Nicotinic acid receptor agonists differentially activate downstream effectors

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Running Title: GPR109A Agonist Directed Signal Transduction

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Summary

Nicotinic acid remains the most effective therapeutic agent for the treatment and prevention of atherosclerosis resulting from low HDL-cholesterol. Nicotinic acid's therapeutic actions are mediated by GPR109A, a G-protein coupled receptor, expressed primarily on adipocytes, Langerhans cells and macrophage. Unfortunately, a severe, cutaneous flushing side-effect limits its use and patient compliance. The mechanism of HDL elevation is not clearly established but assumed to be influenced by an inhibition of lipolysis in the adipose. The flushing side-effect appears to be mediated by the release of prostaglandin D2 (PGD2) from Langerhans cells in the skin. We hypothesized that the signal transduction pathways mediating the anti-lipolytic and PGD2/flushing pathways are distinct, and that agonists may be identified that are capable of selectively eliciting the therapeutic, anti-lipolytic pathway while avoiding the activation of the parallel flush-inducing pathway. We have identified a number of GPR109A pyrazole agonists that are capable of fully inhibiting lipolysis in vitro and in vivo and not only fail to elicit a flushing response, but can antagonize nicotinic acid's ability to elicit a flush response in vivo. In contrast to flushing agonists, exposure of cells expressing GPR109A to the non-flushing agonists fails to induce internalization of the receptor or to activate Erk 1/2 MAP kinase phosphorylation.

Introduction

Nicotinic acid (niacin, vitamin B3, pyridine-3-carboxylic acid) is the most effective therapeutic agent to date for raising high-density lipoprotein (HDL) levels. It also offers protection against other cardiovascular risk factors by lowering very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and lipoprotein(a) (Lp(a)) plasma concentrations (1;2). Although the mechanism by which nicotinic acid raises HDL is not clear, one hypothesis is that it is nicotinic acid’s ability to inhibit lipolysis in adipocytes which results in a decrease in the concentration of free fatty-acids (FFA) available for the liver to use for triglyceride synthesis and VLDL production. The attenuated synthesis of the triglyceride rich VLDL particles in the liver leads to a decreased rate of HDL metabolism via limiting the cholesterol ester transfer protein (CETP)-mediated exchange of cholesterol from HDL to VLDL, and TG from VLDL to HDL (3-6). Another hypothesis is that nicotinic acid inhibits the uptake and subsequent catabolism of Apo-AI containing HDL particles in hepatocytes (7;8).

Identification of a high-affinity nicotinic acid binding site that was localized to adipose, macrophage and spleen tissues, and appeared to function in a G-protein coupled manner (9) led to the molecular identification of the high-affinity nicotinic acid receptor GPR109A (HM74A in humans and PUMA-G in mice; (10-12)). In the adipose, GPR109A mediates an anti-lipolytic response that can attenuate cAMP-stimulated lipolysis (11). A low affinity nicotinic acid receptor has also been identified, referred to as GPR109B or HM74 (11). GPR109B appears to be the product of the gene duplication of GPR109A.
and is greater than 95% identical to GPR109A. A search of available genomes indicates that this receptor is found only in the human and Chimpanzee genomes and is absent in rodents. It is therefore difficult to know whether GPR109B has an endogenous ligand or plays a physiological role.

The therapeutic value of nicotinic acid is limited by its major side-effect, cutaneous flushing. This burning sensation, felt on the face and upper body, is responsible for a large portion of patient non-compliance (13-15). Recent work has begun to elucidate the mechanism by which nicotinic acid induces flushing (16-19). GPR109A has been shown to mediate nicotinic acid-induced flushing through release of prostaglandin D2 (PGD₂) and involves the activation of the DP1 receptor and possibly a PGE₂ receptor (EP2 or EP4; (16;18)). Recent work has further supported the hypothesis that it is GPR109A receptors on Langerhans cells in the skin that mediate nicotinic acid-induced flushing through generation of PGD₂ (17;19).

