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Identification and Functional Characterization of Allosteric Agonists for the G Protein-Coupled Receptor FFA2

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SCFA, short chain fatty acids; GPCR, G protein-coupled receptor; HTS, high-throughput screen; PTX, pertussis toxin; HDL, high-density lipoprotein.

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Abstract

FFA2 (GPR43) has been identified as a receptor for short chain fatty acids (SCFAs) that include acetate and propionate. FFA2 is highly expressed in islets, a subset of immune cells, and adipocytes. Although the potential roles of FFA2 activation in these tissues have previously been described, the physiological functions are still unclear. The potency for SCFAs on FFA2 is low, in the high micromolar to millimolar concentrations. In order to identify better pharmacological tools to study receptor function, we utilized high throughput screening (HTS) to discover a series of small molecule phenylacetamides as novel and more potent FFA2 agonists. This series is specific for FFA2 over FFA1 (GPR40) and FFA3 (GPR41), and it is able to activate both the $G_{\alpha q}$ and $G_{\alpha i}$ pathways *in vitro* on CHO cells stably expressing FFA2. Treatment of adipocytes with these compounds also resulted in $G_{\alpha i}$ -dependent inhibition of lipolysis similar to that of endogenous ligands (SCFAs). Interestingly, these compounds not only act as FFA2 agonists, but also exhibited positive cooperativity with acetate or propionate. The observed allosteric modulation was consistent in all the functional assays that we have explored, including cAMP, calcium mobilization, GTP γ S binding, and lipolysis. Molecular modeling analysis of FFA2 based on human β_2 -adrenergic receptor structure revealed potential non-overlapping binding sites for the endogenous and synthetic ligands, further providing insight into the binding

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pocket for the allosteric interactions. This is the first report describing the identification of novel allosteric modulators with agonist activity for FFA2, and these compounds may serve as tools for further unraveling the physiological functions of the receptor and its involvement in various diseases.

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The superfamily of G protein-coupled receptors (GPCRs) is one of the largest families of proteins in the mammalian genome and shares a conserved structure composed of seven transmembrane helices (Fredriksson et al., 2003, Fredriksson and Schioth, 2005). Historically, the discovery of drugs acting on GPCRs has been extremely successful, with 50% of all recently launched drugs having activities against GPCR targets and annual world wide sales exceeding 50 billion dollars (Lundstrom, 2006). Thus, this family of proteins is attractive for biopharmaceutical research. FFA2 (GPR43) is a member of a subfamily of related GPCRs clustered at chromosome 19q13.1 in humans (Sawzdargo et al., 1997). The subfamily members – FFA1 (GPR40), FFA3 (GPR41), and FFA2 (GPR43) - have recently been identified as receptors for fatty acids (Covington et al., 2006). The three members of this subfamily share ~ 30-40% sequence identity with specificity toward different fatty acid carbon chain lengths, with short-chain fatty acids (SCFAs, six or shorter carbon molecules) activating FFA2 and FFA3, and medium- and long-chain fatty acids activating FFA1 (Rayasam et al., 2007). Although both FFA2 and FFA3 are activated by SCFAs, FFA2 and FFA3 show differences in SCFA specificity, intracellular signaling, and tissue localization. FFA2 can couple to both $G_{\alpha i}$ and $G_{\alpha q}$, while FFA3 couples only to $G_{\alpha i}$ (Brown et al., 2003, Le Poul et al., 2003, Nilsson et al., 2003). In addition, C2 (acetate) and

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C3 (propionate) are the most potent activators of FFA2, whereas C2 is not as potent as C3, C4 or C5 against FFA3 (Brown et al., 2003, Le Poul et al., 2003).

FFA2 expression has been reported to be enriched in immune cells and adipocytes (Brown et al., 2003, Le Poul et al., 2003, Nilsson et al., 2003, Hong et al., 2005, Ge et al., 2008). Given the well established effects of SCFAs on leukocytes, it has been suggested that FFA2 may play a role in various immune and inflammatory responses (Brown et al., 2003, Le Poul et al., 2003, Nilsson et al., 2003). FFA2 is also induced during adipocyte differentiation and exhibits increased levels during high fat feeding in rodents, suggesting that FFA2 may affect adipocyte function as well (Hong et al., 2005). Indeed, it has recently been reported that acetate and propionate may stimulate adipogenesis and inhibit lipolysis in adipocytes via FFA2 activation (Hong et al., 2005, Ge et al., 2008). In addition, we have previously shown that activation of FFA2 results in the reduction of plasma FFA levels *in vivo* (Ge et al., 2008). Given the similarity in activity on adipocytes to the nicotinic acid receptor, 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 (Carlson, 2005), FFA2 could also potentially function to modulate aspects of metabolic disorders. However, due to the low potency of the endogenous ligands, it has previously been challenging to further explore the functions of FFA2 in various diseases.

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To better understand the pharmacological functions of FFA2, we conducted a high through put (HTS) screen and identified a series of phenylacetamide derivatives as FFA2 agonists. Here, we describe the identification and characterization of this novel series of FFA2 ligands and their potential utility in further understanding the receptor function.

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Materials and Methods

Materials: [³⁵S]guanosine 5'-[γ-thio]triphosphate (GTPγS; 1250 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Unlabeled GTPγS was obtained from Roche Molecular Biochemicals (Indianapolis, IN). GDP, forskolin, and sodium acetate were purchased from Sigma (St. Louis, MO). Propionate was obtained from Fluka Chemie (Taufkirchen, Germany) and pertussis toxin was from EMD Chemicals Inc., (New Jersey, NJ). Coelenterazine was from P.J.K. GmbH (Kleinblittersdorf, Germany). HitHunter cAMP XS assay kit was purchased from GE Healthcare. Phenylacetamides 1 and 2 were synthesized at Amgen, Inc. The detailed synthetic routes will be described elsewhere (Wang et al, manuscript in preparation). Phenylacetamides 1 and 2 were completely soluble in dimethyl sulphoxide (DMSO) and were stable at 10 mM concentration. The 10 mM stock solution was then subsequently diluted into media or buffer to final concentrations as indicated in Figures. The final DMSO concentrations in the media of all cell based assays were 0.1 % (v/v) and in GTPγS binding assay was 1 % (v/v). Same final DMSO concentrations were also maintained in no compound control cells or reactions to eliminate any potential solvent effects. No precipitation of compounds were observed either in media or in reaction buffers under the conditions described here.

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Cell line development and aequorin assay: The bicistronic expression plasmid pIRES (Clontech) containing the coding sequence of the human FFA2 receptor was transfected using Fugene6 (Roche Diagnostics GmbH, Germany) in CHO-K1 cells stably expressing the mitochondrial targeted aequorin. Resistant clones were obtained by single colony separation and compared for their response to a reference agonist using the aequorin assay. The cell-based receptor-dependent aequorin assay was used in a high throughput screen of a compound library as described (An et al., 1998) with slight modification. In this assay, agonist induced receptor activation was determined by an increase in cytosolic calcium concentration released from intracellular calcium stores. The increased cytosolic calcium was monitored by luminescence emitted from aequorin.

Lipolysis assay in differentiated 3T3 L1 Cells: 3T3L1 cells (ATCC CL-173) were cultured in DMEM with 10% (v/v) FCS on Corning CellBind 96 well plates. Upon confluence, differentiation was induced by adding 250 nM dexamethasone, 500 μ M isobutylmethylxanthine, 1 μ g/mL insulin, 2 nM T3, and 0.3 μ M BRL into basic culture medium for two days. The cells were then cultured in DMEM with 10% (v/v) FCS with 1 μ g/mL insulin, 2 nM T3, and 0.3 μ M BRL for two days and maintained in basic culture medium thereafter. Fifteen days after induction,

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differentiated adipocytes were pre-incubated with Krebs-Ringer Bicarbonate 25 mM Hepes (KRH Buffer, Sigma #K4002) with 0.01% (w/v) fatty-acid free BSA (Sigma) and 1 unit/ml adenosine deaminase (Biocatalytics Inc, ADA-101) for 2 hrs. Cells were then treated with short chain fatty acids in KRH Buffer for 4 hrs. Glycerol released from lipolysis during treatment was measured by Free Glycerol Reagent (Sigma F6428).

cAMP Inhibition Assay: Inhibition of cAMP response was measured in CHO cells stably expressing human FFA2 via HitHunter cAMP XS assay kit (GE Healthcare). Briefly, cells resuspended (10,000 in 10 μ l/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 CaCl₂, 1.0 mM MgSO₄) with 25 mM HEPES and 0.01% (w/v) BSA were stimulated with forskolin (5 μ M final in 5 μ l/well) in the presence of serially diluted test ligands (5 μ l/well) in a 384-well Optiplate (Perkin Elmer) at R.T. for 30 min before adding antibody and lysis reagents according to manufacturer's protocol. For allosteric activity studies, test ligands were 3-fold serially diluted and added to the above agonist concentration response reactions. 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).

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Membrane Preparation: Membranes were prepared from CHO cells stably expressing FFA2. CHO-FFA2 stable cells were pretreated with or without pertussis toxin (100 ng/ml) for 16 h before harvesting. All the membrane preparation steps were done at 4 °C. Briefly, cells were harvested by centrifugation (10 min at 10,000 g), washed once with phosphate-buffered saline and recentrifuged. Cells were later resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, with 1 mM EDTA) and lysed using 25 strokes of a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation (5 min at 500 g). The supernatant was removed and centrifuged (30 min at 40,000 g). The resulting pellet was resuspended in 20 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, alone) and recentrifuged. Membranes were resuspended at 0.5 to 1.5 mg of protein/ml in binding buffer (20 mM HEPES pH 7.5, 5 mM MgCl₂) and stored at -80°C until use.

