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# The first synthetic agonists of FFA2: Discovery and SAR of phenylacetamides as allosteric modulators

Yingcai Wang [a,*], Xianyun Jiao [a], Frank Kayser [a], Jiwen Liu [a], Zhongyu Wang [a], Malgorzata Wanska [a], Joanne Greenberg [b], Jennifer Weiszmann [b], Hongfei Ge [b], Hui Tian [b], Simon Wong [c], Ralf Schwandner [d], Taeweon Lee [b], Yang Li [b,*]

[a] Department of Chemistry, Amgen Inc., 1120 Veterans Boulevard, South San Francisco, CA 94080, USA
[b] Department of Metabolic Disorders, Amgen Inc., 1120 Veterans Boulevard, South San Francisco, CA 94080, USA
[c] Department of Pharmacokinetics and Drug Metabolism, Amgen Inc., 1120 Veterans Boulevard, South San Francisco, CA 94080, USA
[d] Amgen Research GmbH, Regensburg, Germany

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ABSTRACT

Free fatty acid receptor 2 (FFA2) is a G-protein coupled receptor for which only short-chain fatty acids (SCFAs) have been reported as endogenous ligands. We describe the discovery and optimization of phenylacetamides as allosteric agonists of FFA2. These novel ligands can suppress adipocyte lipolysis in vitro and reduce plasma FFA levels in vivo, suggesting that these allosteric modulators can serve as pharmacological tools for exploring the potential function of FFA2 in various disease conditions.



Free fatty acid receptor 2 (FFA2), also known as GPR43, is a member of a subfamily of closely related G-protein-coupled receptors (GPCR) that can be activated by free fatty acids (FFA) of various chain lengths.[1,2] The other members of the subfamily, FFA1 (GPR40) and FFA3 (GPR41), as well as FFA2, cluster at chromosome 19q13.1 in humans and share ~30–40% sequence identity.[3] Each subfamily member shows a distinct structure–activity relationship (SAR) for FFAs, with short-chain fatty acids (SCFAs, six or fewer carbon molecules) activating FFA2 and FFA3, and medium- and long-chain fatty acids activating FFA1.[4] Acetate and propionate are the most potent natural ligands for FFA2 that can induce coupling to both $G_{\alpha i}$ and $G_{\alpha q}$.[5–7]

Since GPCRs have served as successful targets for a wide range of therapeutic molecules,[8,9] and given the important physiological effects of FFAs, this subfamily of receptors has sparked great interest in recent years as potential novel targets for various diseases.[2] FFA2 expression has been reported to be enriched in islets, various leukocyte populations, adipocytes, and the gastrointestinal tract suggesting its potential role in conditions associated with inflammation and metabolic disorders.[5–7,10,11] We have recently shown that FFA2 expression is induced during adipocyte differentiation and that activation of FFA2 by acetate resulted in inhibition of adipocyte lipolysis in vitro and reduction of plasma FFA levels in vivo.[11] Given the similarity in effects on adipocyte function to the nicotinic acid receptor, GPR109A, and the proposed mechanism for the improvement in lipid profiles observed by niacin treatment via inhibition of lipolysis,[12] we were interested in the potential utility of targeting FFA2 for the treatment of dyslipidemia and other metabolic disorders. Clearly, the identification of pharmacological tools and better understanding of the receptor function will facilitate the development of any potential therapies targeting this receptor.

The potencies of SCFAs for FFA2 are low, in the high micromolar to millimolar concentrations.[2] In order to identify better pharmacological tools to study the physiological functions of the receptor and its involvement in various diseases, we set out to search for more potent FFA2 agonists. Here, the discovery, synthesis, and SAR of phenylacetamides, a novel series of FFA2 agonists are reported. The most promising compounds were also profiled for their pharmacokinetic properties.

A high-throughput screening (HTS) campaign of our internal sample collection led to the identification of compound **1**, which activated FFA2 with a better than 100-fold improvement in

* Corresponding authors.
*E-mail addresses:* yingcaiw@amgen.com (Y. Wang), yangl@amgen.com (Y. Li).

