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Discovery of Hydrolysis-resistant Isoindoline *N*-Acyl Amino Acid Analogs that Stimulate Mitochondrial Respiration

Hua Lin[1, ‡], Jonathan Z. Long[2, ‡], Alexander M. Roche[3], Katrin J. Svensson[2], Florence Dou[3], Mi Ra Chang[1], Timothy Strutzenberg[1], Claudia Ruiz[1], Michael D. Cameron[1], Scott J. Novick[1], Charles M. Berdan[4], Sharon Louie[4], Daniel K. Nomura[4], Bruce M. Spiegelman[3], Patrick R. Griffin[1], Theodore M. Kamenecka[1*]

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ABSTRACT: *N*-acyl amino acids directly bind mitochondria and function as endogenous uncouplers of UCP1-independent respiration. We found that administration of *N*-acyl amino acids to mice improves glucose homeostasis and increases energy expenditure indicating that this pathway might be useful for treating obesity and associated disorders. We report the full account of the synthesis and mitochondrial uncoupling bioactivity of lipidated *N*-acyl amino acids and their unnatural analogs. Unsaturated fatty acid chains of medium length and neutral amino acid head groups are required for optimal uncoupling activity on mammalian cells. A class of unnatural *N*-acyl amino acid analogs, characterized by isoindoline-1-carboxylate head groups (**37**), were resistant to enzymatic degradation by PM20D1 and maintained uncoupling bioactivity in cells and in mice.

INTRODUCTION

The *N*-acyl amino acids are a class of endogenous lipid metabolites with pleotropic bioactivities[1]. Specific members of this metabolite class include *N*-oleoyl serine, *N*-arachidonoyl glycine, and *N*-oleoyl glycine, which have been shown to regulate bone remodelin[2], pain sensation[3], and food intake[4], respectively. At a molecular level, these lipids can act as ligands for ion channels[5] (e.g., TRPV1) or G-protein coupled receptors[6] (e.g., GPR92). To date, over fifty distinct species of *N*-acyl amino acids have been detected in mammalian tissues[7]. However, the full spectrum of biological functions for this class of endogenous lipids remain incompletely characterized.

We have recently identified a novel function for several *N*-acyl amino acids, including *N*-oleoyl leucine, *N*-oleoyl phenylalanine, and *N*-arachidonoyl glycine. These compounds can uncouple mitochondrial respiration, stimulating respiration on isolated mitochondria in both cells and mice[8]. Uncoupling oxidative respiration makes ATP production less efficient resulting in increased metabolic rate, and loss of energy through heat production[9,10]. Key to this discovery was the annotation of the circulating factor peptidase M20 domain containing 1 (PM20D1) as a dominant enzyme that regulates *N*-acyl amino acid levels in vivo. Genetically increased circulating PM20D1 by adeno-associated virus (AAV) delivery to the liver augments circulating *N*-acyl amino acids, leading to increased respiration and blunted weight gain on high fat diet in mice. Direct administration of *N*-acyl amino acids to mice by intraperitoneal (IP) injection also produces increased whole body energy expenditure, with weight loss and improved glucose homeostasis. Mechanistically, *N*-acyl amino acids directly act on isolated mitochondria to increase respiration, potentially by interacting with the SLC25 family of inner mitochondrial transporters[11].

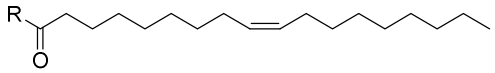
To more fully understand how *N*-acyl amino acids stimulate respiration, we herein disclose a full account of our structure-activity relationship (SAR) studies of *N*-acyl amino acids and their uncoupling bioactivity. The uncoupling activity of this metabolite class is largely restricted to those with neutral amino acid head groups and unsaturated fatty acyl chains of medium length. By exploring unnatural analogs of these metabolites, we identified the proline derivative N-oleoyl-isoindoline-1-carboxylate **37** as a semisynthetic *N*-acyl amino acid analog with exceptional uncoupling activity. **37** possessed uncoupling activity in cells and in mice, but unlike the natural metabolites, was entirely resistant to enzymatic hydrolysis by PM20D1.