[³⁵S]GTPγS binding: Binding assay was done on membranes prepared from CHO cells stably expressing FFA2. The optimum experimental conditions for the concentrations of GDP, MgCl₂, and NaCl in the assay buffer were initially determined. The assay was performed in assay buffer (20 mM HEPES pH 7.5, 5 mM MgCl₂, and 0.1% (w/v) BSA) with 200 mM NaCl, 3 μM GDP, and 5 μg of membranes/well. The reaction was initiated by addition of 0.2 nM [³⁵S]GTPγS in

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the absence or presence of various ligands and incubated at room temperature for 90 min. Nonspecific binding was determined in the presence of 100 μM GTP γS and was always less than 0.2% of total binding. Bound [^{35}S]GTP γS was separated from free [^{35}S]GTP γS by filtration through GF/B filters followed by five washes with 200 μl of cold assay buffer. Filter-bound radioactivity was determined by liquid scintillation counting.

Data Analysis: All results were presented as means \pm SEM of separate experiments, each performed in duplicate. The concentration-dependent increases in FFA2 activation by ligands was expressed as a percent increase above the basal unstimulated binding and analyzed by means of a non-linear regression method using the commercially available program GraphPad PRISM (GraphPad, San Diego, Calif. with modifications suggested by Dr. Arthur Christopoulos) to produce the concentration eliciting half-maximal effect (EC_{50}) and the percent maximal increase above the basal binding ($\%E_{\text{max}}$). The cooperativity factor and LogKB for allosteric modulation was calculated using the following Allosteric Global pEC_{50} equation:

$$\text{part1} = -1 * \log(X + 10^{\text{LogKB}})$$

$$\text{part2} = \log(((10^{\text{Log } \alpha} * X) + (10^{\text{LogKB}})))$$

$$Y = \text{part1} + \text{part2} - \log d$$

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The molar concentration of the modulator and pEC₅₀ values (in the absence and presence of the allosteric modulator) are represented on the X and Y axis respectively.

Molecular modeling. Sequence alignment and homology model were generated using PRIME (Jacobson et al., 2004) as implemented in Maestro (Maestro, 2008). The coordinates of the human β_2 -adrenergic receptor were used as template for model building (Cherezov et al., 2007). Side chain and loop optimization was also performed using PRIME. Final model was energy minimized using MacroModel (MacroModel, 2008) with OPLS-2001 force field (Kaminski et al., 2001). A harmonic constraint was applied to the TMs main chain with a force constant of 50 kcal/mol/Å. Flexible docking was performed using the Induce Fit Docking protocol as implemented in Maestro. Initial docking was done with GLIDE (Friesner et al., 2004) using a receptor and ligand van der Waals scaling of 0.5. The top 20 poses were used as starting geometry for PRIME induce fit with a flexible zone defined by a radius of 5 Å around the ligand poses. Final ligand redocking and scoring was done with GLIDE XP. Each final complex was further optimized in MacroModel with OPLS-2005 force field (Kaminski et al., 2001) and GB/SA (Still et al., 1990) as continuum solvent effect using a dielectric constant of 1. Truncated Newton conjugated gradient (Ponder and Richards, 1987) with a

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convergence threshold on the gradient of 0.05 KJ/Å/mol. During minimization, a 5 Å cavity within the ligand was allowed for free movement and a harmonic constrain of 200 kcal/mol/Å was applied to atoms within 3 Å from the cavity. A final 3 Å frozen shell was include in the calculation, while the remaining residues were excluded. The ternary complexes were built from the respective binary receptor-ligand complexes and the side chains were re-optimized with PRIME. The final geometry was optimized as described above.

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Results

Identification of a novel synthetic small molecule agonist for FFA2. In order to identify novel synthetic ligands for FFA2 as tools to understand receptor function, we have carried out HTS against a chemical library consisting of over 1 million small molecule compounds. Subsequent confirmation and follow-up analyses from HTS resulted in the identification of phenylacetamide 1, (S)-2-(4-chlorophenyl)-3-methyl-N-(thiazol-2-yl)butanamide as a novel and potent FFA2 agonist. Through initial structure activity relationship analysis, an analog, phenylacetamide 2, (S)-2-(4-chlorophenyl)-N-(5-fluorothiazol-2-yl)-3-methylbutanamide, was also shown to have agonistic activity on FFA2. Both ligands showed specificity toward FFA2 and were inactive against a panel of GPCRs that included FFA1, FFA3, GPR109A, GHSR, ETBR, CCR2, CXCR3, CXCR4, and CCR7 at concentrations up to 30 μ M (data not shown). The chemical structures of phenylacetamide 1 and phenylacetamide 2 are shown in Fig. 1A.

Since FFA2 has been reported to couple to both G_{α_i} and G_{α_q} pathways (Brown et al., 2003), the ability of phenylacetamides 1 and 2 to activate both pathways has been explored in G_{α_q} -coupled aequorin and G_{α_i} -coupled cAMP inhibition assays. Since CHO cells normally do not respond to the FFA2 endogenous ligands acetate and propionate, a CHO cell line stably expressing both human FFA2 (hFFA2) and aequorin was constructed to study the effects of various ligands on calcium

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mobilization by hFFA2 activation. In this cell line, the FFA2 endogenous ligands acetate and propionate as well as phenylacetamides 1 and 2 stimulated calcium mobilization in a concentration dependent manner (Fig. 1B). Activation of calcium flux was independent of G_{α_i} , since pertussis toxin (PTX) treatment did not abolish calcium-dependent flash luminescence (data not shown). Both phenylacetamides 1 and 2 induced calcium mobilization with higher efficacy than acetate and propionate in the aequorin assay. Phenylacetamide 1 and phenylacetamide 2 have similar potency, with EC_{50} values of 0.45 μ M and 0.44 μ M against hFFA2 respectively, and were much more potent than acetate and propionate in this assay (Fig. 1B and Table 1).

The effects of FFA2 ligands on G_{α_i} -mediated signalling pathway were also studied in the CHO cell line stably expressing FFA2. Similar to previous reports, acetate and propionate inhibited the forskolin-induced cAMP response with IC_{50} values in the range of 120 - 140 μ M against both the human and mouse receptors (Fig. 1C and Table 1). Both phenylacetamide 1 and phenylacetamide 2 were significantly more potent than endogenous ligands against human and mouse FFA2, with IC_{50} values in the ranges of 0.7 – 0.96 μ M and 0.48 – 0.66 μ M, respectively (Fig. 1C and Table 1). No significant potency difference was detected between human and mouse receptors in CHO cells stably expressing these receptors (Table 1). The observed inhibition of forskolin-induced cAMP response was a direct consequence

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of G_{α_i} -mediated signaling, as this effect was abolished in the presence of PTX (Fig. 1D).

The effects of the synthetic ligands on FFA2 function were further explored in the stimulation of [35 S]GTP γ S binding to hFFA2. The FFA2 endogenous ligands, acetate and propionate, stimulated [35 S]GTP γ S binding in membranes prepared from CHO cells stably expressing hFFA2 in a concentration-dependent manner. The maximal agonist stimulation was observed at approximately 3 mM, corresponding to about 3 fold increases from the basal activity measured in the absence of the agonist (Fig. 1E). Activation of G protein through FFA2 was also observed with the synthetic ligands (phenylacetamides 1 and 2), with a stimulation of 3-3.5 fold over basal, and a slightly higher efficacy than endogenous ligands (Fig. 1E). Agonist-induced [35 S]GTP γ S binding was abolished upon PTX treatment and was not detected from membrane preparations from parental CHO cells, indicating that the effects are indeed mediated through FFA2 coupling to G_{α_i} pathway (data not shown). Therefore, based on the results in Fig. 1, we concluded that phenylacetamides 1 and 2 are agonists for FFA2, and are capable of activating both G_{α_q} and G_{α_i} coupled pathways for receptor function.

Phenylacetamides 1 and 2 activate G_{α_i} pathway and inhibit lipolysis in adipocytes. We have previously shown that activation of FFA2 by endogenous

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ligands in adipocytes could lead to the inhibition of lipolysis (Ge et al., 2008). This effect is similar to that observed for another adipocyte-expressed receptor, GPR109A. Activation of GPR109A by its ligand, nicotinic acid, mediated through $G_{\alpha i}$ pathway, lead to the inhibition of lipolysis (Wise et al., 2003, Tunaru et al., 2003, Soga et al., 2003). Given that the synthetic ligands behaved similarly to endogenous ligands in $G_{\alpha q}$ and $G_{\alpha i}$ mediated assays but with higher potency, we examined the effects of these synthetic ligands on lipolysis in adipocytes. Both phenylacetamide 1 and phenylacetamide 2 inhibited lipolysis in 3T3L1 adipocytes in a concentration dependent manner as measured by the reduction of glycerol production in the media (Fig. 2). Such inhibition was sensitive to PTX treatment, indicating that this was the result of activating $G_{\alpha i}$ -coupled signaling pathway similar to our previously reported effects of acetate and propionate on FFA2 in adipocytes (Ge et al., 2008).

Phenylacetamides 1 and 2 are allosteric agonists for FFA2. Although both endogenous and synthetic ligands behave as agonists in FFA2-mediated activity assays as shown in Fig. 1, given the differences in the chemical structures of these ligands, we were interested in finding if these effects were mediated through ligand binding to the same or different sites on the receptor. To address this question,

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various combinations of ligand treatments in the different assays described below were carried out.

