxylic acid. Taraxerol also fails to inhibit hGOAT acylation activity at concentrations up to 100 μM, which suggests hGOAT inhibition by CDDO is not primarily due to the triterpenoid scaffold structure.

Based on the proposed mode of action for CDDO derivatives binding to their targets through modification of reactive cysteine residues, we hypothesized the α-cyanoenone moiety present in the A ring of CDDO derivatives (green, Figure 4) is required for hGOAT inhibition. This group has been shown to covalently modify nucleophilic thiols in a range of protein targets in a reversible manner.[59, 60] To examine the effect of a Michael acceptor group on hGOAT inhibition, we synthesized a series of minimally functionalized steroid derivatives featuring an α,β-unsaturated ketone in a position analogous to that in CDDO-EA (compounds **9**-**11**, Figure 4). All three of these molecules inhibit hGOAT activity, with the inhibitor potency scaling with the level of activation of the enone towards nucleophilic addition from the most activated α-cyanoenone **9** (IC_{50} = 8 ± 2 μM) to the non-activated enone **11** (IC_{50} = 170 ± 60 μM). We note the α-cyanoenone **9** inhibits hGOAT with potency nearly identical to CDDO-Me **2** and CDDO-EA **3**, indicating the complete triterpenoid scaffold and associate functional groups in the CDDO derivatives are not essential for binding and inhibition of hGOAT.

Using other steroid derivatives, we have demonstrated inhibition of hGOAT exhibits both chemo- and regioselectivity. Removal of the electron-poor alkene leads to loss of inhibition

as shown by compound **12**. Incorporation of an α-bromo ketone as a potential electrophile in compound **13** was similarly unable to support inhibition. Migration of the α,β-unsaturated ketone to the other side of ring A (compound **14**) similarly abrogates inhibition of hGOAT, with the additional alkyl substituent not expected to impact binding to hGOAT based on the tolerance for the larger triterpenoid scaffold in inhibitors **1-4**. Furthermore, the lack of hGOAT inhibition exhibited by estrone (**15**) indicates a planar A ring within a steroid scaffold is insufficient for inhibition of hGOAT, with the electrophilic enone required for activity. These findings indicate hGOAT inhibition requires the presence of a specifically located Michael (conjugate) acceptor group, which is consistent with modification of an enzyme-bound nucleophile within a defined binding pocket on hGOAT.

The equivalent potency of CDDO-EA (**3**) and α-cyanoenone **9** indicate the distal E ring and carboxyl substituent of CDDO-EA are not required for binding to hGOAT. We determined the contribution of the steroid scaffold (purple, Figure 4) to hGOAT binding by measuring inhibition by cyclohexenone and cyclohexenone derivatives (compounds **16–18**) which mimic the A ring substitutions of compounds **9–11**. Both α-cyanocyclohexenone **16** (IC_{50} = 1.2 ± 0.2 mM) and α-bromocyclohexenone **17** (IC_{50} = 500 ± 100 μM) inhibit hGOAT less potently than their steroid analogues **9** and **10**, respectively, while cyclohexenone **18** does not inhibit hGOAT activity at concentrations up to 1 mM. Therefore, the steroid scaffold contributes substantially to inhibitor potency against hGOAT as demonstrated by the ~150-fold enhancement in context of α-cyanoenones **9** and **16**. This enhancement likely arises from a combination of both increased inhibitor association with hGOAT (*better binding*) and a decrease in inhibitor reactivity with other microsomal protein targets (*reduced competition*) due to greater steric congestion from the quaternary center adjacent to the electrophilic β-carbon in α-cyanoenone **9**.[58]

Inhibitor structure-function analysis supports a functionally essential cysteine in hGOAT

The requirement for an α,β-unsaturated ketone and the increased activity of the triterpenoid and steroid α-cyanoenone compounds suggests these compounds could block hGOAT function through alkylation of a nucleophilic cysteine residue involved in hGOAT catalysis. We established the ability of cysteine alkylation to inactivate hGOAT by enzyme incubation with N-ethylmaleimide (NEM), a common thiol-modifying reagent (Figure 5b). NEM efficiently inhibits hGOAT, consistent with the involvement of a functionally essential cysteine residue in ghrelin octanoylation by hGOAT. Should covalent inhibition of hGOAT by CDDO-EA (**3)** involve rapid formation of a noncovalent enzyme-inhibitor complex followed by a slower alkylation of a cysteine sidechain thiol by the α-cyanoenone electrophile,[59] CDDO-EA (**3)** and related compounds should display time dependent inhibition of hGOAT. Reaction monitoring of hGOAT activity using a shorter time course (10 minutes versus 3 hours) and variation of inhibitor preincubation time reveals CDDO-EA (**3)** exhibits such time-dependent inhibition behavior (Figure 5c), consistent with a covalent inhibition mechanism. However, the length of the required reaction time relative to the potential preincubation times and the apparent reversibility of cysteine alkylation by CDDO-EA (see below) render measurement of k_{inact} and K_i values impractical with available assays.