A series of pyrazole derivatives have been reported in the literature that act as partial agonists for the nicotinic acid receptor (20). The authors postulate that tissue selectivity, commonly observed with partial agonists, could be useful in preventing unwanted effects on skin cells and thus reduce or eliminate flushing. In the work presented herein, we set out to test the hypothesis that there are GPR109A agonists that are effective anti-lipolytic agents that do not cause flushing. We find that we can divide GPR109A agonists into two groups, those that elicit an anti-lipolytic and a flushing response, exemplified by nicotinic acid, and those that only elicit an anti-lipolytic response, i.e. non-flushing agonists. This second group of non-flushing compounds significantly decreases plasma free fatty-acids and not only fails to elicit a flushing response, but can block nicotinic acid’s flushing response. The non-flushing agonists are comprised of both full and partial agonists depending on the functional output examined. We further characterized the signaling pathways elicited by these compounds and have shown that compounds that led to a flushing response also induce both Erk 1/2 MAP kinase activation as well as receptor internalization. These non-flushing agonists may have a therapeutic, anti-lipolytic benefit without the unwanted cutaneous flushing side-effect.

**Experimental Procedures**

**Molecular cloning and generation of stable CHO-K1-GPR109A cells:** The GPR109A receptor was cloned and stable CHO-K1 cells were generated as described in (21).

**Animal Use and Protocols:** Animal studies were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (1996). All study protocols were reviewed and approved by Arena Pharmaceutical’s Internal Animal Care and Use Committee. C57Bl/6 mice were purchased from Jackson Labs and jugular vein catheterized Sprague-Dawley rats were purchased from Charles River Labs.

**Measurement of adenylyl cyclase inhibition:** A 96-well Adenylyl Cyclase Activation FlashPlate Assay kit™ (Perkin Elmer) was optimized to measure changes in intracellular cAMP levels due to receptor activation in CHO-K1 stable cell lines expressing GPR109A. CHO-K1 cells were cultured in F-12 Kaighn’s Modified Cell Culture Medium with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 400 μg/ml Geneticin in T-185 cell culture flasks. Cells were harvested from culture flasks and isolated via low speed centrifugation, counted and diluted to a density of 1x10⁶ cells/ml. Compounds were prepared in DMSO and serially diluted with PBS. Cells were added to the test plates containing the compounds at a final cell density of 50,000 cells/well. 5 μM forskolin was added to all wells. The assay plates were placed on a shaker for 1 hr at room temperature (~25°C). Diluted [³²⁵I]-cAMP (supplied with the FlashPlate kit) was added to each well and the plates continued to shake for another 2 hours. The plates were counted on a Wallac Microbeta Counter 1450.

**Human subcutaneous fat lipolysis assay:** Preparation of adipocytes: Cultured human subcutaneous adipocytes were received from Zen Bio., Inc. plated in 96-well plates two
weeks prior to performing the lipolysis assay. Upon arrival, all media was removed and pooled together (ZenBio Adipocyte Maintenance Medium). 150 μL of this media was then re- aliquoted to each well. Cells were kept in a sterile, humidified incubator at 37°C and allowed to recover for 1 week.

*Induction of lipolysis:* On the day of the lipolysis assay, cells were washed twice with 150 μL of Zen Bio Wash Buffer. After the second wash and removal of wash buffer, 75 μL of test compounds were added to each well, in triplicate. Compounds were prepared in Zen Bio Assay Buffer plus 1 μM isoproterenol. Cells were incubated for 5 hrs at 37°C.

*Glycerol assay:* Glycerol was determined using a free glycerol reagent from Sigma (Reagent A). Adipocyte media (50 μL) was removed and transferred to a flat-bottom 96-well plate. Reagent A (50 μL) was then added to each well. After 15 minutes, absorbance was read at OD_540_ on a Spectramax 340PC microplate reader (Molecular Devices). The amount of glycerol released was calculated based on regression analysis of known glycerol concentrations using a Glycerol Standard (Sigma).

*Mouse Non-Esterified Fatty Acid (NEFA) Assay:* Male C57/B16 mice, 8-10 weeks old (~25 g) were food deprived 16 hrs. prior to the experiment. They were then injected intra-peritoneal (i.p.) with vehicle (phosphate buffered saline; PBS) or the compound of interest at the desired concentration (5 ml per kilogram) diluted in PBS. After 10 minutes, the animals were euthanized via CO₂ asphyxiation and blood was drawn by cardiac puncture. The blood was centrifuged on a table top centrifuge (4000 rpm at 4°C for 10 min). Serum was collected in a new microfuge tube and processed as described for the mouse NEFA assay above.