The first indication that phenylacetamide 1 and phenylacetamide 2 have allosteric effects relative to acetate and propionate came from analysis in forskolin-induced cAMP assays. As shown in Fig. 3A, acetate concentration-dependently inhibited forskolin induced cAMP production. The effects of propionate on the acetate concentration response resulted in a decrease in basal cAMP levels without affecting the potency of acetate (Fig. 3A). The decrease in the basal cAMP level is due to the activity of propionate alone on the receptor, and the lack of a shift in potency on acetate upon addition of propionate suggested that propionate and acetate bind to the same or overlapping sites on the receptor. Similar biochemical effects were also observed between phenylacetamide 1 and phenylacetamide 2. The effects from the addition of phenylacetamide 2 to phenylacetamide 1 concentration responses compared to that of phenylacetamide 1 alone was the suppression of basal cAMP levels without changing the potency of phenylacetamide 1, suggesting that these two synthetic ligands are binding to the same or overlapping sites on the receptor (Fig. 3B). However, different results were achieved when endogenous ligands and synthetic ligands were combined together. When phenylacetamide 1 was added to acetate concentration-response studies, not only was there suppression in the basal cAMP levels, but there was

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also a significant left shift in the acetate concentration responses (Fig. 3C). The reverse also holds true, the addition of acetate significantly left-shifted phenylacetamide 1 concentration responses (Fig. 3D). Therefore, if the binding site for endogenous ligands is defined as orthosteric site, then these data suggest that phenylacetamide 1 and phenylacetamide 2 bind to allosteric site on the receptor and induce positive cooperativity with acetate or propionate. The cooperativity factor (α) for phenylacetamide 1 on acetate is 24 ($\text{Log}\alpha = 1.38$) in the $G_{\alpha i}$ -mediated cAMP inhibition assay (Table 2).

To determine whether this positive allosteric effect could also occur in $G_{\alpha q}$ signaling, concentration response curves were generated from various combinations of endogenous ligands and synthetic ligands in aequorin assays measuring calcium fluxes induced by activation of $G_{\alpha q}$ pathway by FFA2. The results show that the potency of acetate was increased in the presence of phenylacetamide 1 with a cooperativity factor of 89 ($\text{Log}\alpha = 1.95$, Fig. 4A). In a reciprocal experiment, acetate increased the potency of phenylacetamide 1 with a cooperativity factor of 24 ($\text{Log}\alpha = 1.38$, Fig. 4B). On the other hand, phenylacetamide 2 did not allosterically modulate the FFA2 receptor at any concentrations of phenylacetamide 1 (Fig. 4C). These results suggest that phenylacetamide 1 can positively modulate acetate activity in $G_{\alpha q}$ -mediated receptor signaling in addition to $G_{\alpha i}$ pathway.

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To further demonstrate that the positive allosteric effects between endogenous and synthetic ligands are not specific to certain assays or downstream effectors, we used a GTP γ S binding assay which measures ligand effects upstream and closer to receptor activation than the amplified downstream effector signaling. Studies of allosteric ligands in [³⁵S]GTP γ S binding for other G $_{\alpha i}$ -coupled receptors have been well documented (Leppik and Birdsall, 2000). The effects of phenylacetamide 1 and phenylacetamide 2 on acetate concentration responses were examined in [³⁵S]GTP γ S binding assay. In the presence of different concentrations of phenylacetamide 1 and phenylacetamide 2, we observed a left shift in potency for acetate (Fig. 5). The cooperativity factor (α) for acetate in the presence of phenylacetamide 1 is 5.2 (Log $\alpha = 0.72 \pm 0.03$) and in the presence of phenylacetamide 2 is 10 (Log $\alpha = 1.0 \pm 0.13$). The concentration-dependent increase in basal activity of phenylacetamide 1 and phenylacetamide 2 in the presence of acetate was due to intrinsic activity of the ligands acting as agonists on the receptor itself. In addition to a change in potency, there was also an increase in the maximal efficacy (E_{\max}) in the presence of either phenylacetamide 1 or phenylacetamide 2 (Fig. 5A and 5B) suggesting that acetate acts as a partial agonist in GTP γ S assays. Similar increase in efficacy of GABA by the allosteric modulator CGP7930 was observed upon activation by [³⁵S]GTP γ S binding on GABA $_{B(1b/2)}$ receptor expressed in CHO stable cell line (Urwyler et al., 2001). To

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further evaluate whether the allosteric effects are reciprocal in nature, we conducted a full concentration response curve of either phenylacetamide 1 or phenylacetamide 2 in the presence of fixed concentrations of acetate and propionate (0.0135 to 10 mM). There was a significant left shift in the potency of phenylacetamide 1 and phenylacetamide 2 in the presence of the endogenous ligands with a cooperativity factor of 10 (Table 3). On the other hand, phenylacetamide 1 did not allosterically modulate the FFA2 receptor in stimulating [³⁵S]GTPγS binding at any concentrations of phenylacetamide 2. Similarly, no positive cooperativity was observed with acetate in the presence of propionate on FFA2 in the GTPγS binding assay (data not shown). These results further support the conclusion that the synthetic ligands, phenylacetamide 1 and phenylacetamide 2, interact allosterically at the FFA2 receptor through distinct site from that of the endogenous ligands.

Phenylacetamide 1 is an allosteric agonist for FFA2 in lipolysis assays. To study the observed allosteric effects of synthetic ligands closer to physiological settings, the effects of acetate and phenylacetamide 1 on each other's concentration response curves were tested in lipolysis assays. Similar to what was observed in the other functional assays, in particular the G_{αi}-coupled assays, the addition of phenylacetamide 1 to acetate concentration response resulted in not only a decrease

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in basal lipolysis rate but also a significantly left-shifted concentration response curves for acetate (Fig. 6A). In the reciprocal experiment, acetate also significantly left-shifted the phenylacetamide 1 concentration response curves in addition to the suppression of the basal lipolysis rate (Fig. 6B). These results further confirmed, in a more native environment, the allosteric activity of phenylacetamide 1 on FFA2.

Molecular modeling of ligand binding to FFA2. In order to gain further insights into the observed positive cooperativity between endogenous and synthetic ligands, a molecular model has been built for FFA2 by homology modeling using the X-ray crystal structure of the human β_2 -adrenergic receptor as a template (Cherezov et al., 2007). The side chains and the extracellular loops were further optimized using PRIME (Jacobson et al., 2004) as described in the Materials and Methods. Although potential amino acid residues involved in FFA2 ligand binding sites are currently unknown, regions and residues that may be important for FFA2 ligand recognition could be suggested based on the recently identified residues that are important for FFA1 ligand recognition (Sum et al., 2007) and the conservation between FFA1 and FFA2 (Fig. 7A). By sequence comparison with FFA1, we focused our efforts in particular in the regions containing Arg180 in TM5, Arg255 in TM7, and His242 in TM6. An area of approximately 10 Å around these residues

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was used for ligand docking with flexibility for all the side chains contained within the region as well as loops of the main chain. Endogenous ligands, acetate and propionate, as well as phenylacetamide 1 were individually docked using this procedure and multiple docking poses were selected for further analysis. The emphasis was then put on putative binding sites with little or no overlap between acetate, or propionate, and phenylacetamide 1. After this analysis, one potential binding mode for the SCFAs and two potential binding sites for phenylacetamide 1 were selected for further evaluation (Fig. 7B).

Flexible docking and energy minimization results for acetate and propionate showed a binding mode where the acid functionality was interacting with both the Arg180 and Arg255 (Fig. 7C). The negatively charged carboxylate interacts with both arginine residues in a bi-dentate like fashion. The aliphatic chain is oriented toward a shallow hydrophobic pocket made of residues from TM6 and TM7. The residues in contact with the hydrophobic moiety of the endogenous ligand include Ser241, Tyr238, and the hydrophobic portions of Lys250 and Arg255. Although we did not model all the SCFAs, the cavity size and proximity of some of these residues to the γ carbon of the propionate suggest that binding of four carbon chain analog could have some steric repulsion especially from Tyr238. Moreover, this cavity will not be able to accommodate FFAs with 5 or more atoms without major conformational changes which in turn could yield high energy and unfavorable

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complexes, therefore, could potentially explain the preference of FFA2 for SCFA (Le Poul et al., 2003).

Flexible docking and minimization yielded two possible binding sites for phenylacetamide 1. A putative binding mode **1** is located in a cavity between TM2, TM3, TM6, and TM7. In this binding mode, the hydrophobic p-chloro phenyl of phenylacetamide 1 is oriented toward the interior of the transmembrane region making several hydrophobic contacts. In addition, the isopropyl group of phenylacetamide 1 is also located in a hydrophobic environment. The amide thiazole moiety, on the other hand, is oriented toward the extracellular loops showing potential polar contacts from the amide carbonyl with the backbone NH of Leu173 (Fig. 7D), although the donor-acceptor angle is not ideal for a strong hydrogen-bond. In this binding mode, Arg255 is in close contact with phenylacetamide 1, forming a possible stacking interaction with the thiazole moiety. Arg255 is also involved in H-bond interaction with the carbonyl backbone of Leu173. We hypothesize that these interactions help to stabilize the complex with phenylacetamide 1 by holding the Leu173 side chain in tight contact with the p-chloro-phenyl ring hence improving the hydrophobic interactions. The second binding mode identified by flexible docking is located in a cavity between TM3, TM5, and TM6 (Fig. 7E). In this orientation, the entire ligand is buried in the transmembrane region. The p-chloro phenyl group of phenylacetamide 1 is

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surrounded by hydrophobic residues whereas the thiazole amide is proximal to His242 making a hydrogen bond interaction with the NH of the side chain. In principle both binding modes for phenylacetamide 1 could potentially explain the allostereism since there is no overlap with the proposed binding site for the SCFAs. However, in order to have positive cooperative effect, the binding mode for phenylacetamide 1 not only should not overlap with the binding of SCFAs, but the hypothetical ternary complex formed should also have improved intermolecular interactions as compared to the corresponding binary states. A comparison between the complexes with phenylacetamide 1, in binding mode **1**, and propionate shows smaller variations on the surrounded side chains. In contrast, the geometry of the complex for phenylacetamide 1 in binding mode **2** showed a significant shift of Arg180 and His242 as compared to the complex with propionate. This shift is necessary for accommodating the synthetic ligand and could have a negative impact on the formation of a stable ternary complex. Therefore, binding mode **1** appears to be more suitable for explaining the allosteric synergism observed between the endogenous ligands and phenylacetamide 1 series.