Previous studies of α-cyanoenones in reaction with thiol nucleophiles such as cysteine show that these molecules act as covalent reversible inhibitors, with a retro-Michael elimination facilitated by the increased acidity of the α-hydrogen geminal to the cyano group.[59, 61] We determined the reversibility of hGOAT inhibition by the α-cyanoenone compounds **3** and **9** by enzyme pretreatment with each inhibitor at three times the measured IC_{50} concentration, followed by a 10-fold dilution into either reaction buffer or buffer containing the same inhibitor concentration as the pretreatment. (Figure 5d). NEM exhibits classical irreversible hGOAT inhibition, with no increase in hGOAT activity following inhibitor dilution. An established GOAT inhibitor, [Dap^3]octanoyl-ghrelin (1-5)-NH_2, serves as an control for reversible inhibition as expected for a product mimetic non-covalent inhibitor.[30, 31] Both CDDO-EA (**3**) and α-cyanoenone **9** display reversible hGOAT inhibition, consistent with previously reported reversibility of CDDO compounds.[59] Taken together, the susceptibility of hGOAT to treatment with NEM and the observed pattern of reversible and irreversible hGOAT inhibition by these Michael acceptors support the requirement for one or more cysteine residues to participate in hGOAT catalysis of ghrelin octanoylation (Figure 5a).

Inhibition of GOAT-catalyzed ghrelin acylation by cysteine alkylation differs between the human and mouse GOAT isoforms

Currently, there are no literature reports regarding the ability of small molecule GOAT inhibitors reduce ghrelin acylation *in vivo,* with only the peptide-based bisubstrate mimic GO-CoA-Tat shown to lower acyl ghrelin levels in cells and animal models.[32, 64] The majority of *in vitro* assays for GOAT activity utilize the mouse isoform of GOAT (mGOAT) in either an enzyme- and cell-based format,[15, 30, 32, 33, 62, 65–69] with mGOAT and hGOAT exhibiting a high level of amino acid sequence homology (79% identity, 92% similarity; Figure 6). Only one example of a direct comparison of inhibitor potency between these closely related enzyme isoforms has been reported, with the [Dap^3]octanoyl-ghrelin (1–5)-NH_2 inhibitor exhibiting similar activity against hGOAT and mGOAT.[15, 30] As previously reported cell lines utilized to investigate ghrelin acylation and GOAT activity utilize mGOAT,[15, 32, 69, 70] the ability of these synthetic triterpenoids to inhibit mGOAT must be established at the enzyme level prior to cell-based studies.

We first tested the ability of CDDO-EA **3** to block mGOAT-catalyzed acylation of our fluorescent $GSSFLC_{AcDan}$ peptide substrate, which mimics the N-terminal sequence of both human and mouse ghrelin. The mouse isoform of GOAT (mGOAT) is inhibited by CDDO-EA **3** less potently than hGOAT, with an ~8-fold higher IC_{50} value in a side-by-side assay (Figure 6a). To investigate the basis for this loss of inhibitor potency against mGOAT, we determined the impact of other cysteine modifying molecules on mGOAT acylation activity through treatment with N-ethyl maleimide and comparison of the steroid derivatives α-cyanoenone **9** and ketone **12** utilized in analysis of hGOAT (Figures 6b–c). In both cases, mGOAT exhibits substantially reduced susceptibility to inhibition to the presence of electrophilic inhibitors compared to hGOAT. In the comparison of α-cyanoenone **9** and ketone **12**, the presence of the cyanoenone Michael acceptor does not enhance mGOAT inhibition, in marked contrast to hGOAT. This suggests the cysteine residue required for enzyme activity in the human enzyme is not present in mGOAT, not required for mGOAT catalysis, or is resistant to modification by alkylating agents. A Clustal Omega alignment of

the hGOAT and mGOAT sequences reveals a number of cysteine residues in the human enzyme that are not conserved in mGOAT (Figure 6d, yellow highlights),[71] with the majority of these non-conserved cysteines lying outside the conserved MBOAT domain in the C-terminal sequence of GOAT.[62, 72–74]

Discussion

Motivated to move beyond substrate- and product-mimetic inhibitors for modulating ghrelin acylation, we have identified a family of synthetic triterpenoids that function as small molecule inhibitors of ghrelin octanoylation by hGOAT. By comparison between the human and mouse isoforms of GOAT, this study establishes these highly homologous enzymes exhibit dramatically different susceptibility to treatment with inhibitors bearing cysteine-reactive electrophilic functional groups. While several classes of small molecule inhibitors of GOAT have been reported in the scientific and patent literature,[33, 35, 36] the CDDO derivatives and associated compounds reported herein bear no resemblance to known GOAT inhibitors and provide new opportunities for developing probes of hGOAT-catalyzed ghrelin octanoylation and potential therapeutic agents targeting ghrelin signaling.