*Rat NEFA Assay:* Jugular vein cannulae of male Sprague-Dawley rats (7-10 weeks old; ~350 g) were flushed with approximately 200 μl saline and animals were food deprived for 16 hr. All blood draws were immediately followed by a 200 μl saline flush to maintain patency of the cannulae. Baseline blood samples were collected via cannula and rats were given vehicle (PBS; 5% Hydroxypropyl-β-cyclodextrin (HPBCD); or 0.5% methylcellulose) or drug via per oral (p.o.) administration (2 ml/kg). Blood was collected at desired time points, transferred to a micro-centrifuge tube, and placed on ice. The blood was centrifuged on a table top centrifuge (4000 rpm at 4°C for 10 min). Serum was collected in a new microfuge tube and processed as described for the mouse NEFA assay above.

*Mole RE FLushing via Laser Doppler:* Male C57/B16 mice (8-10 weeks old; ~25 g) were anesthetized with Nembutal via i.p. injection (80 mg/10 ml/kg). After 10 min the mouse was placed under an LDPI Laser Doppler (PeriScan PIM II; Perimed, Stockholm) and a needle and syringe containing vehicle (PBS; 40% Hydroxypropyl-β-cyclodextrin (HPBCD); or 0.5% methylcellulose) or drug was placed in the intra-peritoneal space and a slight back pressure was applied to prevent premature delivery of compound. The mouse's right ear was turned inside-out to expose the ventral side using forceps. The Laser Doppler was focused in the center of the ventral right ear and adjusted as follows: Repeated data collection; 15X15 Image format; Auto interval start; 20 sec delay; Medium resolution; Very fast scan speed; and 8-9 V intensity (~4.5 cm from ear). After a three minute baseline reading, vehicle or compound was administered into the i.p. space (5 ml/kg through the pre-inserted syringe) and readings continued for approximately 15 min.

For competition assays, a similar protocol was utilized with the exception that immediately following the Nembutal injection the mouse was i.p. injected with vehicle or competing compound. This was followed by establishing baseline and i.p. drug administration (typically 30 mg/kg nicotinic acid) as described above. In some cases, this round of flushing was followed by re-establishing baseline and i.p. PGD₂ (2 mg/5 ml/kg) injection as a positive control.
MAP kinase assays: MAP kinase assays were performed using the phospho-MAP kinase ELISA assay kit from Biosource (Cat# KHO 0091) according to the manufacturer’s specifications. Specifically, CHO-K1 stable cell lines expressing GPR109A were serum-starved overnight. Cells were stimulated with compounds for 5 min at 37°C, the medium was aspirated, and the cells were rinsed with PBS. The cells were scraped in 1 ml PBS and transferred to a microfuge tube. The suspension was centrifuged for 5 min at 3000 rpm and the pellet was resuspended in 200 μl cell extraction buffer (0.1% SDS). The samples were incubated on ice for 30 min then clarified by centrifugation for 10 min, 4°C at 13,000 rpm. Protein concentrations were determined by a modified Bradford analysis and 10 μg of protein was added to each well of a 96-well plate coated with anti-phospho-MAP kinase capture antibody. The samples were incubated for 2 hr at 25°C then extensively washed before incubation with the anti-phospho-MAP kinase detection antibody for 1 hr at 25°C. The samples were washed and then incubated with an HRP-conjugated secondary antibody for 30 min at 25°C. The samples were washed, and then incubated with chromogen in the dark for 20 min at 25°C before stopping the reaction with stop buffer. Absorbance at 450 nm was read on a Spectramax 340PC microplate reader (Molecular Devices).

For the mouse ear MAP kinase assays, mice were injected i.p. with either vehicle or niacin at 100 mg/kg. After 5 min, mice were sacrificed via CO2 asphyxiation and the ears removed. Ears were minced into small pieces and homogenized in lysis buffer using a Brinkman Polytron. Membrane protein was isolated via centrifugation at 20,000 rpm (JA-25.50 rotor; 15 min, 4°C). Membrane pellets were resuspended in 200 μl cell extraction buffer and treated as described above.