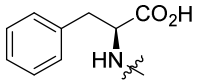
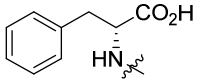
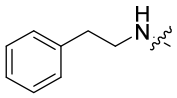
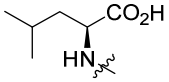
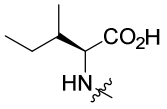
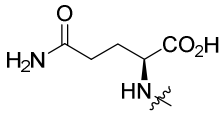
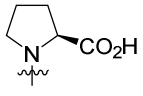
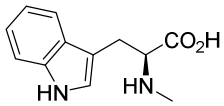
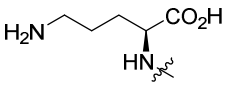
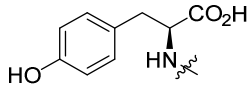
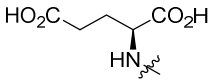
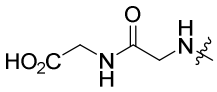
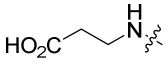
RESULTS AND DISCUSSION

To determine the uncoupling activity of the *N*-acyl amino acid analogs, we tested their effect on cellular respiration of C2C12 mouse myoblast cells. Because these compounds show a time-dependent effect on changes in respiration, we standardized our assay results by reporting the maximal stimulation of respiration as compared to DMSO-control. Further standardizing our respiration assays, all compounds were assayed at 50 μM (see also **Supplemental Figure 1**). This is below the critical micelle concentration for these analogs (see **Supplemental Figure 2**)[12]. Initially, we restricted the scope to oleic acid-conjugated amino acids. As previously reported, C18:1-Phe

possess potent uncoupling bioactivity[8], stimulating respiration to ~160% of baseline levels (**Table 1**, compound **1**). Amino acid stereospecificity was not observed in this uncoupling bioactivity, as both C18:1-D-Phe and C18:1-L-Phe stimulated cellular respiration equally well (**Table 1**, compound **2**). Consistent with our previous report, the amino acid head group carboxylate was absolutely required for activity: removal of the carboxylate moiety entirely abolished uncoupling activity (**Table 1**, compound **3**). Next, by varying the amino acid head group, we found that amino acids containing neutral side chains (e.g., Leu, Ile, Gln, Pro, Trp, compounds **4 – 8**) were potent uncouplers, but those compounds with charged side chains (e.g., Lys, Tyr, Glu, compounds **9 – 11**) were unable to induce respiration (Table 1). Some tolerance for backbone steric modifications were acceptable on the amino acid side. For instance, inclusion of homoglycine or the dipeptide Gly-Gly head group preserved uncoupling bioactivity (**Table 1**, compounds **12** and **13**).

Table 1. Structure and uncoupling bioactivity of compounds with different amino acid head groups.[a]



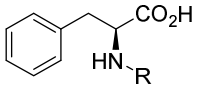
Cmpd	Head group	R	%[b]
1	L-Phe		161 ± 12
2	D-Phe		173 ± 9
3	n/a		104 ± 4
4	L-Leu		178 ± 13
5	L-Ile		167 ± 14
6	L-Gln		164 ± 4
7	L-Pro		182 ± 7
8	L-Trp		147 ± 9
9	L-Lys		108 ± 4
10	L-Tyr		105 ± 13
11	L-Glu		106 ± 7
12	Gly-Gly		206 ± 32
13	Homo-glycine		185 ± 10

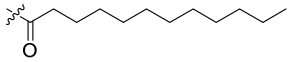
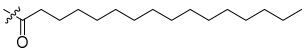
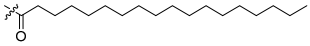
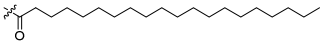
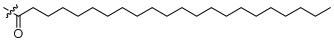
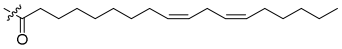
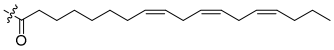
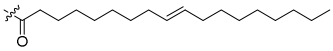
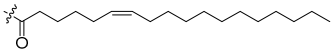
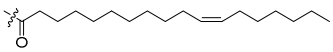
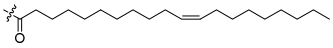
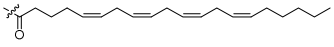
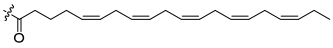
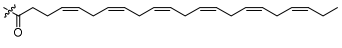
[a]Respiration in C2C12 cells is shown as maximal increases versus basal oligomycin-treated respiration, which is normalized to 100%. Data are shown as means ± SEM, n=3-6/group; [b]stimulation of respiration (baseline = 100%).