Structure-activity analysis of the CDDO-type inhibitors provides the first suggestion for the involvement of a functionally essential cysteine in hGOAT-catalyzed ghrelin acylation, and suggests these synthetic triterpenoids may function as the first reported mechanism-based inhibitors targeting GOAT. The regioselective requirement for an α,β-unsaturated ketone, with inhibitor potency scaling with enone reactivity towards nucleophilic addition, is consistent with inhibitor alkylation of an hGOAT cysteine residue acting as a Michael donor. Catalytic involvement of a cysteine in ghrelin acylation by hGOAT, while defining a new mode of inhibition targeting this enzyme, also presents an opportunity to potentially identify the location of the active site within an MBOAT-family acyltransferase. hGOAT contains a total of 16 cysteine residues (Figure 5a), with several of these cysteines lying in the conserved C-terminal “MBOAT” domain within hGOAT.[62, 72, 74] Mutational analyses of the three protein-modifying members of the MBOAT family (Hhat, PORCN, and GOAT) have revealed functionally required residues but none have implicated cysteine residues as functionally essential.[30, 62, 65, 73, 75–77] While Hhat and PORCN contain palmitoylated cysteine residues,[73, 77] our findings provide the first evidence supporting an enzymatic cysteine residue involved in MBOAT-catalyzed protein acylation. One intriguing possibility involves formation of an octanoyl acyl-enzyme intermediate involving a cysteine residue within GOAT in the course of transferring the octanoyl group to ghrelin (Figure 7), similar to the ping-pong mechanism proposed for protein palmitoylation by DHHC-family palmitoyltransfersases.[78, 79] While the involvement of a cysteine residue directly in hGOAT-catalyzed ghrelin acylation is intriguing, the lack of evidence supporting a functionally essential cysteine residue in mGOAT could also support a model wherein the cysteine residue responsible for inhibition in hGOAT provides an adventitious site for covalent modification by inhibitors in or near the hGOAT active site. Studies to determine the identity and role(s) of functionally required cysteine residues within hGOAT are currently underway and will be reported in due course. Additionally, the marked difference in the ability of cysteine modifying electrophiles to inhibit hGOAT and mGOAT also underscores the

importance of evaluating potential GOAT inhibitors against the human form of GOAT during compound screening and validation.

Our discovery of synthetic triterpenoid inhibitory activity against GOAT reveals an exciting and unanticipated mode of action for these compounds, several of which have been investigated in clinical trials.[38, 54] Previous studies of CDDO derivatives as potential therapeutics have focused on controlling inflammation and oxidative stress in multiple tissues through modulation of multiple cell signaling pathways.[54, 55] Inhibition of ghrelin acylation could explain multiple outcomes observed in rodent and human studies with these compounds, given ghrelin's known roles in regulating body energy balance and glucose metabolism.[4, 9, 11, 32, 80] These outcomes observed during rodent and human studies utilizing CDDO derivatives, such as effects on fat deposition, weight loss, reduction of insulin resistance, and improved glucose tolerance, have been predicted as potential effects of modulating ghrelin signaling.[37–41]

Given the established impact of CDDO derivatives on cell signaling, the broad-ranging effects of these synthetic triterpenoids present a challenge in using these compounds to reduce active ghrelin concentrations. Moreover, the deleterious side effects observed in the BEACON clinical trial utilizing bardoxolone methyl (CDDO-Me, **2**) reinforce the importance of specifically targeting ghrelin acylation without impacting additional physiological pathways.[38, 81] The *in vitro* potency of the relatively unfunctionalized a–cyanoenone steroid **9** illustrates the potential for synthetic modification of this molecular framework to develop new chemical tools for studying GOAT function and ghrelin signaling. Compounds containing the pharmacophores identified in this study can also be designed to combine aspects of these CDDO derivatives with elements involved in ghrelin recognition by GOAT to maximize both potency and specificity.[31, 52, 65]