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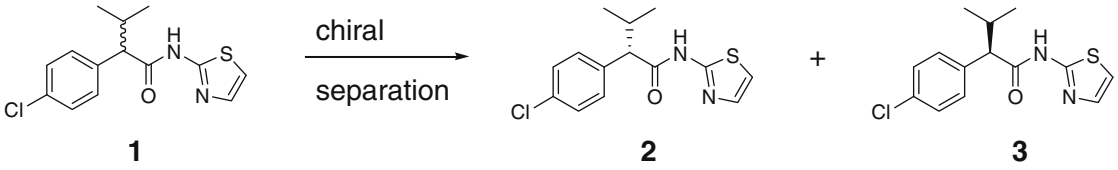
**Table 1**
FFA2 activities for SCFAs and compound **1** and its chiral components **2** and **3**




| Compds | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|
| Acetate | 120 | 100 |
| Propionate | 130 | 100 |
| **1** | 0.8 | 98 |
| **2** | 0.7 | 100 |
| **3** | >100 | <30 |

[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.

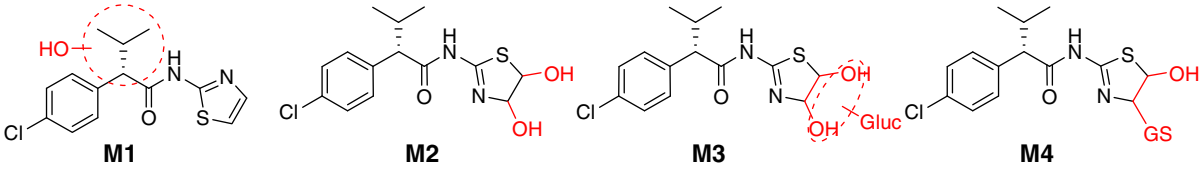



**Figure 1.** Metabolic profiling of compound **2**.

potency in a forskolin-induced cAMP assay (Table 1).[13] Chiral separation of racemic **1** into its enantiomers **2** and **3** revealed that the activity resides in the (*S*) configuration (compound **2**).[14]

Unfortunately, compound **2** displayed a poor pharmacokinetic profile in male Sprague-Dawley rats (**2**: Cl = 6.4 L/h/kg, AUC = 79 μg h/L; 0.5 mg/kg iv) and poor microsomal stability (2% remaining at 30 min with rat liver microsomes). Metabolic profiling of compound **2** in rat and human liver microsomes identified two metabolic soft spots (Fig. 1). First, hydroxylation was seen on the isopropyl group (**M1**). Oxidation of the C=C bond followed by hydrolysis (**M2**), glucuronidation (**M3**) and glutathione conjugation (**M4**) were identified on the thiazole.

To quickly identify good in vivo tool compounds, we set out to improve the PK profiles by focusing our chemistry efforts on modifications in the amide region and the isopropyl group. The SAR is described here first, followed by the discussion on improvement of PK profiles.

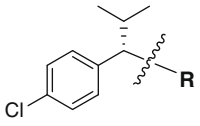
A brief survey of the amide region was conducted, including reversed amide (**5**), sulfonamides (**9** and **10**), and acid **8** (Table 2). However, none of the efforts yielded a replacement for the amide. Compound **4** showed that N-methylation of the amide resulted in a significant loss of activity, thus we focused on secondary amides for our following efforts.

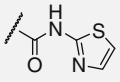
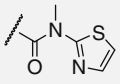
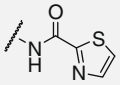
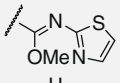
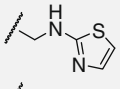
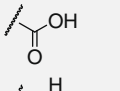
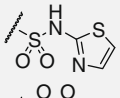
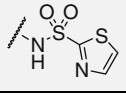
Subsequently, the SAR of the thiazolylamine portion was studied in more detail (Table 3). The primary amide (**11**) and alkyl amides (**12**–**14**, and analogs not shown) lose most of the activity. Phenyl amides (**15** and analogs not shown) are generally not very active. However, 2-pyridyl amides (**18**–**20**) as well as the pyridazin-3-yl amide (**23**) proved to be notably potent.

Exploration of five-membered heterocycles resulted in many interesting findings (Table 4). Incorporating thiadiazoles (**26** and **27**) and a few benzofused heterocycles (**35**–**37**) yielded compounds with good activities. Notably a $CF_3$ group can enhance the activity by sixfold on the 1,3,4-thiadizole (compound **26** vs **25**). An aromatic ring is not required, as exemplified by compound **33**.