For the fatty acid side chain, we observed striking structural requirements regarding length and type of unsaturation for uncoupling bioactivity. By sequentially stepping through increasing fatty acid chain lengths, we found that fatty acyl chains that were too short (e.g., < C12:0) or too long (e.g., >C20:0) possessed no uncoupling activity (**Table 2**, compounds **14 – 18**). Within the "medium" fatty acyl chain range, specific types of unsaturation potently improve the respiration response. For instance, we observed a step-wise increase in uncoupling activity from C18:0-Phe < C18:1-Phe < C18:2-Phe (compound **16** vs. **1** vs. **19**) that, surprisingly, drops off entirely with C18:3-Phe (compound **20**). Other specific olefin perturbations also underscored the importance of specific types and locations of unsaturation. For instance, compared to *cis*-Δ^9-C18:1-Phe (compound **1**), *trans*-Δ^9-C18:1-Phe (**Table 2**, compound **21**) olefin shows essentially no uncoupling bioactivity. Similarly, *cis*-Δ^6-C18:1-Phe and *cis*-Δ^{11}-C18:1-Phe (**Table 3**, compound **22** and **23**) were all inferior to the parent compound **1**.

Given the strong influence of unsaturation on the acyl chain length, we systematically increased unsaturation of C20:0-Phe (compound **17**), a parent compound that initially does not possess uncoupling activity. Again, increasing unsaturation augments uncoupling bioactivity with an apparent optimal unsaturation at C20:4 (**Table 2**, compounds **24 – 26**). A similar strategy to increase unsaturation also converted inactive C22:0-Phe (compound **18**) into a potent uncoupler (C22:6-Phe, compound **27**). The specific requirement for unsaturation is reminiscent of cannabinoid receptor ligands, in which only arachidonoyl- but not other fatty acyl-containing lipids can agonize the receptor13. The unsaturation specificity observed in this *N*-acyl amino acid series (**Table 2**) is consistent with the shape complementarity imposed by interactions with proteinaceous factors that mediate uncoupling. Taken together, our data map out the optimal requirements for uncoupling responses within the *N*-acyl amino acid class: an uncharged carboxylate-containing head group, a medium fatty acid chain length, and specific sites of unsaturation along the acyl chain.

Table 2. Structure and uncoupling bioactivity of compounds with different fatty acid side chains.[a]

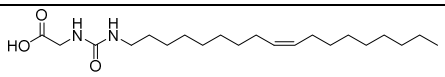
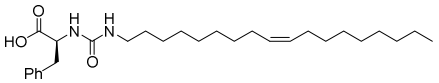


Cmpd	Fatty acid	R	%[b]
14	C12:0		96 ± 4
15	C16:0		180 ± 20
16	C18:0		148 ± 12
17	C20:0		95 ± 8
18	C22:0		100 ± 3
19	C18:2		218 ± 10
20	C18:3		109 ± 12
21	trans-C18:1		107 ± 3
22	Δ6-C18:1		110 ± 12
23	Δ11-C18:1		115 ± 8
24	C20:1		103 ± 3
25	C20:4		200 ± 12
26	C20:5		259 ± 43
27	C22:6		166 ± 11

[a]Respiration in C2C12 cells is shown as maximal increases versus basal oligomycin-treated respiration, which is normalized to 100%. Data are shown as means ± SEM, n=3-6/group; [b]stimulation of respiration (baseline = 100%).

Given the structure activity relationships we observed by simply varying the two sides of the amide bond, we next explored an alternative bond linkage between the fatty acid and amino acid to identify those that may possess superior properties as compared to the naturally occurring metabolites, with the ultimate goal of developing improved mitochondria-uncoupling analogs that might be useful for the treatment of obesity and metabolic disease[14,15]. Towards this end, we synthesized two urea analogs of C18:1-Gly and C18:1-Phe that we hypothesized might be less metabolically labile compared to the natural amides, and tested their effects on cellular respiration. Both urea analogs failed to induce respiration on cells, demonstrating a potential preference for the presence of an amide bond in the bioactivity of this class of compounds (**Table 3**).