Detection of GPR109A internalization: COS-7 cells were transiently co-transfected with NH2-termally-HA-epitope tagged human or mouse GPR109A and human β-arrestin2 and plated on glass cover-slips in 24 well plates (1x10^5 cells/well). Receptor internalization was assayed at 48 hours after transfection. Cells were washed 3 times with PBS and incubated on ice for 1 hour with Alexa 488-conjugated anti-HA-antibody 16B12 (Molecular Probes) at 5 μg/ml in OPTI-MEM. Indicated concentrations of compounds were then added to the cells and incubated at 37°C for various lengths of time. Cells were washed 3 times with PBS and fixed with formaldehyde. Cover-slips were then mounted on slides for visualization.

Mouse Mixed Epidermal Cell Culture: Mouse epidermal cells were isolated and cultured as described in (19). PGD2 ELISA Assays: The PGD2 content of culture medium was assayed as described in (19) using 2 x 10^6 cells/ml. For the assay, 50 μl of cell suspension was added to 400 μl pre-warmed (37°C) RPMI containing 20 μM MEK inhibitor PD98059 or vehicle (DMSO) and incubated at 37°C, 5% CO2 for 15 min. 50 μl RPMI containing 1 mM nicotinic acid, 10 μM indomethacin or vehicle were added to each well and the cells were further incubated for 30 min and treated as described in (19).

Results

Compounds used in the assays described herein are shown in Figure 1. CHO-K1 cells were stably-transfected with GPR109A and cellular cAMP was measured in the intact cells. Compounds were added at the indicated concentrations to measure their ability to inhibit 5 μM forskolin-stimulated cAMP levels, and nicotinic acid was used as the full-agonist positive control. 5-methyl-3-carboxyl-pyrazole (3a), 3-methyl-5-carboxyl-isoxazole (4a), 5-isopropyl-3-carboxyl-pyrazole (2a), and 5-meta-fluorobenzyl-3-carboxyl-pyrazole (1c) inhibited forskolin stimulated cAMP production to the same extent as nicotinic acid (i.e. were full-agonists; Fig. 2). By contrast, 5-meta-chlorobenzyl-3-carboxyl-pyrazole (1a) and 5-meta-bromobenzyl-3-carboxyl-pyrazole (1b) were able to inhibit only 65-67% of the forskolin stimulated cAMP production (i.e. were partial-agonists; Fig. 2). These compounds had the same potency and efficacy when applied to cAMP assays using cells stably transfected with human, rat or mouse GPR109A. The partial agonists 1a and 1b were applied to [3H]nicotinic acid...
acid competition binding assays and were able to fully displace $[^{3}H]$nicotinic acid binding (data not shown).

We examined the ability of these compounds to inhibit human adipocyte lipolysis in an *in vitro* model. Human subcutaneous adipocytes were cultured and differentiated as described in Experimental Procedures. Lipolysis was stimulated with 1 μM isoproterenol and compounds were added to determine their ability to inhibit lipolysis. Figure 3 shows the results of the adipocyte lipolysis assays. All of the compounds tested inhibited isoproterenol-stimulated lipolysis with varying potencies. All but one compound, 4a, appeared to inhibit lipolysis to the same extent as nicotinic acid (*i.e.* full inhibition; Fig. 3). Compound 4a was only able to decrease glycerol production to 50% of the control value (Fig. 3) indicating that it is a partial agonist in this assay.

We next examined the effect of these GPR109A agonists on plasma free-fatty acid concentrations *in vivo* in male C57Bl/6 mice. Again, all of the compounds examined were able to acutely depress plasma free fatty-acid concentrations to an equivalent or greater extent than nicotinic acid 10 min after p.o. agonist administration (Fig. 4). This effect appeared to be dose-dependent for each of the agonists examined. The potency of the compounds varied but did not necessarily reflect the predicted, *in vitro*, potency of the agonists. This more likely reflects the bioavailability and/or compound half-life.

When we applied the GPR109A agonists to an acute, *in vivo* rat model of plasma free fatty-acid measurement, we found similar results. All of the compounds examined dose- and time-dependently depressed plasma free fatty-acids levels (Fig. 5). The maximal extent of plasma free fatty-acid inhibition was similar for all of the agonists (up to ~75% inhibition of baseline values).

