Symmetrically substituted dichlorophenones inhibit N-acyl-phosphatidylethanolamine phospholipase D

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N-Acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD) (EC 3.1.4.4) catalyzes the final step in the biosynthesis of N-acyl-ethanolamidates. Reduced NAPE-PLD expression and activity may contribute to obesity and inflammation, but a lack of effective NAPE-PLD inhibitors has been a major obstacle to elucidating the role of NAPE-PLD and N-acyl-ethanolamidate biosynthesis in these processes. The endogenous bile acid lithocholic acid (LCA) inhibits NAPE-PLD activity (with an IC_{50} of 68 μM), but LCA is also a highly potent ligand for TGR5 (EC_{50} 0.52 μM). Recently, the first selective small-molecule inhibitor of NAPE-PLD, ARN19874, has been reported (having an IC_{50} of 34 μM). To identify more potent inhibitors of NAPE-PLD, here we used a quenched fluorescent NAPE analog, PED-A1, as a substrate for recombinant mouse Nape-pld to screen a panel of bile acids and a library of experimental compounds (the Spectrum collection). Muricholic acids and several other bile acids inhibited Nape-pld with potency similar to that of LCA. We identified 14 potent Nape-pld inhibitors in the Spectrum Collection, with the two most potent (IC_{50} ~ 2 μM) being symmetrically substituted dichlorophenones, i.e. hexachlorophene and bithionol. Structure–activity relationship assays using additional substituted dichlorophenones identified key moieties needed for Nape-pld inhibition. Both hexachlorophene and bithionol exhibited significant selectivity for Nape-pld compared with nontarget lipase activities such as Streptomyces cro
cromofuscus PLD or serum lipase. Both also effectively inhibited NAPE-PLD activity in cultured HEK293 cells. We conclude that symmetrically substituted dichlorophenones potently inhibit NAPE-PLD in cultured cells and have significant selectivity for NAPE-PLD versus other tissue-associated lipases.

N-Acyl-ethanolamidate (NAE) biosynthesis and signaling appear to play important roles in regulating energy balance, inflammation, stress responses, and addiction (1–6). Saturated and monounsaturated NAEs like N-palmitoyl-ethanolamide (C16:0NAE) and N-oleoyl-ethanolamide (C18:1NAE) act on GPR55 and GPR119, respectively, as well as on peroxisome proliferator-activated receptor α (7–13). Pharmacological administration of these compounds enhances the resolution of inflammation, induces satiety, and protects against the development of obesity on high-fat diet (14–17). In contrast, N-arachidonoyl-ethanolamide (C20:4NAE, anandamide) is a polyunsaturated NAE that acts at endocannabinoid receptors (CB1 and CB2) to exert pleotropic effects on food intake, anxiety, nociception, inflammation, locomotion, and memory (7, 18).

Even with recent advances, much about the regulation of NAE levels and their contributions to biological processes remains poorly understood. The first step of NAE biosynthesis requires the transfer of the appropriate O-acyl chain from phosphatidylcholine to the ethanolamine headgroup of a phosphatidylethanolamine (PE) to form N-acyl-phosphatidylethanolamines (NAPEs) (19). Five calcium-independent PE N-acyltransferases (PLA1T–5) and one calcium-dependent PE N-acyltransferase (PLA2G4e) that catalyze this transfer have been identified in humans (19–20). The second step of NAE biosynthesis, conversion of NAPE to NAEs, can be directly catalyzed by NAPE phospholipase D, a member of the zinc metallo-β-lactamase family (Fig. 1) (21–23). NAPE-PLD homologs are found in mammals, reptiles, worms, and yeast, suggesting that it has highly conserved functions in normal physiology (12, 21, 24, 25). Surprisingly, global deletion of the Nape-pld in mice from birth causes marked changes in multiple lipid pathways yet only partially reduces NAE levels in most tissues (26–29). Several alternative pathways for the conversion of NAPE to NAE have now been identified (Fig. 1) (24, 30, 31). Despite the identification of these enzymes, how biosynthesis of individual NAEs is controlled remains unclear. For instance, refeeding of

2 The abbreviations used are: NAE, N-acyl-ethanolamide; NAPE, N-acyl-phosphatidylethanolamine; PLD, phospholipase D; LCA, lithocholic acid; PE, phosphatidylethanolamine; HTS, high-throughput screening; NGS, N-octyl-β-D-glucoside; Ihh, inhibitor compound; 1-AT, 1-arachidonoyl-thio
glycerol; DMEM, Dulbecco’s modified Eagle’s medium; Hi-FBS, heat-inac
tivated fetal bovine serum; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe
tyltetrazolium bromide.