Identifying potent GOAT inhibitors is an essential step towards validation and exploitation of the ghrelin-GOAT system for therapeutic targeting. In this work, we demonstrate that synthetic triterpenoids containing a α-cyanoenone moiety can efficiently block ghrelin acylation by hGOAT through a covalent reversible inhibition mechanism involving cysteine alkylation. This susceptibility to treatment with cysteine modifying electrophiles is not observed in the mouse form of GOAT, revealing an important distinction in the activity and behavior of these closely related enzyme isoforms. Previous and ongoing clinical trials employing CDDO-type molecules have established the suitability of these orally available compounds for human studies, and our findings strongly suggest ghrelin signaling and associated physiological pathways should be directly monitored in clinical studies employing these compounds. We are hopeful our discovery of this new class of small molecule hGOAT inhibitors will accelerate inhibitor development targeting ghrelin octanoylation, potentially leading to therapeutics for treating diabetes, obesity, and other health conditions impacted by ghrelin signaling.

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Abbreviations

GOAT	ghrelin *O*-acyltransferase
hGOAT	human ghrelin *O*-acyltransferase
mGOAT	mouse ghrelin *O*-acyltransferase
AcDan	acrylodan
DMSO	dimethyl sulfoxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
HPLC	high pressure liquid chromatography
MALDI	matrix assisted laser desorption ionization
IC_{50}	half maximal inhibitory concentration
MAFP	methyl arachidonyl fluorophosphonate
Dap-C8	[Dap^3]octanoyl-ghrelin (1–5)-NH_2 GOAT inhibitor

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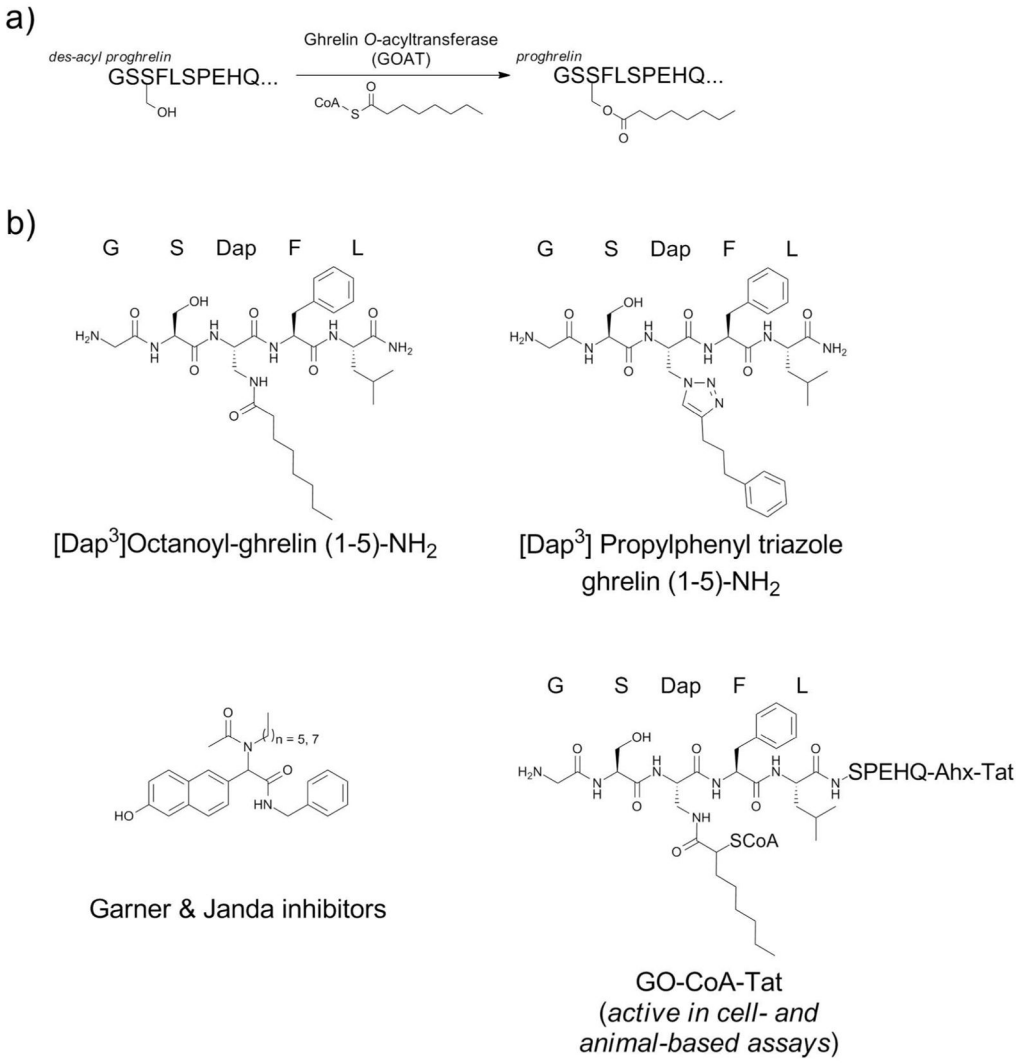