To explain the selectivity of phenylacetamide 1 and phenylacetamide 2 over FFA3, we examined the residues lining the potential binding mode **1** in the modeled FFA2 structure and identified several residues in close contact with the ligand that are different between the two FFA receptors. These residues include Ile66 and

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Ser256, which are replaced by Met62 and Ile252 in FFA3, respectively. The significant size and property differences of these FFA3 residues could cause significant steric hindrance and contribute to the observed selectivity. In addition, Asp170 in FFA2 is replaced by Ala170 in FFA3 which breaks a potential intramolecular H-bond in the EL2 loop that is involved in the binding of phenylacetamide 1. These potential differences in the binding pocket are shown in Fig. 7F, where the FFA2 residues have been mutated to the corresponding FFA3 residues to highlight the potential steric hindrance.

The hypothetical ternary complex using binding mode 1 for phenylacetamide 1 and the binding site identified for propionate is shown in Fig. 7G. A model for this ternary complex was obtained by merging the individual ligands into their respective binding sites followed by geometry optimization for residues in a 7 Å radius around each ligand. In this complex, propionate has similar bi-dentate salt bridge interaction as in the binary complex. In addition, the interaction of the amide carbonyl oxygen of phenylacetamide 1 with the backbone NH of Leu173 has a much better geometry for a hydrogen-bond than in the corresponding binary complex due to the displacement of Arg255 toward the propionate. Furthermore, the thiazole amide moiety rotates around 90 degrees relative to the binary complex and this further enhances the H-bond interaction. Based on these rearrangements around the ligand binding site, we hypothesize that the thiazole amide moiety of

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phenylacetamide 1 helps to better orient the side chain of Arg255 toward the propionate ion by inserting between the extracellular loop 2 (EL2) and the phase of Arg255 side chain. At the same time, the presence of the SCFA attracts Arg255 allowing a better hydrogen-bond interaction for phenylacetamide 1. Overall these effects should bring more stability to the ternary complexes which could result in a corresponding positive cooperative effect.

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Discussion

Acetate and propionate have previously been identified as endogenous ligands for FFA2 (Brown et al., 2003). However, no potent synthetic small molecule ligands specific for FFA2 have been reported. In this report, we describe the identification and characterization of two phenylacetamide derivatives, phenylacetamide 1 and phenylacetamide 2, as novel synthetic allosteric agonists for FFA2. Similar to the activities of endogenous ligands, the synthetic ligands can induce FFA2 coupling to both G_{α_i} and G_{α_q} signaling pathways in multiple assay formats (Fig. 1). In addition to the greatly improved potency of the synthetic ligands over the endogenous ligands, the synthetic ligands show higher efficacy than acetate and propionate in G_{α_q} coupled aequorin assay (Fig. 1B) and slightly increased efficacy in more G_{α_i} coupled [35 S]GTP γ S binding assays (Fig. 1E). The differences in efficacy between endogenous and synthetic ligands in different assays could be the result of differences in coupling efficiencies of G_{α_i} vs G_{α_q} subunits to FFA2 for the two distinct classes of ligands. The synthetic ligands also activated the receptor in its native setting by inhibiting lipolysis in adipocytes via G_{α_i} dependent pathway similar to what has been described for endogenous ligands (Ge et al., 2008). All of these results strongly support the conclusion that the synthetic ligands are agonists for FFA2.

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To gain more insight into how these endogenous and synthetic ligands activate the receptor, we carried out various experiments using different combinations and concentration of ligands. It was interesting to note that the endogenous and synthetic ligands showed positive cooperativity with each other in either upstream or downstream pathways. We observed a greater degree of cooperativity in cAMP assay and aequorin assay, which may be due to amplification of the response through the receptor compared to [³⁵S]GTPγS binding assays. A positive allosteric effect is defined by a cooperativity factor of greater than 1 ($\alpha > 1$) (Christopoulos and Kenakin, 2002, Langmead and Christopoulos, 2006). Phenylacetamide 1 significantly potentiated (24-89 fold, Log α = 1.38-1.95) the functional potency of orthosteric ligand acetate to FFA2 in cAMP inhibition and Ca²⁺ stimulation assays. This positive cooperativity would not be observed from ligands binding to the same site on the receptor. Indeed, in the inhibition of forskolin-induced cAMP assay, the addition of propionate did not affect the potency of acetate concentration responses; rather, only the base line was reduced due to the activity of propionate alone in the assay (Fig. 3). A similar relationship was observed between phenylacetamide 1 and phenylacetamide 2, suggesting that ligands of each class share similar binding sites on the receptor (Fig. 3). A more definitive method to study whether ligands have overlapping binding sites on a receptor is to use radiolabeled ligands, which have been used extensively to study allosterism on

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other GPCRs (Trankle et al., 2003, Trankle et al., 2005, Prilla et al., 2006). Although the potency of our synthetic ligands does not allow the development of a radioligand binding assay to directly measure binding sites on the receptor, these results still strongly support the conclusion that synthetic ligands bind to an allosteric site on the receptor and that they show positive cooperativity with endogenous ligands, similar to allosteric modulators reported in other GPCRs, including calcium sensing receptor (Nemeth et al., 2004), A1 adenosine receptor (Kollias-Baker et al., 1997), metabotropic glutamate receptor (Hemstapat et al., 2006), and others (May et al., 2007).

Such positive cooperativity was also observed in a native cell line. We and others have previously shown that activation of FFA2 in adipocytes by endogenous ligands, acetate and propionate, results in the inhibition of lipolysis via $G_{\alpha i}$ signaling pathway (Hong et al., 2005, Ge et al., 2008). In this adipocyte lipolysis assay, phenylacetamide 1, in addition to being a FFA2 agonist, also showed positive cooperativity to acetate. The addition of phenylacetamide 1 resulted in a significant left shift in acetate concentration response curve, and the reciprocal combination also generated similar cooperativity (Fig. 6). These results suggest that the observed allosteric effects of synthetic ligands are not *in vitro* assay artifacts, but could have significance in receptor function in a more physiological setting.

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To further understand the nature of allosteric interaction between endogenous and synthetic ligands, a molecular model was built for FFA2 by homology modeling using the X-ray crystal structure of the human β_2 -adrenergic receptor (Cherezov et al., 2007) as a template. Based on the identified residues that are important for FFA1 ligand interaction and the homology between FFA1 and FFA2, putative ligand binding pockets for endogenous ligands and the synthetic ligands were identified (Fig. 7). Analysis of the optimized ternary complex between receptor, propionate, and phenylacetamide 1, highlighted a number of important residues that could influence the binding of these two classes of ligands and provided a possible explanation for the observed positive cooperativity. However, this model needs to be verified experimentally, and could be tested by site-directed mutagenesis of the residues identified. Therefore, on the basis of the sequence alignment and molecular modeling described here, Arg180 and Arg255 could be selected to explore their roles in binding with the carboxylate group of the SCFAs, and Ile66, Phe89, Leu173, Tyr238 and Val259 could be selected to explore their involvement in hydrophobic interactions with phenylacetamide 1, as suggested in the proposed binding mode **1**.

It has been known that allosteric agonists affect signaling in their own right, which opens new avenues in understanding receptor structure, pharmacology and physiological function. Positive allosteric agonists offer an attractive therapeutic

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approach for the activation of GPCRs because they would be efficacious either in the presence or absence of endogenous agonist and they may elicit less tachyphylaxis and/or receptor desensitization than competitive agonists (Langmead and Christopoulos, 2006). They could also potentially offer greater selectivity on the receptor by binding to a non-conserved region on the receptor from a family of related receptors and provide a greater magnitude of physiological response (May et al., 2007).

The identification of a novel allosteric site on FFA2 for the first time is of great importance in understanding FFA2 physiological functions. The presence of this allosteric site on the FFA2 receptor can be used to explore and develop new compounds with greater efficacy and potency that selectively target diseases related to this receptor. Furthermore, the allosteric site provided the platform for the identification of FFA2 selective ligands over related subfamily members such as FFA3, which is also a SCFA receptor. However, a potential cooperative effect of any novel synthetic ligands with either endogenous ligands or substances that could be converted to acetate or propionate should be a particularly important consideration for future *in vivo* pharmacology study designs. For example, the primary fate of ethanol metabolism by the liver is the conversion and release of acetate into circulation. Plasma levels of acetate could increase up to 20 fold and into millimolar concentrations after alcohol ingestion (Lundquist et al., 1962, Siler

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et al., 1999). Therefore, the use of ethanol as vehicle in animal model studies, or the administration of a drug against FFA2 in humans, with or without alcohol consumption, could potentially alter the effects and outcome of any studies using FFA2. In conclusion, we have identified novel synthetic allosteric agonists for FFA2, these and potentially other allosteric agonists represent a new and exciting avenue for research and possible therapeutic intervention on FFA2.