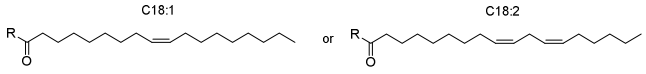
Table 3. Structure and uncoupling bioactivity of unnatural urea-containing N-acyl amino acid analogs.[a]

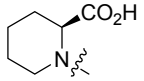
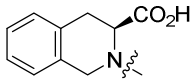
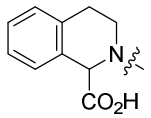
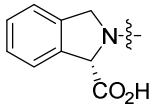
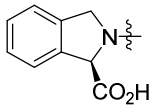
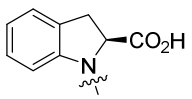
Cmpd	Structure	%[b]
28		104 ± 3
29		97 ± 5

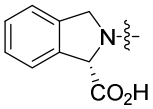
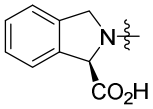
[a]Respiration in C2C12 cells is shown as maximal increases versus basal oligomycin-treated respiration, which is normalized to 100%. Data are shown as means ± SEM, n=3-6/group; [b]stimulation of respiration (baseline = 100%).

We also surveyed unnatural amino acid head groups. We were intrigued by the potent uncoupling observed with C18:1-Pro (compound **7**) and noted that this derivative with a cyclic head group is the most structurally distinct from the other *N*-acyl amino acids tested. We therefore synthesized and tested a variety of unnatural proline and homoproline *N*-acyl amino acid derivatives with oleate as a fatty acyl chain. Testing these unnatural analogs in cellular respiration assays revealed that they all stimulated uncoupling to a maximum of ~150-240% of DMSO control (**Table 4**, compounds **30** – **35**). Derivatization of these isoindoline head groups with linoleoyl instead of oleoyl acyl groups also produced potent uncouplers (**Table 4**, compounds **36** and **37**). These analogs **30** – **37** demonstrate that the uncoupling bioactivity of *N*-acyl amino acids can extend beyond structures containing only the proteinogenic amino acids.

Table 4. Structure and uncoupling bioactivity of compounds with unnatural proline- and homoproline-drived head groups.[a]

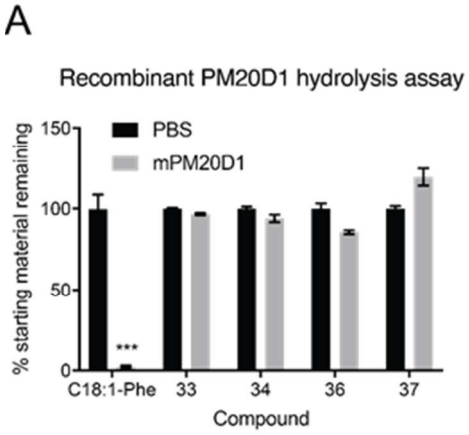


Cmpd	Fatty acid	R	%[b]
30	C18:1		174 ± 3
31	C18:1		187 ± 19
32	C18:1		216 ± 17
33	C18:1		240 ± 24
34	C18:1		190 ± 12
35	C18:1		146 ± 5

36	C18:2		185 ± 18
37	C18:2		190 ± 12

[a]Respiration in C2C12 cells is shown as maximal increases versus basal oligomycin-treated respiration, which is normalized to 100%. Data are shown as means ± SEM, n=3-6/group; [b]stimulation of respiration (baseline = 100%).