Figure 1. Ghrelin *O*-acyltransferase (GOAT) as a target for blocking ghrelin signaling

a) Ghrelin octanoylation catalyzed by GOAT. b) Structures of reported GOAT inhibitors. Ahx denotes aminohexanoate; Tat denotes a Tat peptide sequence (-YGRKKRRQRRR).

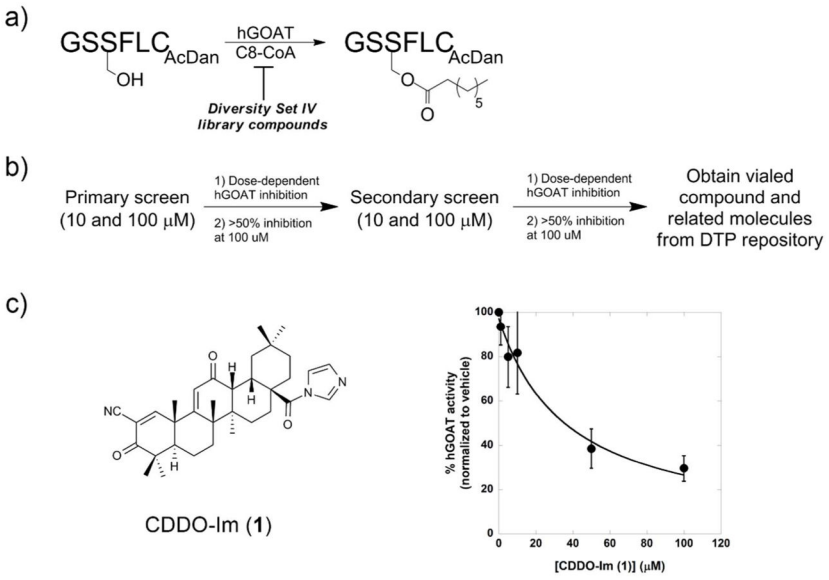

Figure 2. Screening of Diversity IV library compounds reveals a novel small molecule hGOAT inhibitor

a) Fluorescence-based hGOAT activity assay used for compound screening. B) Protocol for screening assay to identify hGOAT inhibitors; b) Structure of CDDO-Im (**1**), the initial hit from the Diversity IV library; c) Inhibition of hGOAT octanoylation activity by CDDO-Im (**1**). Reactions were performed and analyzed to determine percent activity as described in the inhibitor assay protocol included in the Experimental section. Error bars reflect the standard deviation from a minimum of three independent measurements.

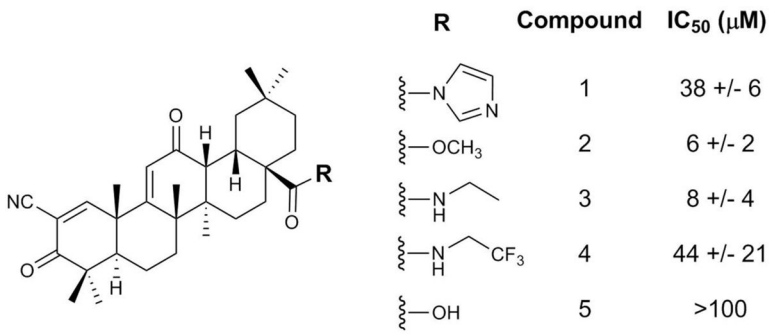

Figure 3. Multiple CDDO derivatives effectively inhibit hGOAT

Structures and IC_{50} values for CDDO derivatives with substitutions at the carboxyl group at position 28: R = imidazole (CDDO-Im, **1**); R = methyl ester (CDDO-Me, **2**); R = ethylamide (CDDO-EA, **3**); R = trifluoroethylamide (CDDO-TFEA, **4**); R = carboxylic acid (CDDO, **5**).