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Footnotes:

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Figure legends

Figure 1. Biological activity of phenylacetamide 1 and phenylacetamide 2 in *in vitro* receptor functional assays. (A) Compound structures of phenylacetamide 1 and phenylacetamide 2. (B) Response of Ca^{2+} release in CHO cells stably expressing hFFA2. Aequorin-mediated detection of Ca^{2+} release was measured. Data from four independent experiments were combined and normalized to percent of phenylacetamide 2 maximum activity value. The average RLU measured for phenylacetamide 2 is 34,172,570. (C) Inhibition of cAMP response in CHO cells stably expressing hFFA2. Inhibition of forskolin-induced cAMP response was measured in the presence of different concentrations of ligands as indicated. Data from four independent experiments were combined and normalized to percent of control (absence of ligand) expressed over concentration responses. The average basal RLU value measured is 25,280. (D) PTX-sensitive response of phenylacetamide 1 in cAMP inhibition. Data is representative of 3 independent experiments, determined in duplicates. (E) Effect of FFA2 ligands on the binding of [^{35}S]GTP γ S to CHO membranes expressing FFA2 receptor. Binding of [^{35}S]GTP γ S was measured in the presence of different concentrations of ligands as indicated. Data is represented as percentage over basal activity (absence of the ligand) and are combined from three independent experiments. The average basal

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binding value corresponded to 0.14 – 0.19 pmol/mg protein. All data points were determined in duplicate.

Figure 2. The synthetic ligands, phenylacetamide 1 and phenylacetamide 2, inhibit lipolysis in adipocytes. Differentiated 3T3L1 cells were treated with (A) phenylacetamide 1 or with (B) phenylacetamide 2 in the presence or absence of PTX. Lipolysis was measured by the release of glycerol in the culture medium. Data are representative of 3 independent experiments, determined in duplicate.

Figure 3. Allosteric activity of phenylacetamide 1 and phenylacetamide 2 in cAMP assays. Inhibition of forskolin-induced cAMP response was measured in the presence of different concentrations of agonists; (A) acetate vs. propionate, (B) phenylacetamide 1 vs. phenylacetamide 2, (C) acetate vs. phenylacetamide 1, and (D) phenylacetamide 1 vs. acetate. Data was combined from three independent experiments and expressed as percent of no ligand control. The average basal RLU measured is 27,570.

Figure 4. Allosteric modulation of acetate by phenylacetamide 1 in aequorin assay. (A) The allosteric effect of phenylacetamide 1 on acetate-induced calcium flux. (B) The positive cooperating effect of acetate on phenylacetamide 1-induced

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calcium flux. (C) The lack of cooperating effect of phenylacetamide 2 on phenylacetamide 1-induced calcium flux. Data was derived from two independent experiments and normalized to percent of the maximal response from phenylacetamide 1 with an average RLU of 35,896,085.

Figure 5. Allosteric modulation of acetate by phenylacetamide 1 and phenylacetamide 2 in GTP γ S binding assay. The allosteric effects of (A) phenylacetamide 1 and (B) phenylacetamide 2 on acetate in the stimulation of [³⁵S]GTP γ S binding was determined. Data was combined from three independent experiments performed in duplicate and was expressed as percent of control (in the absence of ligand). The average control values measured corresponded to 0.14 – 0.19 pmol/mg protein.

Figure 6. Allosteric modulation of acetate by phenylacetamide 1 in Lipolysis assay. Differentiated 3T3L1 cells were treated with (A) acetate concentration response at fixed concentrations of phenylacetamide 1, or with (B) phenylacetamide 1 concentration response at fixed concentrations of acetate. Lipolysis was measured by the release of glycerol in the culture medium. Data are representative of 3 independent experiments, determined in duplicate.

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Figure 7. Molecular modeling of ligand binding on FFA2. (A) Sequence alignment for FFA1, FFA2, and FFA3. (B) Model of the human FFA2 and putative binding sites for endogenous ligand Propionate, cyan, and allosteric synthetic agonist Phenylacetamide 1, magenta. Both ligands are in close proximity but do not share the same binding site. (C) Putative binding mode for propionate (cyan). Salt bridge interactions between the carboxylate anion of the ligand and guanidinium cation groups of R180 and R255 are shown in dashed lines. Lipophilic chain is in a gauche conformation and oriented toward S241 and between R255 and K250 side chains. Additional intra-molecular H-bond interactions between R255, H242 and Y238 help to stabilize the complex. (D) Putative binding mode **1** for synthetic agonist Phenylacetamide 1 (magenta). Hydrogen bond interactions between the L173 backbone and Phenylacetamide 1 as well as the guanidinium cation group of R255 are shown in dotted lines. The lipophilic p-chloro phenyl group is surrounded by several hydrophobic residues, shown in green, from TM2 (I66), TM3 (F89), TM6 (Y238), TM7 (V259) and the EL2 loop (L173). (E) Putative binding model **2** for synthetic agonist Phenylacetamide 1 (magenta). Hydrogen-bond interactions are shown in dotted lines. (F) Putative binding mode **1** for synthetic agonist Phynylacetamides 1 (magenta). The lipophilic p-chloro phenyl group is surrounded by several hydrophobic residues. In CPK light blue is shown the corresponding different

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residues in FFA3. M62 and to some extent I252 of FFA3 show overlaps with the ligand binding that may cause significant steric hindrance. (G). Model for a ternary complex of the endogenous ligand propionate (cyan) and synthetic agonist Phenylacetamide 1 (magenta). Hydrogen bond interactions between the L173 backbone and Phenylacetamide 1 is shown in dotted line. Salt bridge interactions between propionate and conserved arginine residues are shown in dashed lines. The packing interaction between Phenylacetamide 1 and R255 is also indicated.

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Table 1. Summary of FFA2 potencies. Data are average IC₅₀/EC₅₀ values ± standard error (S.E.) from 4 independent experiments.

Ligand	hFFA2		mFFA2	
	cAMP (IC ₅₀ , μM)	Aequorin (EC ₅₀ , μM)	cAMP (IC ₅₀ , μM)	Aequorin (EC ₅₀ , μM)
Acetate	123.8±9.2	3032 ±1083	131.0±9.7	1153±159.4
Propionate	125.2±9.9	2862 ±988	120.2±10.7	709.7±99.5
phenylacetamide 1	0.70±0.07	0.45±0.07	0.96±0.07	1.27±0.10
phenylacetamide 2	0.48± 0.04	0.44±0.07	0.66±0.07	0.62±0.06

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Table 2. Summary of positive cooperativity of FFA2 ligands in cAMP inhibition assays. The logKB values and cooperativity factors of the ligands are calculated by fitting the data to allosteric global pEC₅₀ equation (See Material and Methods). Data represented is mean ± S.E. from 3 independent experiments performed in duplicate.

Ligands	Log KB	Log α (cooperativity factor)
Acetate +phenylacetamide 1	-6.0 ± 0.14	1.38 ± 0.11
Phenylacetamide 1+acetate	-3.5 ± 0.91	1.95 ± 0.91
Phenylacetamide 1 + phenylacetamide 2	-7.05 ± 0.07	-0.10 ± 0.15
Acetate+propionate	-5.7 ± 1.1	0.3 ± 0.04

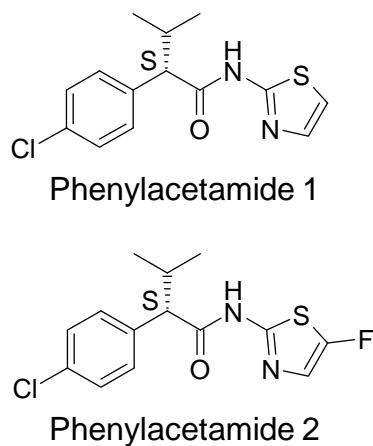
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Table 3. Stimulation of [³⁵S]GTPγS binding by FFA2 agonists in the presence of different concentrations of allosteric ligands on FFA2 receptor. The logKB values and cooperativity factors of the ligands are calculated by fitting the data to allosteric global pEC₅₀ equation (See Material and Methods). Data represented is mean ± SEM from 2-3 independent experiments performed in duplicate.

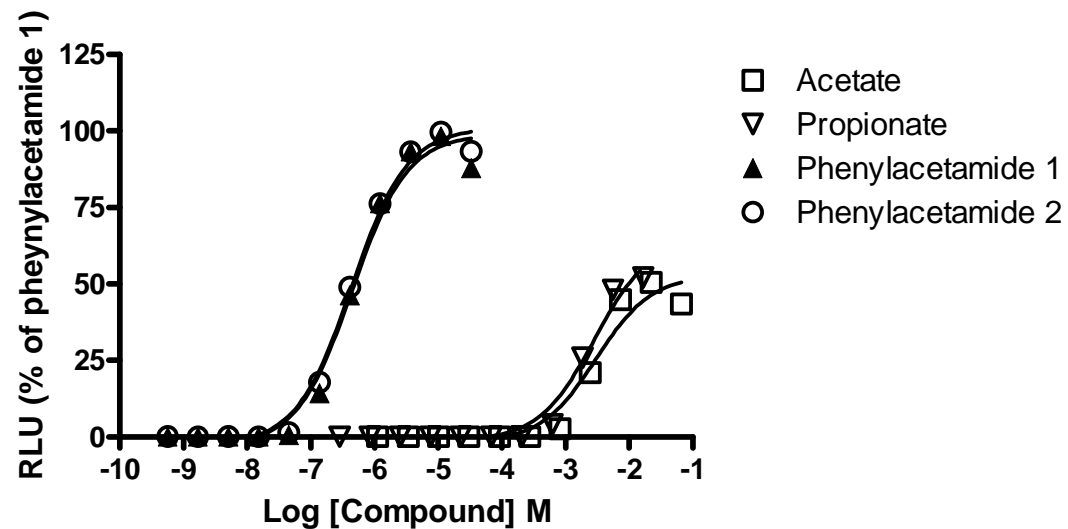
Ligands	Log KB	Log α (cooperativity factor)
Acetate +phenylacetamide 1	-6.38 ± 0.73	0.72 ± 0.03
Acetate +phenylacetamide 2	-6.2 ± 0.39	1.0 ± 0.133
Propionate +phenylacetamide 1	-6.0 ± 0.66	1.1 ± 0.27
Propionate+phenylacetamide 2	-6.67 ± 0.90	0.6 ± 0.07
Phenylacetamide 1+acetate	-3.5 ± 0.37	1.14 ± 0.3
Phenylacetamide 2+acetate	-3.0 ± 0.05	1.13 ± 0.08
Phenylacetamide 1+propionate	-3.5 ± 0.14	0.97 ± 0.02
Phenylacetamide 2+propionate	-3.3 ± 0.57	1.2 ± 0.08