The cutaneous flushing induced by these agonists was assessed via Laser Doppler (which measures blood flow, and thus vasodilation) on the ears of male C57Bl/6 mice. Figure 6 illustrates that some of the GPR109A agonists described above led to a flushing response (nicotinic acid, 3a and 4a) while others did not (1a, 1b, 1c and 2a; Fig. 6). When compound 1a, a non-flushing GPR109A agonist, was pre-injected into the mice 10 min prior to baseline (13 min prior to nicotinic acid injection), nicotinic acid was unable to elicit a flushing response (Fig. 7). When similar experiments were performed with a follow-up PGD2 injection, the animals responded with a concomitant full flushing response (data not shown). This data indicates that the non-flushing compound 1a is able to interact with receptors on the cells responsible for mediating the flushing response and antagonizes the nicotinic acid-mediated flushing effect (Fig. 7).

Anti-phospho Erk 1/2 MAP kinase ELISA assays were performed on the GPR109A stably transfected CHO cells. Interestingly, of the compounds tested, only the flush-inducing compounds nicotinic acid, 5-methyl-3-carboxyl-pyrazole (compound 3a) and 3-methyl-5-carboxyl-isoxazole (compound 4a) stimulated MAP kinase activation (Fig. 8). On the other hand the non-flush-inducing compounds 5-meta-chlorobenzyl-3-carboxyl-pyrazole (1a) and 5-isopropyl-3-carboxyl-pyrazole (2a) failed to significantly increase Erk 1/2 MAP kinase activity (Fig. 8).

It was postulated that β-arrestin recruitment and receptor internalization are required for MAP kinase activation (as proposed by Daaka, *et al.* (22)). To test this, we examined the ability of these compounds to differentially internalize epitope-tagged GPR109A in transiently-transfected COS-7 cells. Figure 9 shows the dose-dependent effects of nicotinic acid and 5-meta-chlorobenzyl-3-carboxyl-pyrazole (compound 1a) on the cellular localization of HA-tagged GPR109A. Following 30 min exposure of the cells to 1, 10, and 100 μM nicotinic acid or the isoxazole agonist 4a, anti-HA immuno-reactivity failed to significantly increase receptor internalization. On the other hand, compound 1a, a non-flushing agonist, failed to effect any change in anti-HA immuno-reactivity, confirming the hypothesis that this agonist fails to drive receptor internalization (Fig. 9, middle column). When applied to other GPR109A agonists described herein, the qualitative assessment of HA-tagged GPR109A immuno-reactivity revealed that, again, nicotinic acid stimulation results in receptor internalization while non-flushing compounds 2a, and 1a-c fail to internalize receptor immuno-reactivity (Fig. 10).
Recent work examining the mechanism of niacin-induced flushing has revealed that it is mediated by GPR109A (16), requires the release of PGD2 and activation of DP1 receptors in the skin (18), and finally, that the PGD2 release is mediated by epidermal Langerhans cells (LCs) (17;19). Based on the results shown herein we would speculate that GPR109A agonists activate a MAP kinase-mediated release of PGD2 from LCs, leading to the cutaneous vasodilation responsible for the flushing response. As shown in Figure 11A, mice given an i.p. niacin injection had a profound, 3-fold elevation of phospho-MAP kinase in the ear (where flushing is measured in the mouse model). When mouse epidermal cells were isolated and cultured, niacin treatment led to a significant PGD2 release, which was attenuated by pretreatment with the MEK inhibitor, PD98059 (Fig. 11B). This effect was also seen in cultured human LCs where both the MEK inhibitor and indomethacin (a cyclooxygenase inhibitor), were able to attenuate PGD2 release (Fig. 11C). While in vivo use of the MEK inhibitor to prevent flushing was confounded by its inability to inhibit MAP kinase production in this model, the above results are consistent with a MAP kinase requirement for flushing.

Discussion

The results presented herein indicate that there are GPR109A agonists that, like nicotinic acid, are capable of stimulating the G_i-mediated inhibition of adenylyl cyclase, inhibit isoproterenol-stimulated lipolysis in adipocytes and decrease plasma free fatty-acids in both mice and rats, but that lack the ability to induce receptor internalization and Erk 1/2 MAP kinase activation. We find that the compounds that fall into this group fail to elicit a flushing response and, in fact, can antagonize the flushing response elicited by nicotinic acid. These results suggest that it may be possible to tailor anti-lipolytic compounds that fail to induce the unwanted flushing side-effect present in nicotinic acid preparations.