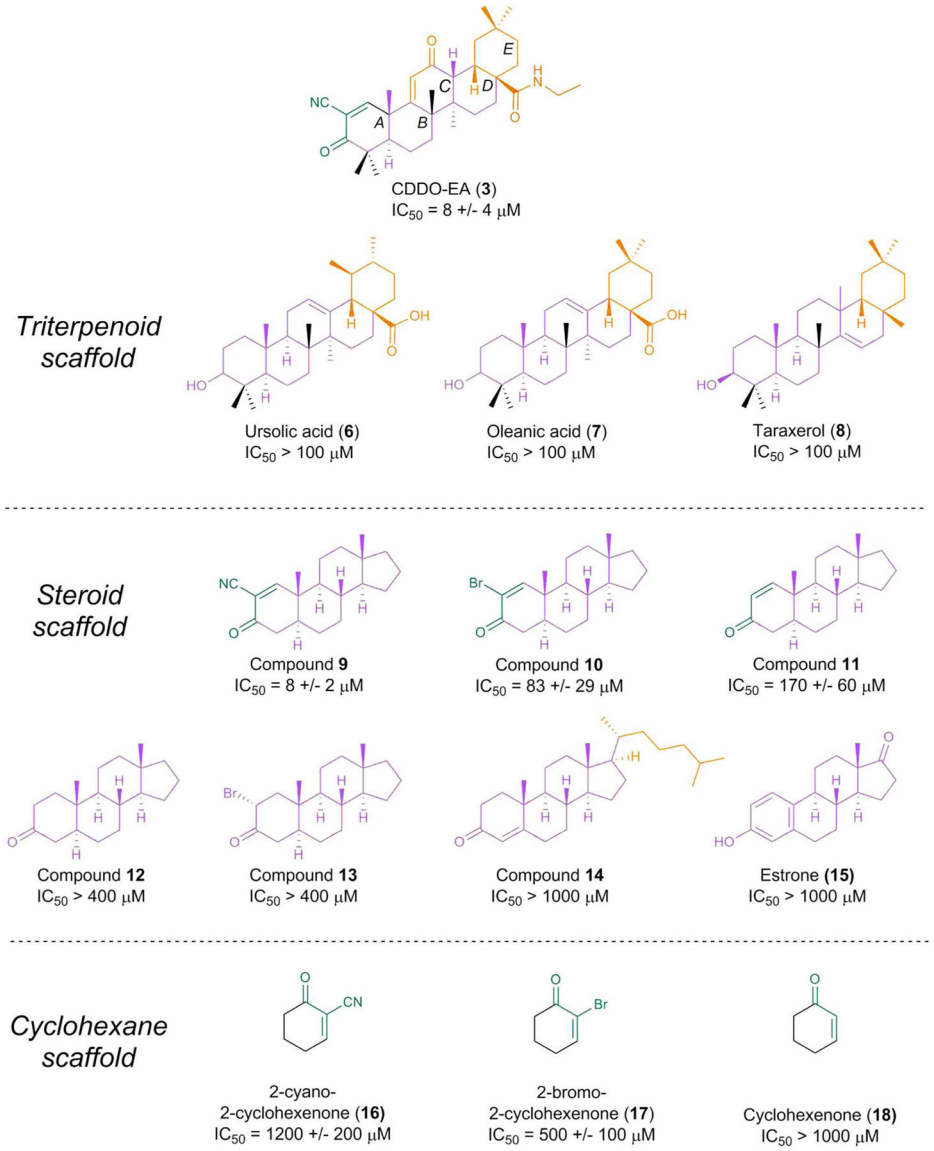

Figure 4. Structure-activity analysis reveals multiple pharmacophores contribute to synthetic triterpenoid inhibition of hGOAT

Compounds tested as inhibitors of hGOAT octanoylation activity, organized by overall hydrocarbon skeleton family (triterpenoid, steroid, or cyclohexane parent structure) and colored to reflect potential pharmacophores (α,β-unsaturated ketone, green; steroid scaffold, purple; CDDO derivative functional groups in rings C-E, orange) Measured IC_{50} values are provided for each compounds, with lower limits established based on compound solubility and lack of inhibition observed at the highest experimentally accessible concentration. Errors reflect standard deviations from a minimum of three determinations. Reactions were performed and analyzed to determine percent activity and IC_{50} values as described in the inhibitor assay protocol included in the Experimental Section. Synthetic protocols for compounds **9-18** are provided in the Online Materials and Methods and Supplementary Information sections.