N-acyl amino acids can be hydrolytically inactivated through cleaveage by PM20D1, a bidirectional amidase that cleaves fatty acid amides to liberate free fatty acids and amino acids[8]. Given the unusual cyclic structures of **30** – **37**, we hypothesized that these analogs might have lower rates of hydrolysis, and therefore higher stability. We therefore incubated purified, recombinant murine PM20D1 with a canonical *N*-acyl amino acid substrate, *N*-oleoyl phenylalanine, or one of the unnatural *N*-acyl amino acids. We observed >90% hydrolysis of *N*-oleoyl phenylalanine to oleic acid in our assay conditions, demonstrating that this enzyme efficiently cleaves naturally occurring *N*-acyl amino acids. Remarkably, all unnatural *N*-acyl amino acids tested were completely resistant to PM20D1-mediated hydrolysis (**Fig. 1a**). Consistent with these observations, when endogenously occurring *N*-acyl amino acids or unnatural analogs were incubated with mouse liver microsomes, the unnatural analogs also maintained exceptionally long half-lives (**Fig. 1b**): as a group, the average half-life of the natural *N*-acyl amino acids was 8 min, whereas the average for *N*-acyl analogs with unnatural head groups was 64 min. The tertiary amide structures in **30**-**37** may also contribute to improved metabolic stability relative to the secondary amides tested. These data therefore demonstrate that changing the amide head group can alter the downstream metabolism of *N*-acyl amino acid analogs.



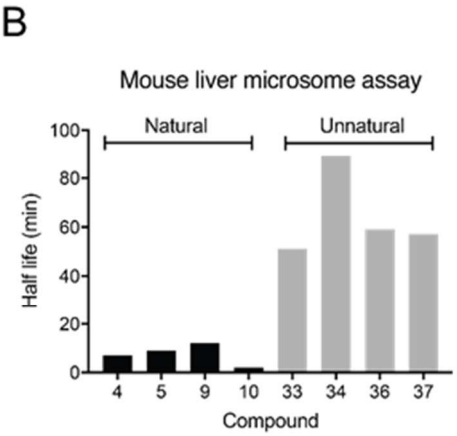


Fig. 1. (A) Amount of the indicated compound remaining in the presence of PBS or recombinant, murine PM20D1 after incubation at 37ºC for 1 h. Data are shown as means ± SEM, n = 3/group. *** $P < 0.001$. (B) Half-life (min) of the indicated compound after incubation in mouse liver microsomes. "Natural" indicates endogenously present *N*-acyl amino acid whereas "unnatural" indicates an *N*-acyl amino acid analog with a non-proteinogenic head group.

Lastly, we tested the uncoupling bioactivity of unnatural N-acyl amino acid analogs in mice. Towards this end, we adopted a previously used assay in which mice rendered obese by high fat diet feeding (diet-induced obesity, DIO) are given N-acyl amino acids daily by IP administration. Synthetic chemical uncouplers such as 2,4-dinitrophenol have been shown to blunt weight gain in this model[15], and administration of N-acyl amino acid led to weight loss[8]. For these experiments, we selected compound **37** because this compound showed excellent uncoupling bioactivity (**Table 4**) and resistance to hydrolysis (**Fig. 1**). DIO mice (initial weight, 46.8 ± 0.6 g) were treated with increasing doses of compound **37** (5, 10, and 25 mg/kg/day IP) daily for 7 consecutive days (**Fig. 2a**). Though no differences in weight were observed at 5 mg/kg vs. vehicle-treated mice, both 10 and 25 mg/kg doses of compound **37** led to dose-dependent blunting of body weight gain (net weight change, 0.2 ± 0.2 g and –1.2 ± 0.6 g, respectively, vs. 1.1 ± 0.1 g for vehicle-treated mice, $P < 0.05$ for each comparison). Food intake was not statistically significantly reduced in mice at these higher doses (**Fig. 2b**). However, ad lib blood glucose 2 h after the last compound injection was significantly lower in mice treated with 25 mg/kg compound **37** versus vehicle-treated mice (172 ± 7 mg/dl versus 204 ± 7 mg/dl, respectively, **Fig. 2c**). These data therefore indicate that unnatural *N*-acyl amino acid **37** maintains uncoupling bioactivity in vivo.