The effect of substituents on the thiazole was studied in somewhat more detail (Table 5). The 4-position appears to be less toler-

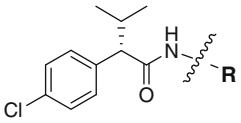
**Table 2**
FFA2 activities of amide derivatives

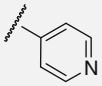
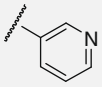
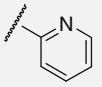
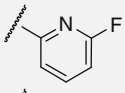
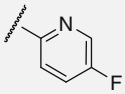
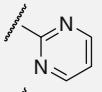
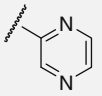
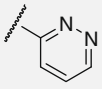



| Compds | R | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|---|
| **2** |  | 0.7 | 100 |
| **4** |  | 84 | 37 |
| **5** |  | >100 | <30 |
| **6** |  | >100 | <30 |
| **7** |  | >100 | <30 |
| 8[c] |  | >100 | <30 |
| 9[c] |  | >100 | <30 |
| **10** |  | >100 | <30 |

[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.
[c] Racemic mixture.

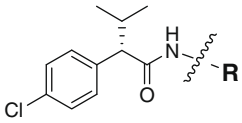
**Table 3**
Amide SAR I: alkyl and six-membered aromatic rings

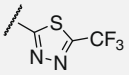
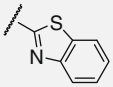
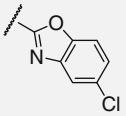
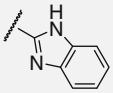


| Compds | R | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|---|
| **11**[c] | H | >100 | <30 |
| **12** | Me | 11 | 66 |
| **13** | *t*-Bu | >100 | <30 |
| **14** | Bn | >100 | <30 |
| **15** | Ph | >100 | <30 |
| **16** |  | 56 | 40 |
| **17**[c] |  | 49 | 73 |
| **18** |  | 1.4 | 92 |
| **19** |  | 1.1 | 84 |
| **20** |  | 1.3 | 93 |
| **21**[c] |  | 53 | 50 |
| **22**[c] |  | 5.7 | 115 |
| **23** |  | 2.3 | 101 |

[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.
[c] Racemic mixture.

**Table 4**
Amide SAR II: five-membered rings



| Compds | R | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|---|
| **2** |  | 0.7 | 100 |
| **24** |  | 25 | 92 |
| **25** |  | 12 | 115 |
| **26** |  | 1.9 | 115 |
| **27** |  | 2.0 | 99 |
| **28** |  | 20 | 55 |
| **29**[c] |  | >100 | <30 |
| **30** |  | >100 | <30 |
| **31**[c] |  | 36 | 90 |
| **32** |  | 57 | 48 |
| **33** |  | 2.1 | 109 |
| **34** |  | 20 | 96 |
| **35**[c] |  | 3.0 | 91 |
| **36** |  | 4.3 | 88 |
| **37**[c] |  | 5.2 | 84 |

[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.
[c] Racemic mixture.

ant of substituents, especially those of bigger size (**39**–**41**). However, the 5-position can tolerate many groups, irrespective of their electronic properties (**43**–**48**).

Although compounds **49** and **50** (Table 5) may suggest that polar groups are not preferred, a more careful study revealed that many polar groups are tolerated (Table 6, compounds **57**, **60**–**63**).

Turning our attention to the α-substitution revealed that small alkyl groups are preferred (Table 7). The most desired alkyl groups have three or four carbons. The (*R*)-configured isomers are generally much less active than the corresponding (*S*)-enantiomers.

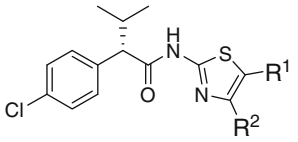
Geminal dialkyl groups were explored in an effort to remove the stereocenter (Table 8). Notably, cyclobutyl (**77**) appears to be a good replacement and demonstrated that a stereocenter is not required to maintain agonist activity.

The phenyl SAR was briefly studied as shown in Table 9. Replacement of the chlorine with a sulfone (**81**) was not tolerated and some pyridines (**82** and **83**) are less active.

By blocking the two metabolic soft spots of compound **2**, namely the isopropyl group and the amide region, we were able to improve the PK properties significantly (Table 10). The AUC increased steadily in both rat and mouse as X was changed from H to F then Cl and Ph, and when R was changed from an isopropyl to a *t*-butyl group.