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rodents leads to an increase in jejunal C18:1NAE levels but also leads to a decrease in jejunal C20:4NAE levels (32), despite the notion that both NAEs theoretically share the same biosynthetic machinery. Modulating the NAE-biosynthetic enzymes with appropriate tool compounds should help elucidate the contribution of individual enzymes to the biosynthesis of individual NAEs under various physiological and pathophysiological conditions and could lead to the development of effective therapies for a range of clinical conditions including obesity, inflammation, chronic pain, and addiction (33, 34).

There is currently a lack of specific inhibitors or activators to directly assess the contribution of NAPE-PLD to NAE biosynthesis under various conditions. One endogenous bile acid, lithocholic acid (LCA), has been reported to inhibit NAPE-PLD (IC$_{50}$ = 68 μM) (35). Although its bioavailability and relatively low toxicity allow LCA to be administered in vivo, LCA is also a potent ligand for the bile acid receptor Tgr5, with an EC$_{50}$ of 0.53 μM (36), so it cannot be used as a selective inhibitor of NAPE-PLD. More recently, ARN19874 was reported to be a selective NAPE-PLD inhibitor, with an IC$_{50}$ of 34 μM (37). The absorbance, distribution, metabolism, and excretion properties of this compound and whether it has efficacy in vivo have not been reported. We therefore performed high-throughput screening (HTS) for modulators of recombinant mouse Nape-
pld using a small chemical library consisting of a collection of American and European drugs with known absorbance, distribution, metabolism, and excretion characteristics (the Spectrum Collection) and identified 14 Nape-pld inhibitors with IC_{50} < 20 μM. The two most potent compounds were symmetrically substituted dichlorophenes that showed at least 75-fold specificity toward Nape-pld over other lipases tested and effectively inhibited NAPE-PLD in HEK293 cells.

**Results and discussion**

An optimal HTS assay for changes in NAPE-PLD activity requires high reproducibility for replicate samples and sufficient dynamic range to reliably detect activators and inhibitors. Fluorogenic lipid substrates have been previously used successfully in HTS assays for modulators of lipase activity (38, 39). PED-A1 is a quenched fluorogenic NAPE analog that has previously been used to measure phospholipase A1 activity in vitro and in tissue samples (40). To test whether PED-A1 could be used as a substrate for NAPE-PLD, we incubated PED-A1 with hexahistidine-tagged recombinant mouse Nape-pld and measured the development of fluorescence. Incubation of PED-A1 with active Nape-pld resulted in a rapid rise in fluorescence, whereas incubation with Nape-pld that had been inactivated by boiling did not (Fig. S1A). The K_{D} of recombinant Nape-pld for PED-A1 (3.0 ± 0.5 μM) was determined by varying the PED-A1 concentration incubated with 0.1 μM Nape-pld (Fig. S1B). To ensure that the assay would tolerate small variations in buffer composition, we determined the concentration range of N-octyl-β-D-glucoside (NOG, used to stabilize Nape-pld) and DMSO (used as vehicle for PED-A1 and for screening compounds) in which Nape-pld activity did not differ by more than 10% from maximal activity. For NOG, this was 0.2–0.6% (w/v), and for DMSO, this was 1.0–3.7% (w/v) (data not shown). Based on these results, a final concentration of 0.4% NOG (w/v) and 1.6% DMSO (v/v) was used in all subsequent assays. To assess the assay’s ability to reproducibly distinguish between normal and inhibited signals across replicated wells in 384-well plate, 100 μM LCA or vehicle was added in checkerboard fashion to a 384-well plate, and initial rate of fluorescence change (t = 30–100 s) and steady-state fluorescence (t = 419–420 s) was determined (Fig. S2). The Z' factor calculated for LCA (0.45–0.47) demonstrated that the signal windows were sufficiently separated to observe hits in the screen, regardless of plate position.

LCA is currently the only known endogenous inhibitor of NAPE-PLD (35). There are a variety of primary and secondary bile acids besides LCA, and these acids differ from each other in respect to the number, position, and stereochemistry of their hydroxyl groups, as well as their conjugated moieties. To determine whether other bile acids serve as endogenous inhibitors of Nape-pld, we screened a panel of 19 primary or secondary bile acids (50 μM each) (Fig. 2). The LCA conjugates glycolithocholic and tauroliothocholic acid were slightly less potent Nape-pld inhibitors than LCA, which suggests that there is some flexibility in the length of the negatively charged moiety needed for inhibitory interactions with Nape-pld. Taurine conjugates of deoxycholic acid (taoursodeoxycholic acid and taurohyodeoxycholic acid) were nearly as potent as LCA, whereas their nonconjugated forms (deoxycholic acid and ursodeoxycholic acid) had no inhibitory effects. Finally, several muricholic acids (α-, β-, and tauro-α-muricholic acids) were slightly less potent than LCA. These results identify a number of new endogenous inhibitors of Nape-pld that may be relevant to Nape-pld activity in the intestinal tract where bile acid concentrations are high.