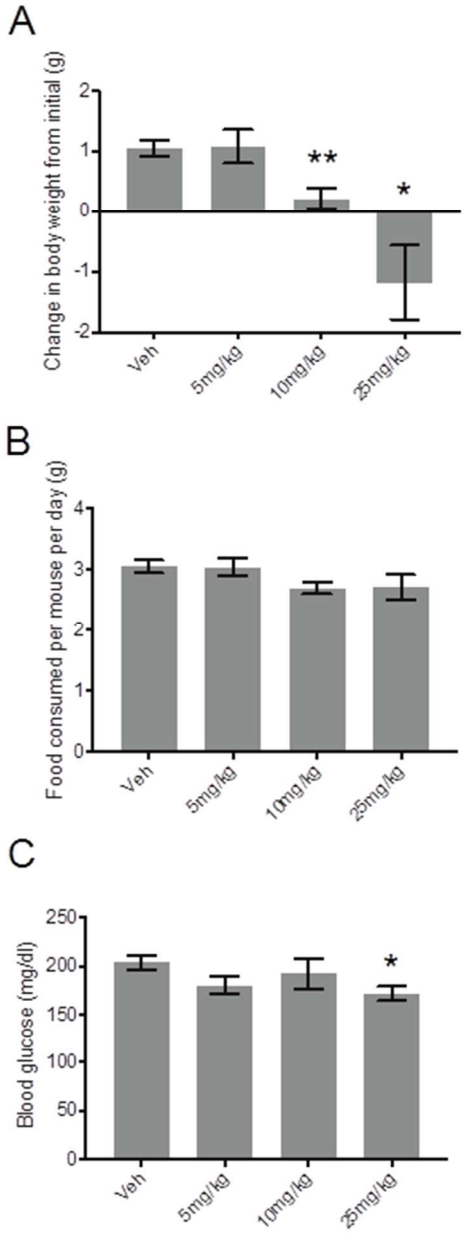
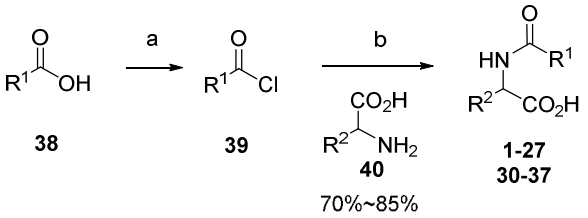


Fig. 2. (A-C) Total change in body weight (A), food intake (B), and ad lib blood glucose (C) of diet-induced obese mice (19 weeks high fat diet) after 7 days daily treatment with the indicated dose (IP) of compound **37**. Prior to compound administration, mouse weights were not different between the groups (average weight across all groups, 46.8 ± 0.6 g). Data are shown as means ± SEM, n=5/group. *, $P < 0.05$; **, $P < 0.01$ for the indicated dose versus vehicle-treated mice.

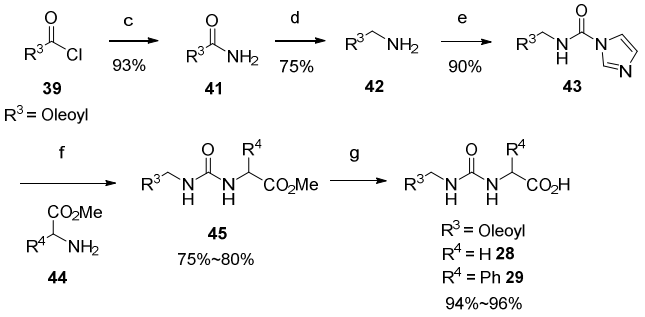
CHEMISTRY

Compounds were synthesized as depicted in Scheme 1 following well-known procedures. Commercially available acid chlorides **39** (using General procedure A), or those generated from acid **38** with oxalyl chloride (using General procedure B), were coupled with the corresponding natural or unnatural amino acids **40** to provide *N*-acyl amino acid analogs **1**-**27** and **30**-**37** (**Scheme 1**). Oleoyl chloride reacted with ammonia hydroxide to give primary amide **41**, which was reduced with $LiAlH_4$ to afford primary amine **42**. Imidazole intermediate **43** was obtained by treatment of amine **42** with CDI, which then reacted with amino esters **44** in the presence of DIPEA. The resulting urea esters **45** were hydrolyzed with LiOH to provide the desired urea analogs **28** or **29** in good yields (using General procedure C) (Scheme 2).

Scheme 1. Synthesis of Natural and Unnatural *N*-Acyl Amino Acid Analogs[a]



Scheme 2. Synthesis of Urea Analogs[a]



[a]Reaction conditions: (a) oxalyl chloride, DMF, DCM; (b) K_2CO_3, acetone/H_2O or DIPEA, DCM; (c) NH_4OH, THF; (d) $LiAlH_4$, THF; (e) CDI, DCM; (f) DIPEA, DCM; (g) LiOH, then HCl.