Compounds[15] were synthesized as illustrated by synthesis of compound **58** (Scheme 1). Peterson olefination of (1,3-dithian-2-yl)trimethylsilane[16] with 1-(4-chlorophenyl)-2,2-dimethylpropan-1-one (**84**) delivered 2-(4-chlorophenyl)-3,3-dimethylbutanoic acid (**86**) as a racemic mixture after methanolysis/hydrolysis.[17] Chiral

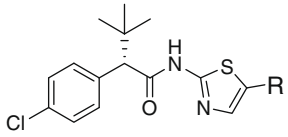
**Table 5**
Amide SAR III: thiazoles

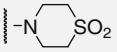


| Compds | $R^1$ | $R^2$ | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|---|---|
| **2** | H | H | 0.7 | 100 |
| **38** | H | Me | 1.4 | 87 |
| **39** | H | $CF_3$ | 17 | 123 |
| **40** | H | *t*-Bu | 7.9 | 45 |
| 41[c] | H | 3-Pyr | >100 | <30 |
| **42** | Me | Me | 1.8 | 97 |
| **43** | Me | H | 0.8 | 99 |
| **44** | F | H | 0.5 | 103 |
| **45** | Cl | H | 0.7 | 100 |
| **46** | $CF_3$ | H | 1.5 | 95 |
| **47** | CN | H | 2.0 | 105 |
| **48** | $CO_2Me$ | H | 2.0 | 98 |
| **49** | $CO_2H$ | H | 79 | 42 |
| **50** | $CONH_2$ | H | 71 | 59 |

[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.
[c] Racemic mixture.

**Table 6**
Amide SAR IV: 5-thiazoles



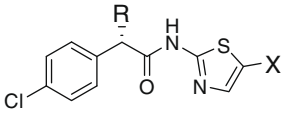
| Compds | R | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|---|
| **51** | F | 0.7 | 92 |
| **52** | Cl | 1.0 | 97 |
| **53** | Br | 0.7 | 95 |
| **54** | CN | 1.5 | 106 |
| **55** | $CF_3$ | 1.5 | 95 |
| **56** | SMe | 0.5 | 109 |
| **57** | $SO_2Me$ | 3.4 | 107 |
| **58** | Ph | 0.7 | 111 |
| **59** | 4-Cl-$PhCH_2$ | 25 | 100 |
| **60** | $NMe_2$ | 1.6 | 112 |
| **61** | Morpholine | 1.6 | 111 |
| **62** | 4-Methylpiperazine | 3.8 | 113 |
| **63** |  | 2.4 | 118 |
| **64** |  | 1.2 | 113 |

[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.

separation gave the (*S*)-isomer **87**.[18] The acid **87** was then coupled with 5-phenylthiazol-2-yl amine through its acid chloride to obtain compound **58**, under a condition (step d) where no racemization was observed.[19]

We have previously shown that compounds **2** and **44** are allosteric agonists of FFA2, and we have suggested that these synthetic

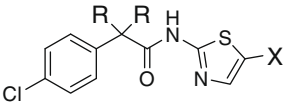
**Table 7**
α-Substitution SAR



| Compds | R | X | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|---|---|
| **65** | H | H | >100 | <30 |
| **66** | Me | H | 46 | 83 |
| **67** | Et | H | 5.0 | 105 |
| **68** | *n*-Pr | H | 5.5 | 108 |
| **2** | *i*-Pr | H | 0.7 | 100 |
| **69** | *c*-Pr | F | 1.2 | 103 |
| **51** | *t*-Bu | F | 0.7 | 91 |
| **70** | *i*-Bu | H | 0.6 | 68 |
| **71** | *c*-Pr-$CH_2$ | H | 8.8 | 72 |
| **72** | $MeOCH_2CH_2$ | H | 170 | 53 |
| **73** | allyl | H | 1.1 | 88 |
| **74** | 4-Cl-Ph | H | >100 | <30 |

[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.

**Table 8**
Gemi-dialkyl substitutions



| Compds | R, R | X | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|---|---|
| **75** | Me, Me | F | 50 | 57 |
| **76** | *c*-Pr | H | >100 | <30 |
| **77** | *c*-Bu | F | 1.5 | 89 |
| **78** | *c*-Pentyl | H | 5.0 | 88 |
| **79** | *c*-Hexyl | H | 8.5 | 81 |

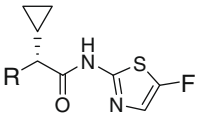
[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.