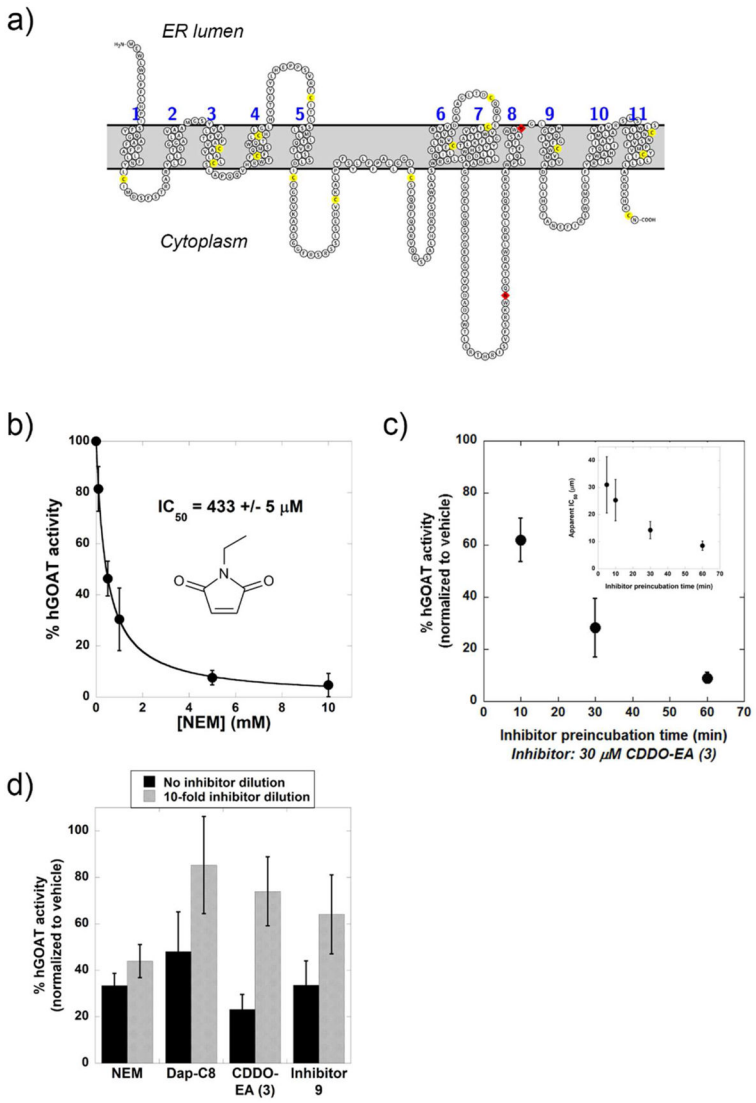

Figure 5. hGOAT inhibition profile supports the involvement of a catalytically essential cysteine residue

a) Topological model of hGOAT, with cysteine residues highlighted in yellow. The conserved functionally essential residues N307 and H338 are denoted in red. This model was constructed by comparison to the experimentally developed topology model for mouse GOAT using the Protter online server.[62, 63] b) Inhibition of hGOAT octanoylation activity by N-ethylmaleimide (NEM, structure shown in inset). c) Time dependence of hGOAT inhibition by CDDO-EA (**3**). hGOAT activity was measured as a function of preincubation time in the presence of 30 μM CDDO-EA. Inset: IC_{50} values for CDDO-EA (**3**) inhibition of hGOAT activity as a function of inhibitor preincubation time. d) Inhibitor dilution assays reveal irreversible hGOAT inhibition by NEM and reversible inhibition by CDDO-EA (**3**) and α-cyanoenone steroid **9**. Dap-C8 denotes the GS(octanamide-Dap)FL product-mimetic GOAT inhibitor used as a control for reversible inhibition.[30, 31] Errors bars reflect the standard deviation from a minimum of three determinations.