CONCLUSIONS

Here we provide a detailed account of the synthesis and structure activity relationships of the *N*-acyl amino acids and their uncoupling activity on mammalian cells. Our data reveal the structural requirements for the uncoupling bioactivity of this class of endogenous metabolites, which include a strict requirement for neutral carboxylate-containing head groups, medium chain fatty acids with specific unsaturation, and an amide bond connecting the two. Through exploration of unnatural *N*-acyl amino acid analogs, we identify *N*-acyl isoindoline **37** as a potent, hydrolysis-resistant compound that displays uncoupling bioactivity in cells and in vivo. Projecting forward, compound **37** or other hydrolysis-resistant *N*-acyl amino acid analogs may serve as useful probes for understanding the full spectrum of bioactivities associated with the *N*-acyl amino acid family of lipids. Such compounds may also serve as leads for alternative structural classes that may have widened therapeutic windows or altered beneficial and adverse profiles compared with the more "classical" chemical uncouplers such as 2,4-dinitrophenol.

EXPERIMENTAL SECTION

All solvents and chemicals were reagent grade. Unless otherwise mentioned, all reagents and solvents were purchased from commercial vendors and used as received. Flash column chromatography was carried out on a Teledyne ISCO CombiFlash Rf system using prepacked columns. Solvents used include hexane, ethyl acetate (EtOAc), dichloromethane and

methanol. Purity and characterization of compounds were established by a combination of HPLC, TLC, mass spectrometry, and NMR analyses. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX-400 (400 MHz) spectrometer and were determined in chloroform-*d* or DMSO-d^6 with solvent peaks as the internal reference. Chemical shifts are reported in ppm relative to the reference signal, and coupling constant (J) values are reported in hertz (Hz). Thin layer chromatography (TLC) was performed on EMD precoated silica gel 60 F254 plates, and spots were visualized with UV light or iodine staining. Low resolution mass spectra were obtained using a Thermo Scientific ultimate 3000/ LCQ Fleet system (ESI). High resolution mass spectra were obtained using a Thermo Scientific EXACTIVE system (ESI). All test compounds were greater than 95% pure as determined by qNMR on a Bruker Avance DPX-400 (400 MHz) spectrometer.

General procedure A. To the amino acid (2 eq.) in acetone and water (0.1 M, 1:1, v/v) was added K_2CO_3 (3 eq.) and acyl chloride (1 eq.) at 0 ^{o}C. The reaction mixture was stirred at room temperature overnight and was then acidified with HCl (1 M) until pH<3. The mixture was extracted with ethyl acetate, washed with brine, dried (Na_2SO_4) and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography (silica gel, ethyl acetate/hexanes) to give the desired product.

General procedure B. To a solution of fatty acid (1 eq.) in DCM was added oxalyl chloride (1.2 eq.) and one drop of DMF at 0 ^{o}C. The reaction was aged at room temperature for 2 hours and then concentrated *in vacuo*. The crude residue was dissolved in DCM and added to a suspension of amino acid (1.5 eq.) and DIPEA (2 eq.) and DCM. The reaction mixture was stirred at room temperature overnight and was then acidified with 1.0M HCl to pH<3. The result mixture was extracted with DCM, washed with brine, dried (Na_2SO_4), and concentrated. The crude residue was purified by flash chromatography on silica gel to give the product.