The concept that a receptor agonist may selectively activate only a subset of signal effectors distinct from other agonists via a common receptor has been proposed for a number of G protein-coupled receptors (GPCRs). This phenomenon may result if the effectors being examined are sequential and the agonists used have differing intrinsic efficacies resulting in differential signal strength. One agonist may induce a first effector sufficient to overcome a threshold necessary to activate a second, downstream effector signal, while another agonist is capable of eliciting a measurable, first effector signal that is not sufficient to activate the second effector pathway.

Alternatively, it has been proposed that receptors such as GPCRs may, upon activation, have more than one active conformation. In this model one agonist would induce a conformation capable of activating multiple, parallel effector pathways, while another agonist induces a receptor conformation only capable of eliciting a subset of signaling effectors. This model of pleiotropic agonist signaling has been termed “agonist directed trafficking of receptor signals” (23).

The idea that different agonists may induce different receptor conformations or may have different signal strengths, and therefore divergent signaling pathways, has been suggested by a number of groups for a variety of GPCRs including the α2A-adrenergic receptor, 5HT2 receptors, adenosine-A1, and the nicotinic acid receptor (20;24-28) and reviewed by T. Kenakin (23). In these studies it is suggested that use of one agonist or agonist class may elicit cellular and pharmacological activities, distinct from activities elicited by another agonist or agonist class.

These concepts have been adopted by a number of groups for the use of partial agonists, compounds that bind receptors but in which activation does not translate to a fully efficacious response, to elicit a desired response without an undesirable side-effect. For example, it was shown by Tan et al. that use of an α2A-adrenergic receptor (α2AAR) partial agonist, moxonidine, has an anti-hypertensive effect in wild-type C57Bl/6 mice while avoiding the sedative side-effect elicited by the α2AAR full-agonist clonidine (24). In this study it is important to note that different agonists elicit in vitro responses with varying degrees of efficacy as a function of the different effectors examined (e.g. GTPγS binding, MAP kinase activation, etc.). In addition, the receptor density and downstream effectors are going to vary in different tissues and therefore the relative efficacies of agonists will also vary accordingly.
The authors conclude that partial agonists can evoke response-selective pathways and may be used to provide response-selective therapies (24).

As described in the introduction, a series of pyrazole derivatives have been reported in the literature that act as partial agonists for [35S]GTPγS binding via the nicotinic acid receptor in rat adipocyte and spleen membranes (20). The authors postulate that tissue selectivity, commonly observed with partial agonists, could be useful in preventing unwanted, flush-inducing side-effects on skin cells while maintaining the ability to inhibit lipolysis.

In examination of the compounds presented herein, when we look at agonist effects at inhibiting cAMP we identified four full-agonists (compounds 1c and 2-4a) and two partial agonists (compounds 1a and 1b), of these, three are non-flushing compounds (one full agonist and both of the partial agonists). These effects are independent of the species of the receptor as the potency and efficacy of these compounds were essentially identical in human, rat, or mouse stably transfected cells. In the in vitro lipolysis assay one of the compounds, 4a, is a partial agonist, yet this compound is a potent flush inducer. It should be noted that these compounds have little or no affinity to GPR109B, the low-affinity niacin receptor. In addition, as mentioned in the introduction, GPR109B does not appear to have a rodent ortholog. Therefore, differential effects mediated by the panel of molecules presented herein cannot be explained on the basis of non-selectivity.

Therefore, the separation of downstream effector signals in order to achieve a therapeutic effect without the unwanted side-effect does not necessarily require the development of partial agonists. Rather, the drug design and identification process should be focused on identification of agonists that potently stimulate responses downstream of effectors known to mediate therapeutic responses, and that lack the ability to stimulate effectors that mediate untoward side-effects. Obviously, because the mechanism of therapeutic action of nicotinic acid has yet to be identified, one concern would be that eliminating the side-effect may also eliminate the desired therapeutic effect. Pre-clinical and clinical studies need to be done comparing the therapeutic efficacy of compounds that fall into the two categories; those that elicit flushing, MAP kinase activation, and internalization in addition to eliciting an anti-lipolytic response versus those that solely elicit an anti-lipolytic response.

**Abbreviations:** Ab, antibody; FFA, free fatty-acid; GPCR, G protein coupled receptor; HDL, high-density lipoprotein; i.p., intraperitoneal; LCs, Langerhans cells; LDL, low-density lipoprotein; MAPK, Mitogen-activated protein kinase; NA, nicotinic acid; NEFA, non-esterified fatty-acid; p.o., per oral; PGD2, prostaglandin D2; TG, triglyceride; VLDL, very low-density lipoprotein.