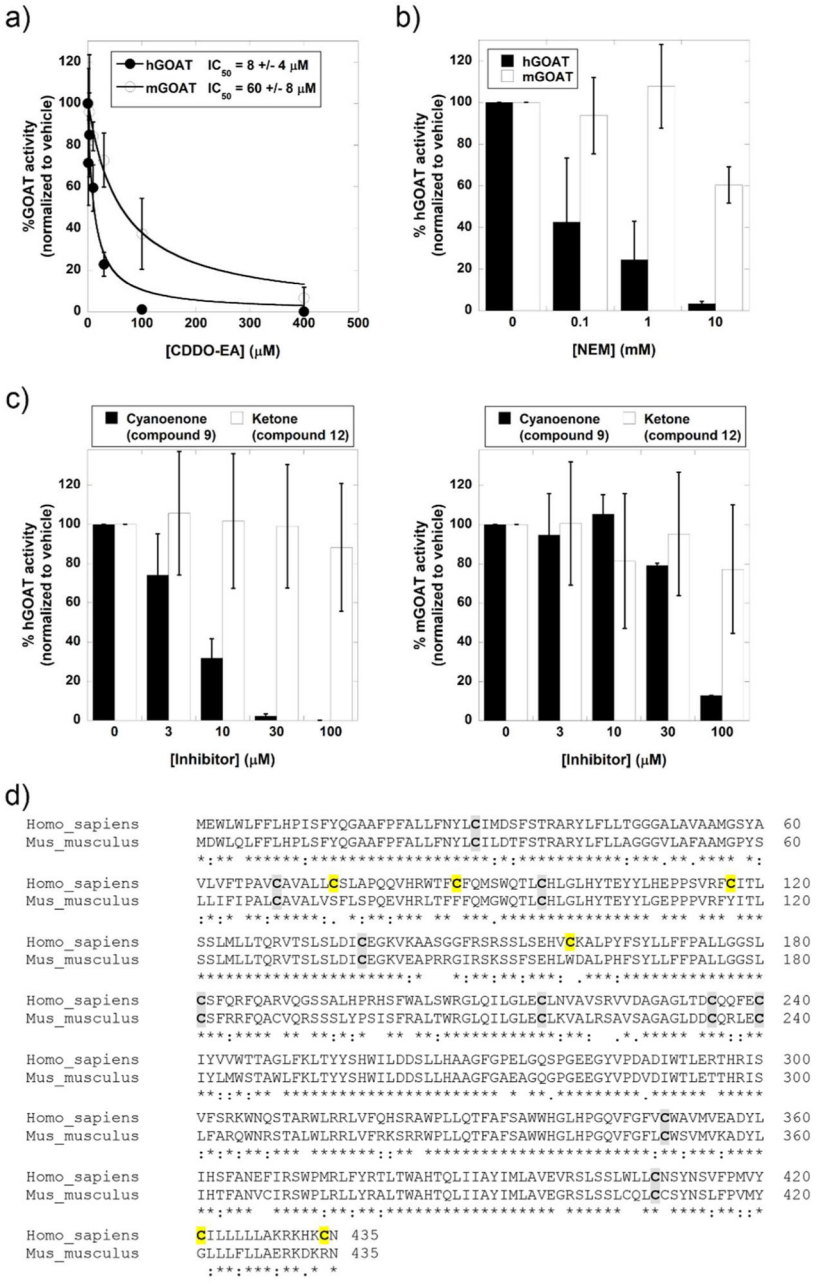

Figure 6. hGOAT and mGOAT exhibit dramatically different susceptibility to inhibition by cysteine modifying agents

a) Inhibition of hGOAT and mGOAT octanoylation activity by CDDO-EA **3**; hGOAT, filled circles; mGOAT, open circles. b) Inhibition of hGOAT and mGOAT octanoylation activity by N-ethylmaleimide (NEM). hGOAT, black bar; mGOAT, white bar. c) Inhibition of hGOAT (left) and mGOAT (right) octanoylation activity by α-cyanoenone **9** and ketone **12**. α-cyanoenone (compound **9**, black bar); ketone (compound 12, white bar), treatment with ketone **12**. d) Clustal Omega alignment of hGOAT and mGOAT sequences. Cysteine residues are indicated in bold, with cysteines conserved in both isoforms highlighted in gray and cysteine residues unique to hGOAT highlighted in yellow. Reactions were performed

and analyzed to determine percent activity and IC_{50} values as described in the Experimental section. Error bars reflect the standard deviation from a minimum of three independent measurements.

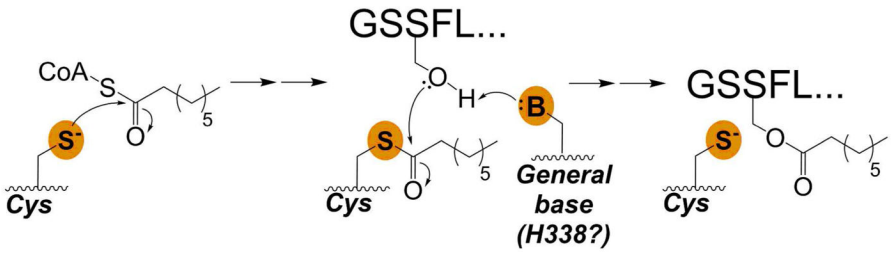

Figure 7. A potential mechanism for hGOAT-catalyzed ghrelin octanoylation employing a cysteine acyl-enzyme intermediate

Following formation of an octanoyl-enzyme intermediate, transfer of the octanoyl group to the serine acylation site near the N-terminus of ghrelin can be catalyzed through involvement of a general base such as the conserved and functionally essential H338 histidine residue within hGOAT.[30, 65]