General procedure C. Step 1: To a 0 ^{o}C solution of oleoyl chloride (1 g) in THF (20 mL) was added ammonia hydroxide (10 eq.). The mixture was allowed to warm to room temperature and stirred for 3 hours. The mixture was filtered to give the desired oleamide **41** as a colorless solid (796 mg, 86%). Step 2: To a suspension of $LiAlH_4$ (2 eq.) in THF was added oleamide in one portion at 0 ^{o}C. The mixture was heated to reflux overnight. Then the mixture was quenched with water (3 drops) at 0 ^{o}C, followed by 1 M NaOH solution and then stirred at room temperature for 1 hour. The suspension was filtered through celite. The filtrate was diluted with ethyl acetate, washed with water and brine, dried (Na_2SO_4) and concentrated to give (*Z*)-octadec-9-en-1-amine **42** (726 mg, 95%) which was used for the next step without further purification. Step 3: To a solution of (*Z*)-octadec-9-en-1-amine **42** in DMF was added DIEA (2 eq.) and CDI (1.5 eq.) at 0 ^{o}C. The mixture was stirred at room temperature overnight. The mixture was then diluted with ethyl acetate, washed with $NaHCO_3$, brine, dried (Na_2SO_4) and concentrated. Purification of the crude residue on silica gel afforded (*Z*)-*N*-(octadec-9-en-1-yl)-1H-imidazole-1-carboxamide **43** (795 mg, 81%). Step 4: To a solution of (*Z*)-*N*-(octadec-9-en-1-yl)-1H-imidazole-1-carboxamide **43** in DMF was added DIEA (2 eq.) and amino ester **44**. The mixture was stirred at room temperature overnight. Then the mixture was diluted with ethyl acetate, washed with $NaHCO_3$, brine, dried (Na_2SO_4) and concentrated in vacuo. The crude residue was purified on silica gel to give intermediate **45** (75-80%). Step 5: To a solution of intermediate **45** in THF and H_2O (1:1) was added LiOH (5 eq.). The mixture was stirred at room temperature for 3 hours. The mixture was then acidified with HCl (1M) until pH<3. Then the mixture was extracted with ethyl acetate, washed with brine, dried (Na_2SO_4) and concentrated to give the desired compound **28** or **29** (94-96%).

PM20D1 hydrolysis assays. 10 nmol of the indicated compound was incubated in 100 µl PBS (100 µM initial substrate). Reactions were initiated by the addition of PBS or mPM20D1 (5 µl). After 1 h at 37ºC, reactions were quenched with 600 µl of a 2:1 v/v mixture of chloroform and methanol with 10 nmol d_{31}-palmitate as an internal standard. The reactions were vortexed and the organic layer was transferred to a sample vial for analysis by LC-MS. For separation of polar metabolites, normal-phase chromatography was performed with a Luna-5 mm NH_2 column (50 mm × 4.60 mm, Phenomenex). Mobile phases were as follows: Buffer A, acetonitrile; Buffer B, 95:5 water/ acetonitrile with 0.1% formic acid or 0.2% ammonium hydroxide with 50 mM ammonium acetate for positive and negative ionization mode, respectively. The flow rate for each run started at 0.2 ml/min for 2 min, followed by a gradient starting at 0% B and increasing linearly to 100% B over the course of 15 min with a flow rate of 0.7 ml/min, followed by an isocratic gradient of 100% B for 10 min at 0.7 ml/min before equilibrating for 5 min at 0% B with a flow rate of 0.7 ml/min. MS analysis was performed with an electrospray ionization (ESI) source on an Agilent 6430 QQQ LC−MS/MS. The capillary voltage was set to 3.5 kV, and the fragmentor voltage was set to 100 V. The drying gas temperature was 325 °C, the drying gas flow rate was 10 l/min, and the nebulizer pressure was 45 psi. Monitoring of hydrolysis starting materials and products was performed by scanning a mass range of m/z 50-1200. Peaks corresponding to the liberated fatty acids (products) or the intact starting material was integrated.

ASSOCIATED CONTENT

Supporting Information.

The Supporting information is available free of charge on the ACS Publications website at DOI:

Full characterization data on all analogs including Molecular Formula Strings, cell respiration and microsome stability assays, PM20D1 generation, Seahorse experimentals, critical micelle concentration measurements, and *in vivo* details.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

UCP1, uncoupling protein 1; TRPV1, transient receptor potential cation channel subfamily V member 1; GPR92, G-protein coupled receptor 92; SLC25, solute carrier family 25; DMSO, Dimethyl sulfoxide; CDI, Carbonyldiimidazole; DIPEA, N,N-Diisopropylethylamine; DMF, Dimethylformamide; DCM, Dichloromethane; THF, Tetrahydrofuran.

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TABLE OF CONTENTS GRAPHIC