Figure 1

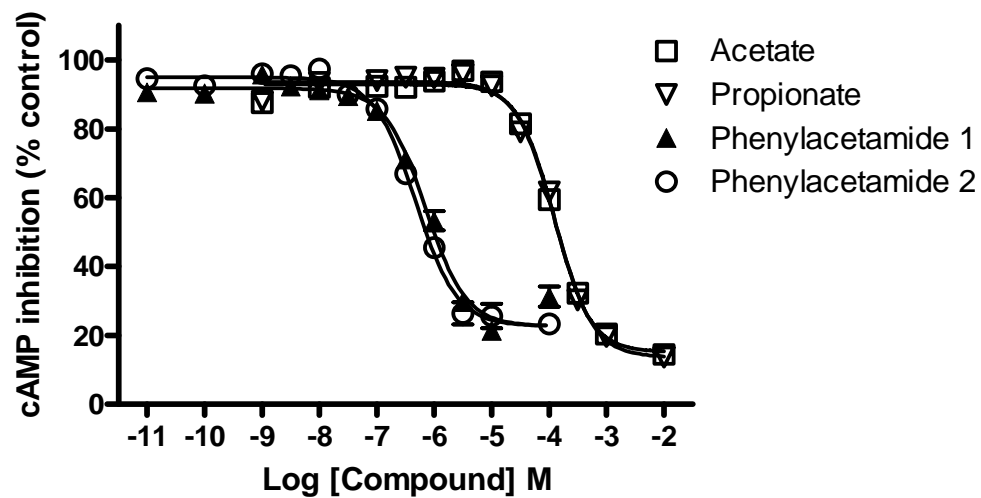
A.



B.



C.



D.