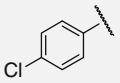
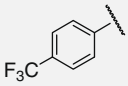
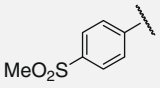
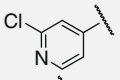
ligands bind to the receptor at a site distinct from binding site of SCFAs.[20] Because of the structural difference of compound **58** compared to compounds **2** and **44**, we also tested the combination of acetate and compound **58** in forskolin-induced cAMP assays, and found that the addition of **58** significantly left-shifted acetate dose responses (Fig. 2). On the other hand, the addition of **58** did not change the $IC_{50}$ of compound **2** in the cAMP assay (Fig. 3).

These findings suggest that these phenylacetamides bind to an allosteric site on the receptor and induce positive cooperativity with natural ligands. Molecular modeling analysis of FFA2 based on the human $\beta_2$-adrenergic receptor structure and subsequent mutagenesis studies revealed potential non-overlapping binding sites for the endogenous and synthetic ligands, further providing insight into the nature of the allosteric interactions.[20,21] Allosteric modulators present a new avenue for potential therapeutic intervention on FFA2. They may potentially offer greater receptor selectivity by binding to non-conserved regions of the receptor among a family of related receptors and may offer other potential advantages such as differential effects on tachyphylaxis and efficacy.[22,23] Therefore, positive allosteric agonists offer an attractive therapeutic approach for the activation of GPCRs.

Since our compounds can also activate mouse FFA2, with the potency of 1.0, 1.4, and 0.7 μM for compounds (**2**, **43** and **44**), respectively, in cAMP response in CHO cells stably expressing

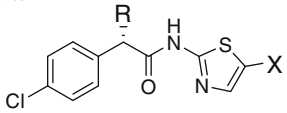
**Table 9**
Phenyl SAR



| Compds | R | Human FFA2 cAMP $IC_{50}$, μM[a] | Human FFA2 cAMP efficacy[b] (%) |
|---|---|---|---|
| **69** |  | 1.2 | 103 |
| **80** |  | 1.1 | 108 |
| **81** |  | >100 | <30 |
| **82** |  | 16 | 120 |
| **83** |  | 45 | 75 |

[a] Values are means of four experiments, standard deviation is ±30%. See Ref. 13 for assay protocol.
[b] Efficacy relative to acetate.

**Table 10**
Pharmacokinetic properties

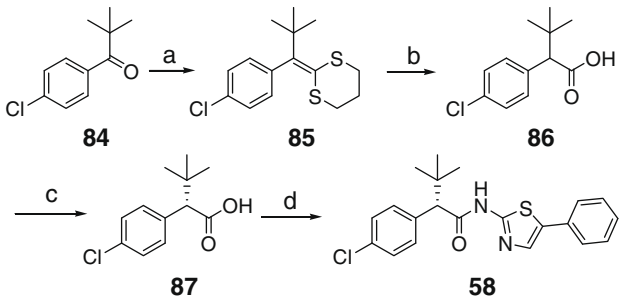


| Compds | | **2** | **44** | **45** | **52** | **58** |
|---|---|---|---|---|---|---|
| R | | *i*-Pr | *i*-Pr | *i*-Pr | *t*-Bu | *t*-Bu |
| X | | H | F | Cl | Cl | Ph |
| Rat iv[a] | CL (L/h/kg) | 6.4 | 2.6 | 2.0 | 0.48 | 0.32 |
| | AUC (μg h/L) | 79 | 194 | 249 | 1030 | 1670 |
| | MRT (h) | 1.1 | 3.8 | 4.2 | 9.0 | 9.1 |
| | Vdss (L/kg) | 7.0 | 9.8 | 8.3 | 4.4 | 2.7 |
| Mouse PO[b] | $C_{max}$ (μg/L) | 5 | 83 | Not tested | 569 | 1871 |
| | AUC (μg h/L) | 10 | 62 | | 4550 | 18585 |
| | MRT (h) | 1.8 | 0.93 | | 7.6 | 9.2 |
| | $T_{max}$ (h) | 1.0 | 0.33 | | 2.0 | 2.0 |

[a] Dose at 0.5 mg/kg.
[b] Dose at 5 mg/kg.

mouse FFA2, we next tested compound effect on adipocyte lipolysis. Our compounds inhibited lipolysis in 3T3L1 adipocytes in a concentration-dependent manner (Fig. 4). Such inhibition was sensitive to pertussis toxin treatment,[20] indicating that this was the result of activating the $G_{\alpha i}$ coupled signaling pathway, similar to our previously reported effects of acetate and propionate on FFA2 in adipocytes.[11]