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**Figure Legends**

**Figure 1.** Structures of GPR109A agonists presented herein with compound designations indicated below each structure.

**Figure 2.** Inhibition of forskolin stimulated cAMP in stably-transfected CHO-K1 cells. GPR109A agonists were added at the indicated concentrations to measure their ability to inhibit forskolin (5 μM) stimulated cAMP production. Nicotinic acid was used as the full agonist, positive control. Values represent mean % inhibition of forskolin stimulated cAMP production normalized to nicotinic acid ± sem. IC$_{50}$ values are shown in the table above. Height represents the % maximal inhibition of forskolin stimulated cAMP relative to nicotinic acid.

**Figure 3.** Human subcutaneous adipose *in vitro* lipolysis. Human subcutaneous adipocytes were treated with 1 μM isoproterenol to stimulate lipolysis and then exposed to agonists at the indicated concentrations. Values are the mean % of control (isoproterenol stimulated) ± sem of four or more experiments each run in triplicate (EC$_{50}$ and replicate values are indicated in the legend (n)).

**Figure 4.** Mouse *in vivo* lipolysis (NEFA) assays. Male C57Bl/6 mice were p.o. injected with vehicle (PBS) or compounds as indicated and blood was collected 10 min post-treatment. Values are the mean plasma FFA concentration (mM) ± sem of three or more experiments each run in triplicate as indicated (n). ***, p<0.001; **, p< 0.01; *, p<0.05 via One-way ANOVA with Bonferroni’s Multiple Comparison Post-hoc Test.

**Figure 5.** Rat *in vivo* lipolysis (NEFA) assays. Male Sprague-Dawley rats were p.o. injected with vehicle (PBS) or compounds as indicated and blood was collected via jugular vein catheter at the indicated time points post-treatment. Values are the mean % baseline plasma FFA concentration ± sem of four or more independent experiments as indicated (n).

**Figure 6.** Mouse flushing via Laser Doppler. Baseline perfusion was established for 3 min in the ventral right ear of C57Bl/6 mice. Mice were then given i.p. injections of individual GPR109A agonists. Values are the mean % change from baseline, pre-injection values ± sem of 3-26 individual animals as indicated in the key at right.
Figure 7. Competition of nicotinic acid-induced flush in mice. C57Bl/6 mice were given i.p. injections of vehicle or compound 1a 10 min prior to determining baseline (co-administered with the Nembutal anesthetization). Baseline was established followed by i.p. injection of nicotinic acid. Values are the mean % change from baseline, pre-nicotinic acid injection values ± sem of 11 or 5 animals as indicated in the key at right. Pre-injection of compound 1a antagonized nicotinic acid’s ability to induce a flushing response.

Figure 8. Presence or absence of Erk 1/2 MAP Kinase stimulation in stably-transfected CHO-K1 cells. Values are the mean fold-change relative to control ± sem. The table inset above summarizes the EC50 and experimental number (n) for Erk 1/2 MAP kinase activation for the compounds tested. NR indicates no response for which an appropriate EC50 could be calculated (i.e. goodness of fit R2 < 0.4).

Figure 9. Qualitative assessment of GPR109A internalization following agonist exposure. The dose-dependent internalization of GPR109A was examined using immunofluorescence microscopy on transiently-transfected COS-7 cells following 30 min exposure to indicated doses of nicotinic acid (left), compound 1a (middle), or compound 4a (right). Data shown is representative of at least three independent experiments.

Figure 10. Qualitative assessment of GPR109A internalization following 10 μM agonist exposure for 30 min. Internalization of GPR109A was examined using immuno-fluorescence microscopy on transiently-transfected COS-7 cells following 30 min exposure to 10 μM of indicated compounds. Data shown is representative of at least three independent experiments.