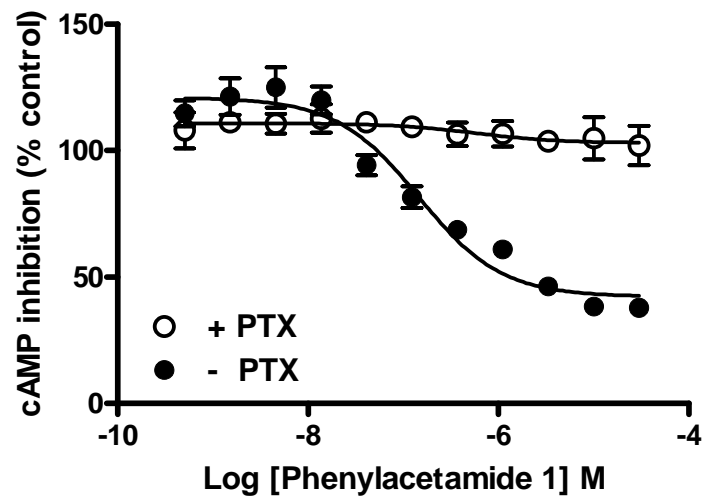


Figure 1

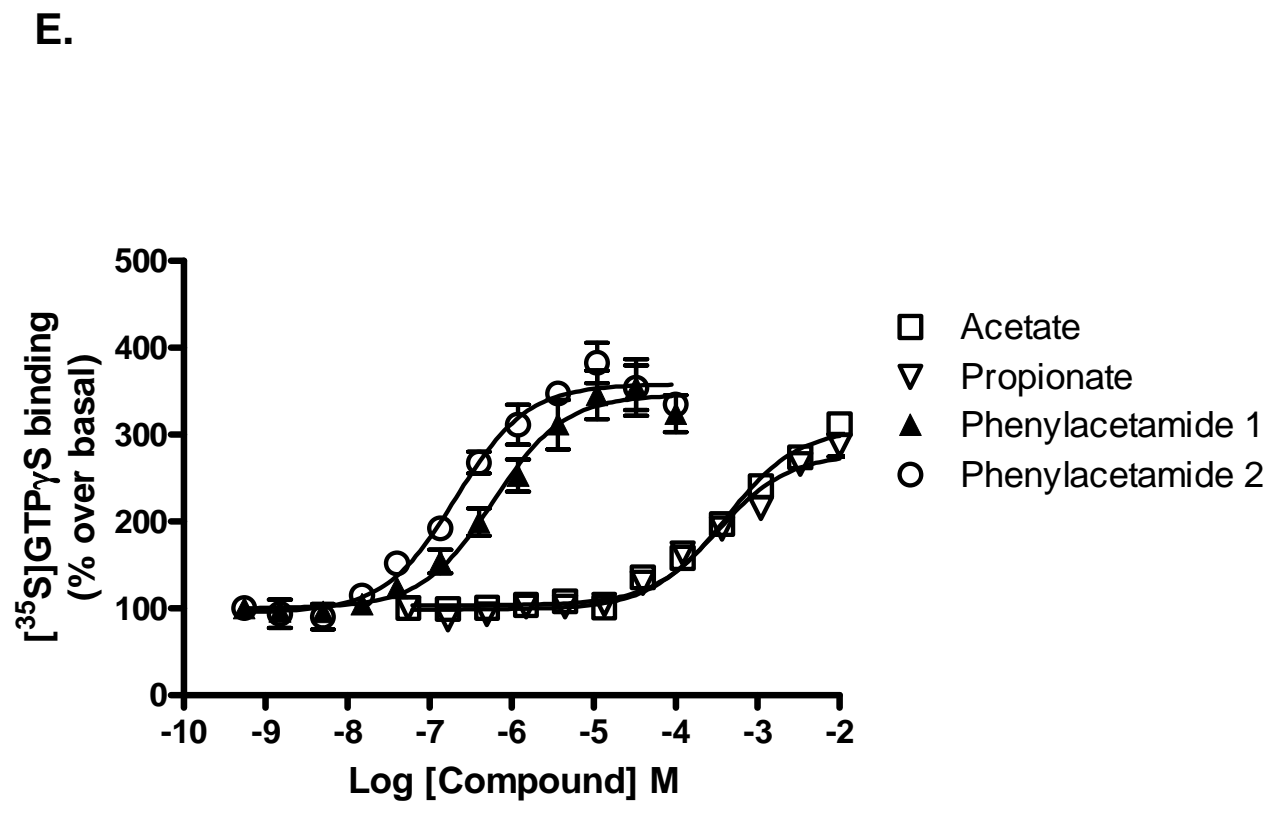


Figure 2

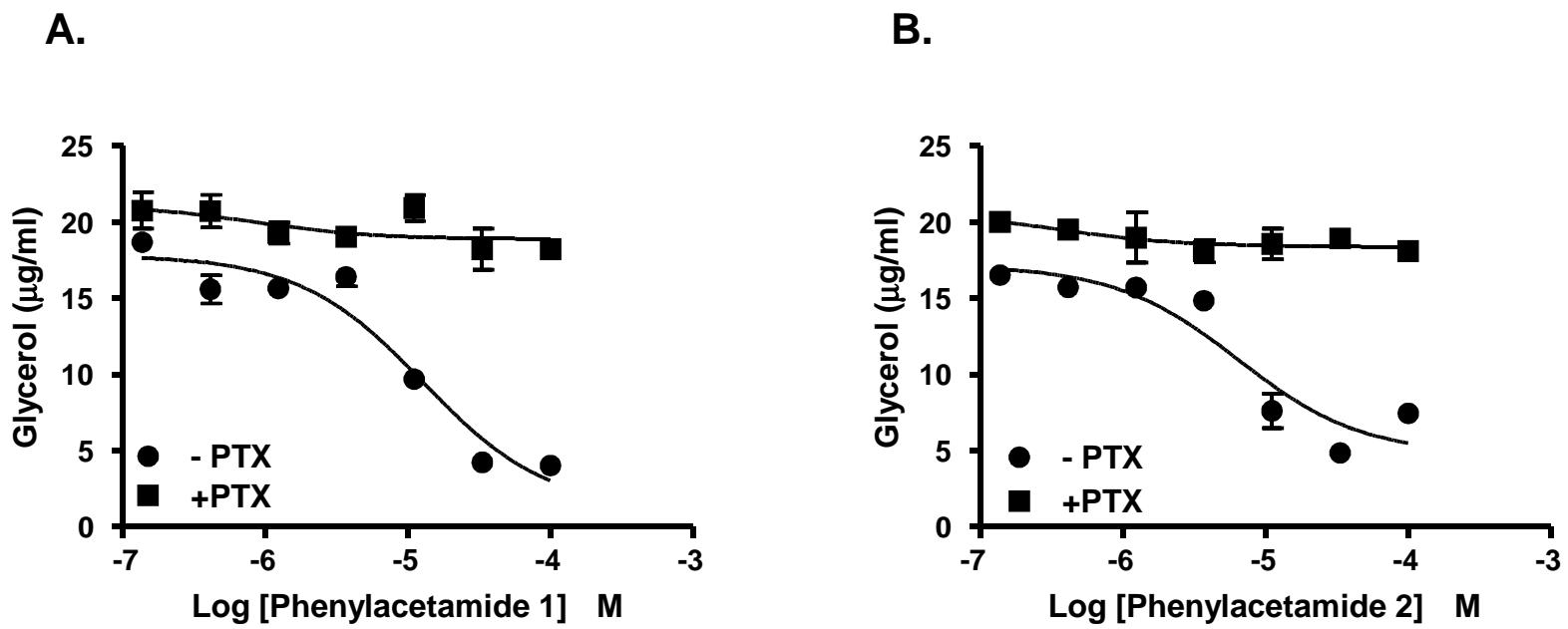


Figure 3

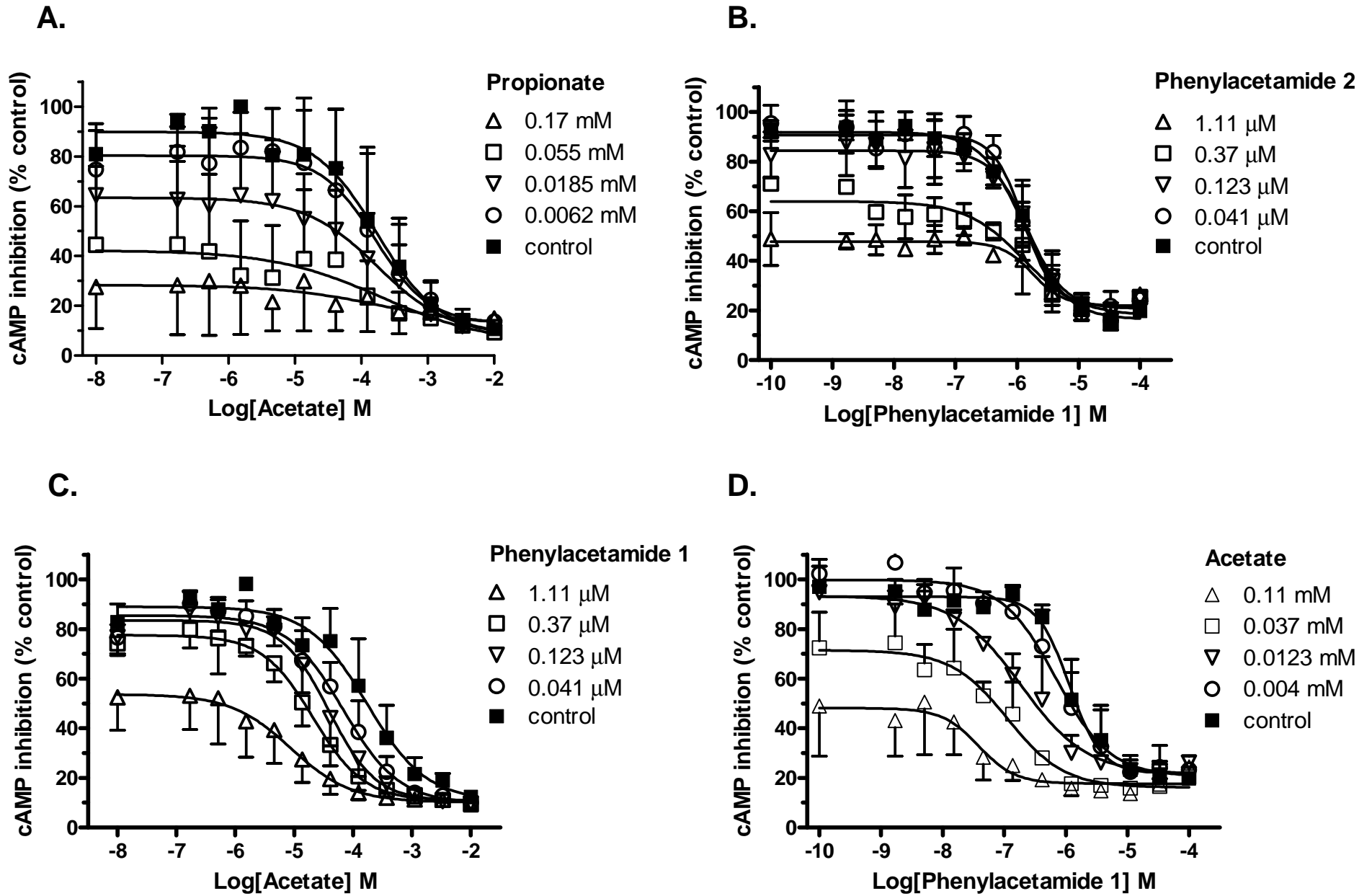
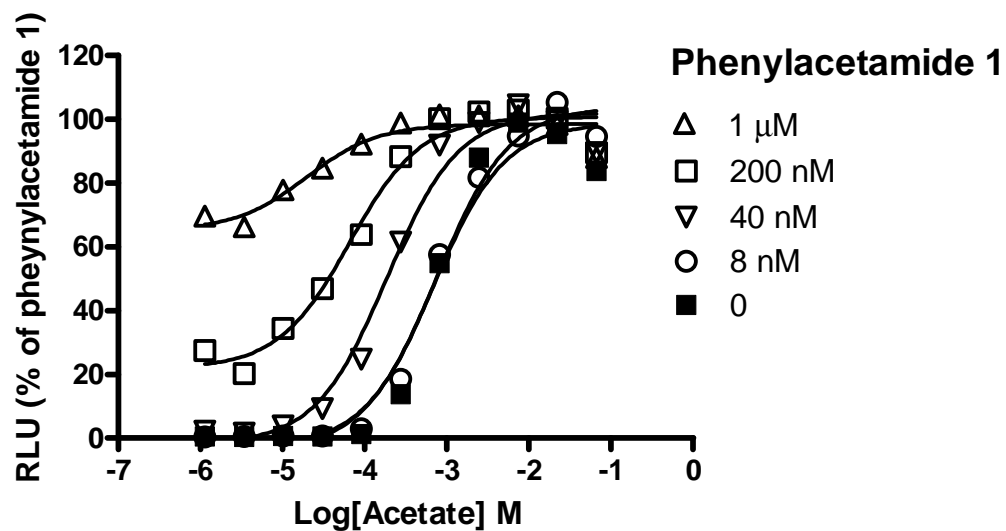
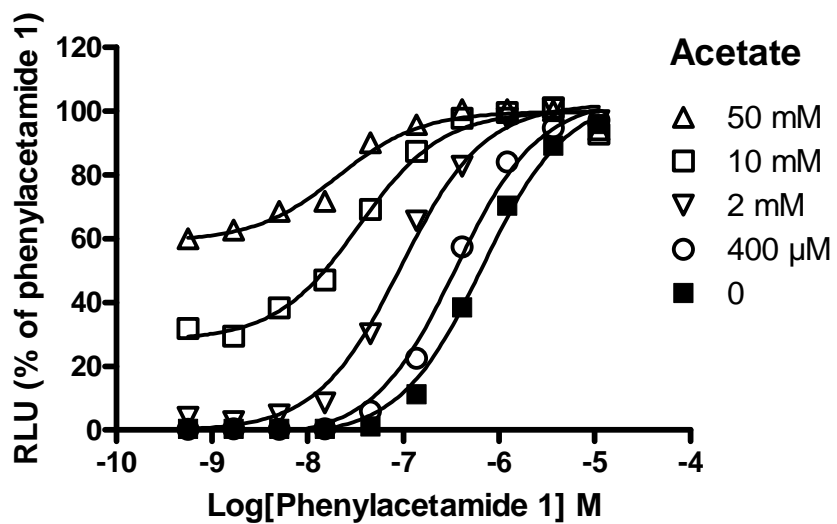


Figure 4

A.



B.



C.