The acute effects of compound **44** on plasma FFA were tested in C57BL6 mice (Fig. 5).[24] Compound **44** reduced the plasma FFA level in wild type (WT) mice but not FFA2 knock-out (KO) mice[25] similar to our previous observation on the effects of acetate on FFA levels in mice,[11] suggesting that these synthetic ligands could serve as in vivo pharmacological tools to probe the receptor function as well. More importantly, we showed[11] that the activation of FFA2 by acetate did not induce flushing, an adverse effect associated with the activation of GPR109A.[26]




**Scheme 1.** Reagents and conditions: (a) (1,3-dithian-2-yl)trimethylsilane, butyllithium, THF, 0–23 °C, 3 h, 95%; (b) (i) mercury(II) chloride, MeOH/$H_2O$, 85 °C, 2 h; (ii) LiOH, EtOH/$H_2O$, 23 °C, 16 h, 60%, two steps; (c) chiral separation on OJ-H, 45%; (d) DMF, oxalyl chloride, 2,4,6-collidine, 5-phenylthiazol-2-amine, −15 °C, 2 h, 23%, >99% ee.

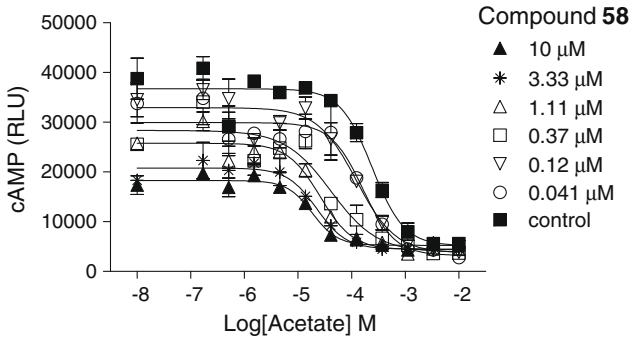



**Figure 2.** Acetated-mediated cAMP responses in the presence of **58**. Values are means of two experiments, standard deviation is ±30%.

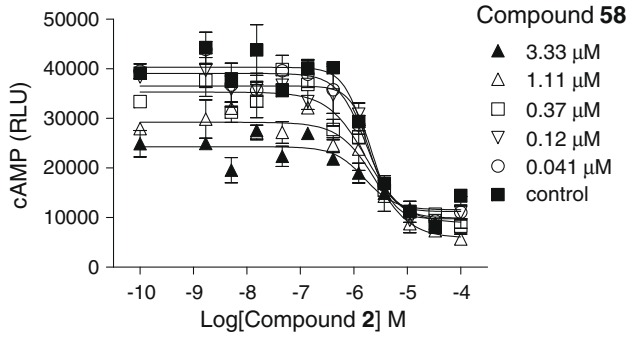



**Figure 3.** Compound **2**-mediated cAMP responses in the presence of **58**. Values are means of two experiments, standard deviation is ±30%.

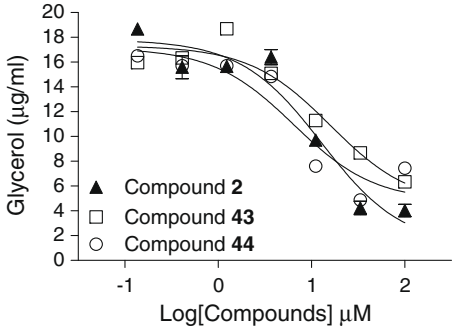



**Figure 4.** Inhibition of lipolysis in adipocytes. Values are means of three experiments, standard deviation is ±30%.

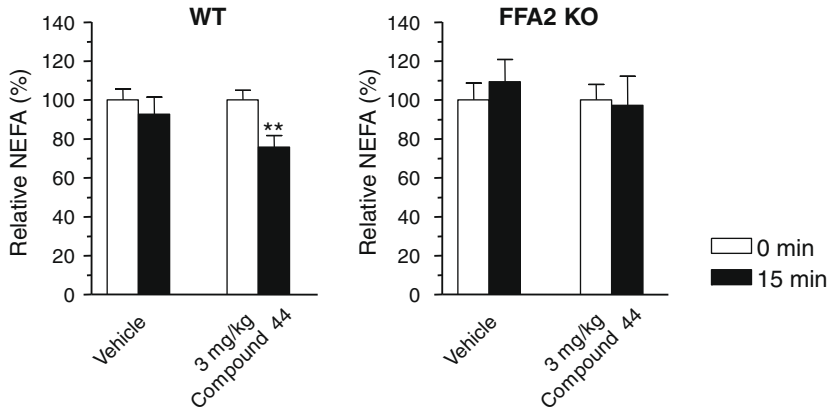



**Figure 5.** Compound **44** reduces plasma FFA levels in WT but not KO mice. Ten animals per group. $^{**}p$ Value less than 0.01 using *T* test.