Figure 11. Niacin-mediated MAP kinase and PGD2 production in mouse skin and human Langerhans cells. (A) Niacin mediates a > 3-fold stimulation of phospho-Erk 1/2 MAP kinase in the ears of mice i.p. injected with 100 mg/kg niacin relative to vehicle treated mice. Data shown is the mean ± standard deviation of values derived in triplicate from two mice. (B) Mouse epidermal cells were isolated and cultured. Cells were incubated for 15 min. with 20 μM PD98059 or DMSO vehicle control and then treated with 1 mM niacin for 30 min. Niacin-mediated PGD2 released into the culture medium was measured, basal PGD2 values were subtracted out as background, and values were normalized to the maximal PGD2 release elicited in the individual experiments. Values are the mean ± sem of four independent experiments. P-
value determined by student’s t-test. (C) Human Langerhans cells were incubated for 15 min. with 20 μM PD98059, indomethacin or DMSO vehicle control and then treated with 1 mM niacin for 30 min. Niacin-mediated PGD₂ release was attenuated by pre-treatment with either PD98059 or indomethacin.
Figure 1

Nicotinic acid

1a

2a

3a

4a

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| IC<sub>50</sub>   | Inhibition | N  |
|-----------------|------------|----|
| 4.2 μM          | 65%        | 10 |
| 1.7 μM          | 67%        | 2  |
| 2.2 μM          | 96%        | 8  |
| 2.6 μM          | 98%        | 4  |
| 434 nM          | 100%       | 12 |
| 1.3 μM          | 100%       | 12 |
| 67.3 nM         | 100%       | 9  |

**Figure 2**

[Graph showing log[Drug] vs. % Inhibition of forskolin-stimulated cAMP (relative to niacin)]

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Figure 3

- △ 1a (EC$_{50}$ = 72.5 μM; n=5)
- ● 1b (EC$_{50}$ = 102.7 μM; n=4)
- △ 1c (EC$_{50}$ = 32.6 μM; n=4)
- ○ 3a (EC$_{50}$ = 1.4 μM; n=6)
- ★ 4a (EC$_{50}$ = 5.3 μM; n=5)
- ■ NA (EC$_{50}$ = 0.9 μM; n=11)
### Figure 4

#### Mouse Plasma [FFA] (mM)

| Compound | Veh | 1 | 10 | 100 | 1 | 10 | 100 | 1 | 10 | 100 | 1 | 10 | 100 |
|----------|-----|---|----|-----|---|----|-----|---|----|-----|---|----|-----|
| Nicotinic acid | -   | (21) | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) |
| 1a       | Veh | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) | (6) |
| 1b       | Veh | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) |
| 1c       | Veh | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) | (5) |
| 2a       | Veh | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) |
| 3a       | Veh | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) | (3) |

* denotes statistical significance compared to Veh.
Figure 5

- Vehicle (n=13)
- Niacin (100mg/kg; n=9)
- 1a (1mg/kg; n=4)
- 1a (10mg/kg; n=6)
- 1a (100mg/kg; n=4)
- 1b (10mg/kg; n=5)
- 2a (10mg/kg; n=4)
- 4a (1mg/kg; n=7)
Figure 6

- □ NA (30 mg/kg; n=26)
- □ 3a (100 mg/kg; n=13)
- ● 4a (100 mg/kg; n=6)
- ▲ 1a (50 mg/kg; n=3)
- ● 1b (50 mg/kg; n=4)
- ○ 1c (200 mg/kg; n=6)
- ○ 2a (100 mg/kg; n=5)
- + Vehicle (n=5)
Figure 7

- Vehicle + NA (30 mg/kg) n=11
- 1a (100 mg/kg) + NA (30 mg/kg) n=5

Flush (% change from baseline) vs. Time (Min)
Figure 8

|                  | Flushing compounds | Non-flushing compounds |
|------------------|--------------------|------------------------|
| **EC$_{50}$**    | NA (n=16)          | 3a (n=4)               |
|                  | 580 nM             | 900 nM                 |
|                  | 1a (n=4)           | 2a (n=10)              |
|                  | NR                 | NR                     |

Phospho-Erk 1/2 (fold increase)

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Figure 9

Nicotinic acid

0.1 μM

1 μM

10 μM

100 μM

1a

4a
Figure 10

DMSO

Nicotinic acid

2a

1a

1b

1c
Figure 11

A

Vehicle | Niacin

phospho-MAPK (U/ml)

B

% Maximal PGD<sub>2</sub> Released

Niacin (1mM): + +
PD98059: - +

p = 0.026

C

[PGD<sub>2</sub>] (pg/ml)

Niacin (1mM): + + +
PD98059: - + -
Indomethacin: - - +

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Nicotinic acid receptor agonists differentially activate downstream effectors
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