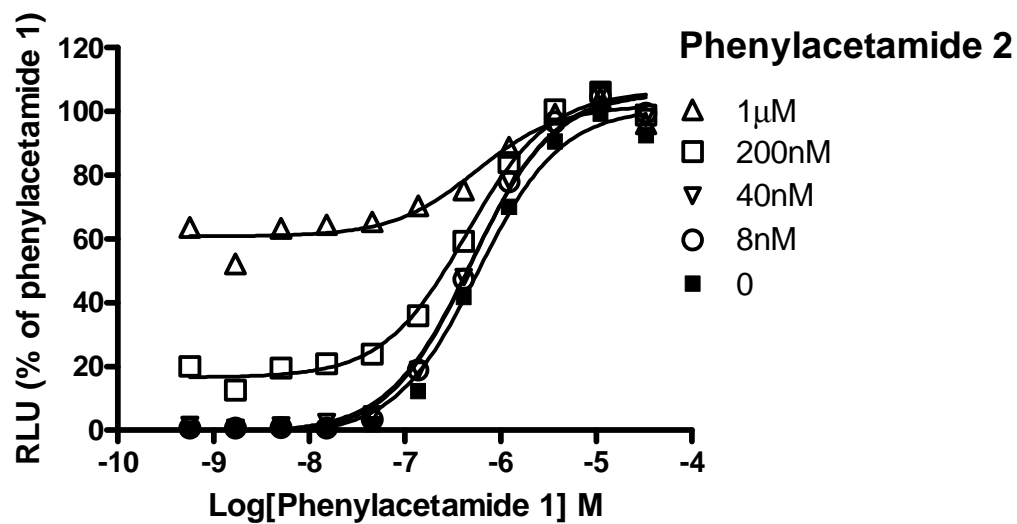
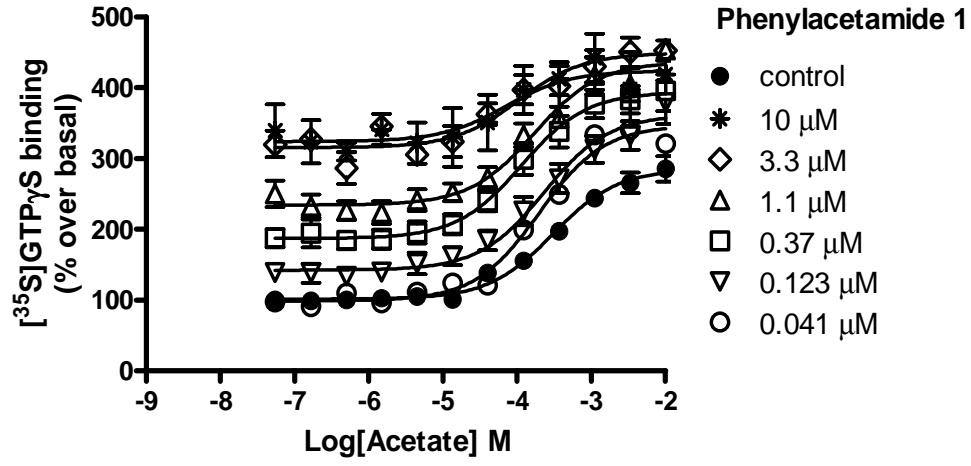


Figure 5

A.



B.

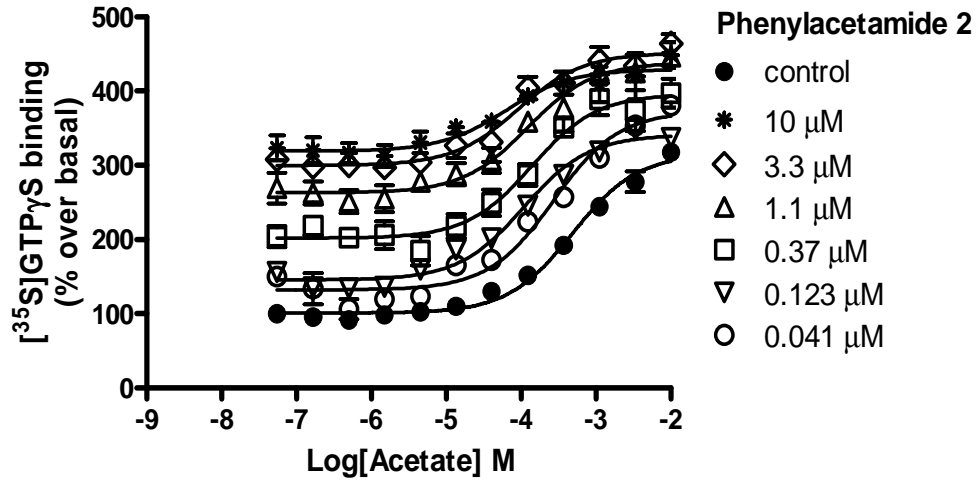


Figure 6

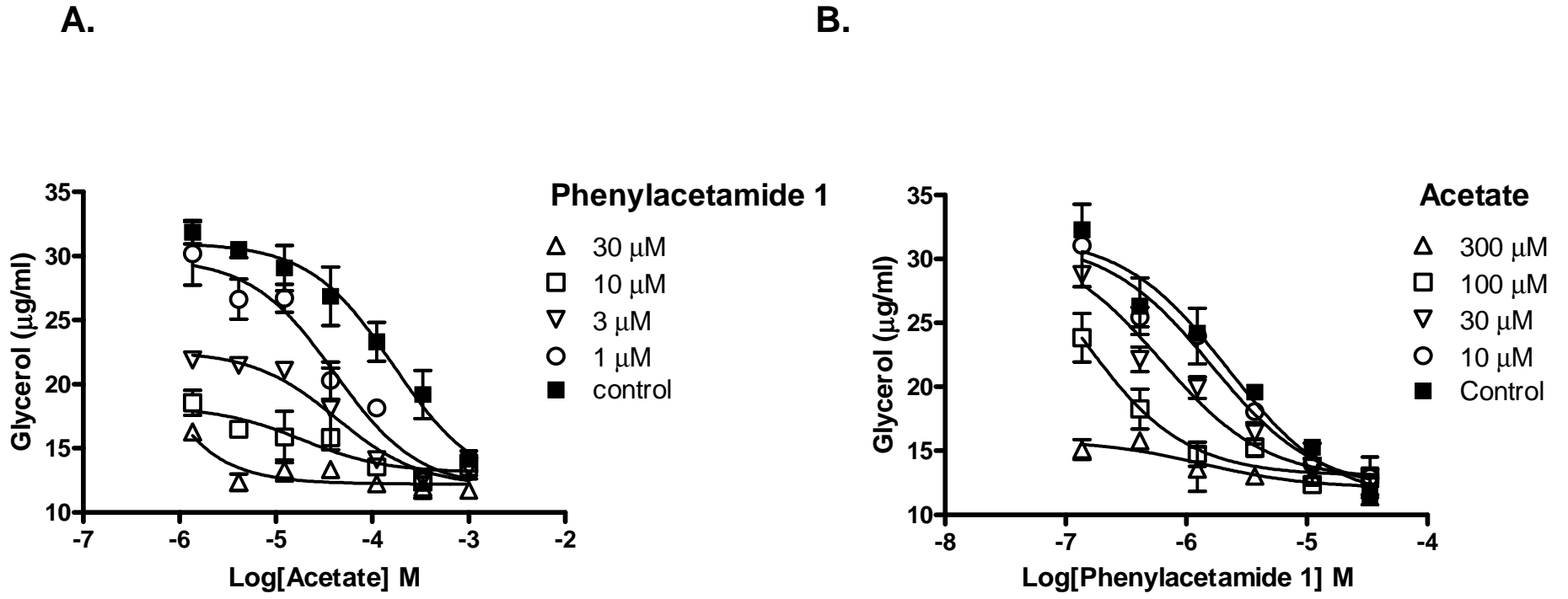


Figure 7

A.

```

                                     TM1                                     TM2
TM      =====
FFA3   MDTGPDQSYFSGNHWFVFSVYLLTFLVGLPLNLLALVVFVVGKLR-RPVAVDVLNLTALTA
FFA2   MLPDWKSS-----LILMAYIIIFLTGLPANLLALRAFVGRIRQPQPAPVHILLSLTL
FFA1   MDLPPQ-----LSFGLYVAAFALGFPLNVLAIRGATAHARL-RLTPSLVYALNLGC
*      :  :  * :  *  * : *  * : * :  . :  :  :  . :  * . *
      TM2                                     TM3
=====                                     =====
FFA3   SDLLLLLFLPFRMVEAANGMHWPLPFILCPLSGFIFFTTIYLTALFLAAVSIERFLSVAH
FFA2   ADLLLLLLLPLFKIIEAASNFRWYLPKVVICALTSFGFYSSIYCSTWLLAGISIERYLGVAF
FFA1   SDLLLTVSLPLKAVEALASGAWPLPASLCPVFAVAHFFPLYAGGGFLAALSAGRYLGAAF
:**** :  ** :  : ** .  *  **  : * :  . . :  : *  : ** : *  * : * . * .

                                     TM4
                                     =====
FFA3   PLWYKTRPRLGQAGLVSVACWLLASAHCSVVYVIEFSG-----DISHS-QGTNGTCYLE
FFA2   PVQYKLSRRPLYGVIAALVAWVMSFGHCTIVIVQYLN-----TTEQVRSGNEITCYEN
FFA1   PLGYQAFRRPCYSWGVCAAIWALVLCGLVFGLEAPGGWLDHSNTSLGINTPVNGSPVC
* :  * :  *  .  . . * :  *  : *  : :  .  .  .  .

                                     TM5                                     TM6
                                     =====                                     =====
FFA3   FRKDQLAILLPVRLEMAVVLVFPVPLIITSYCYSRVLVWILGRG--GSHRRQRRVAGLLAA
FFA2   FTDNQLDVVLVVRLELCLVLFIPMAVTIFCYWRFVWIMLSQPLVGAQRR-RAVGLAVV
FFA1   LEAWDPASAGPARFSLSLLLFFLPLAITAFVYVGLRALARSG--LTHRRKLRAAVVAGG
:  :  :  * . * : : : * : * : *  :  :  :  : * *  * . . :

      TM6                                     TM7
      =====                                     =====
FFA3   TLLNFLVCFGPYNVSHVVGYICGESP-AWRIYVTLTLLSTLNSCVDPFVYFSSSGFQADFH
FFA2   TLLNFLVCFGPYNVSHLVGYHQKSP-WWRSIAVVFSSLNASLDPLLFFYFSSSVVRRAFG
FFA1   ALLTLLLCVGPYNASNVASFLYPNLGGSWRKLGLITGAWSVVLNPLVTGYLGRGPGLKTV
: * . : * : * . * * * . * : :  :  **  :  . :  .  : * :  :

FFA3   ELLRRLCGLWGQWQQEESMELKEQKGGEEQRADRPAERKTSEHSQCGTGGQVACAES
FFA2   RGLQVLRNQSSLLGRRGKDTAEGTNEEDRGVQGEQEGMPSSDFTTE-----
FFA1   CAARTQGGKSQK-----

```

Figure 7