Sustained reduction in plasma FFA was reported to improve glucose tolerance in patients.[27] Given the similarity in activity on adipocytes to GPR109A, and the beneficial effects of nicotinic acid treatment on raising high-density lipoprotein (HDL) levels, improvements in multiple cardiovascular risk factors, and overall reduction in mortality,[12] FFA2 could also potentially function to modulate aspects of metabolic disorders. These results suggest a potential role and possible advantage for FFA2 in regulating plasma lipid profiles and perhaps aspects of the metabolic syndrome.

In summary, we discovered phenylacetamides as the first class of non-SCFA agonists of FFA2. These novel FFA2 allosteric modulators induce positive cooperativity with natural SCFAs. Our compounds inhibit adipocyte lipolysis in vitro and reduce plasma FFA levels in vivo, suggesting a similar role of FFA2 to GPR109A. Significant improvement in rodent PK profiles was achieved through optimization focusing on metabolic soft spots. These compounds (e.g., **52** and **58**) may serve as good tools for further unraveling the physiological functions of the receptor and its involvement in various diseases.

## Acknowledgments

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13. Inhibition of cAMP response was measured in CHO cells stably expressing human FFA2 via HitHunter cAMP XS assay kit (GE Healthcare). In brief, cells re-suspended (10,000 in 10 μl/well) in Hank's balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 1.3 mM $CaCl_2$, and 1.0 mM $MgSO_4$) with 25 mM HEPES and 0.01% (w/v) BSA were stimulated with forskolin (5 μM final in 5 μl/well) in the presence of serially diluted test ligands (5 μl/well) in a 384-well Optiplate (Perkin–Elmer) at room temperature for 30 min before adding antibody and lysis reagents according to manufacturer's protocol. The plates were further incubated in the dark overnight after adding detection solution, and read in CLIPR (Molecular Devices) for 1 min per plate. Data were expressed as Relative Luminescence Unit (RLU).
14. Separated on ChiralPak AD-H column using 5% 2-propanol in hexanes. Retention times are 10.4 min for compound **3** and 13.8 min for compound **2**.
15. The purity of final compounds was evaluated by HPLC and determined to be >95%. The structures were confirmed by $^1$H NMR and LC–MS.
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18. Separated on ChiralCel OJ-H using 15% 2-propanol in hexanes. Retention times are 11.8 min for (*S*)-enantiomer **87** and 17.2 min for its (*R*)-enantiomer.
19. The chiral center was preserved under the coupling condition (step d in Scheme 1). The ee (enantiomeric excess) of **58** was determined to be >99%, by comparing to a mixture of *S*,*R* enantiomers (*S*/*R* = 3:1) that was obtained by running the same reaction using DBU (instead of 2,4,6-colidine) at room temperature. Retention times are 19.7 min for (*S*)-enantiomer **58** and 35.5 min for its (*R*)-enantiomer on ChiralPak AD-H using 5% 2-propanol in hexanes.
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24. Age-matched male FFA2 knockout (KO) or wild type (WT) mice (C57Bl/6 background) were used in the studies. Mice were fasted overnight before the experiment. Compound or vehicle alone (10% dimethyl acetamide, 10% ethanol, 30% propylene glycol, 50% saline) was intraperitoneally (ip) injected (10 ml/kg) into mice at indicated doses. Blood samples were collected before and 15 minutes after injection by tail bleeding. Plasma FFA levels were measured using a Wako HR Series FFA-HR (2) kit following manufacturers instructions.
25. At a high dose (20 mg/kg), however, compound **44** also reduced FFA levels in FFA2 KO mice, although to a lesser extent than in WT animals. This could be due to some off-target effects of compound **44**, which was not extensively profiled against other targets. Compound **44** was inactive against a small panel of GPCRs that included FFA1, FFA3, GPR109A, GHSR, ETBR, CCR2, CXCR3, CXCR4, and CCR7 at concentrations up to 30 μM.
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