We next screened 2,388 biologically active and structurally diverse compounds (the Spectrum Collection compound library) at 10 μM final concentration of each compound. For all compounds, a B score (41) for both initial rate of fluorescence change and steady-state fluorescence value was calculated. Compounds with a B score 3 standard deviations above (activators) or below (inhibitors) the mean (or 3 median absolute deviations from the median) were classified as potential hits after confirmation by manual inspection (Fig. S3). Structures known to potentially interfere with the assay because of fluorescence quenching were eliminated from further study. Hits were then reassayed to confirm the original findings. Although all 14 of the potential inhibitors demonstrated reproducible inhibition in the confirmation assay, none of the 12 potential activator compounds demonstrated reproducible activation.

To determine whether the reduction in fluorescence by any of the 14 potential inhibitor compounds was due to direct quenching of BODIPY fluorescence rather than inhibition of Nape-pld, these compounds were incubated with BODIPY FL C5, the phospholipase A1 cleavage product of PED-A1. For each hit compound, the fluorescence of BODIPY FL C5 after treatment with 10 μM compound for 30 min was compared...
with vehicle treatment. None of the 14 hit compounds altered fluorescence by more than 10% (Fig. S4), indicating they were bona fide inhibitors of Nape-pld. All 14 inhibitor compounds (Inh 1–14) were then purchased from commercial sources to validate the putative library compound and analyzed by MS to confirm identity and purity, and concentration–response curve studies were performed to establish potency. The IC_{50} values of these compounds ranged from 1.6 to 19.1 μM, so that each was potentially more potent in vitro than ARN19874 (Table 1) (37).

The effects of Inh 1 to 14 on cell viability were tested in HEK293 cells. The HEK293 cell line was chosen because it has been extensively used as a model for transfection and inhibition studies. Cytotoxicity of each compound was tested at a concentration five times that of their calculated IC_{50} in Table 1 (5× IC_{50}). At these concentrations, six of the fourteen compounds gave cell viability >70%: Inh 1 (hexachlorophene), Inh 2 (bithionol), Inh 4 (ebeselen), Inh 7 (estradiol valerate), Inh 12 (theaflavin mongallates), and Inh 14 (dioxybenzone) (Fig. 3).

Our finding that estradiol valerate (Inh 7, IC_{50} = 7.8 μM) inhibited Nape-pld activity is of interest both because it shares significant structural homology to bile acids such as LCA and because estradiol valerate is a widely used birth control agent because of its potent estrogen receptor agonist activity. Although this finding suggests the possibility that medicinal chemistry could be used to modify estradiol valerate to improve potency and reduce estrogen receptor activity, it is unclear what further modifications of this compound could be undertaken to this end. Several closely related estradiol analogs, including estradiol, estradiol dicacetate, estradiol cypionate, and estratriol, were tested in the original screen of 2,388 compounds but did not show significant inhibitory activity at 10 μM. Therefore, development of estradiol valerate was not pursued further.

Our two most potent inhibitors, Inh 1 (hexachlorophene) and Inh 2 (bithionol), share a symmetrically substituted dichlorophene structure, with Inh 2 having a thioether linker rather than an alkane linker, as well as lacking the 5/5'-chboro groups of Inh 1. Because Nape-pld acts as a homodimer, the potency of these symmetrical compounds may arise from acting at the dimer interface (e.g. by binding similar residues on both Nape-pld monomers). We used commercially available dichlorophene analogs to carry out limited structure–activity relationship studies. We first tested whether the two hydroxyl groups contributed to inhibition. Inh 15, in which chloro groups replace the hydroxyl groups, had no inhibitory activity (Table 2). Inh 16 (chlorophene), with a hydroxyl and a chloro group on only one of the two aromatic rings had only very weak inhibitory activity. Inh 17 (dichlorophen) and Inh 18 (fenticlor) have a hydroxyl and chloro group on both aromatic rings, and both these compounds were ~10-fold less potent than Inh 1 and Inh 2. Inh 19, in which both hydroxyl groups of Inh 17 are replaced with methoxy groups, gave no measurable inhibitory activity, supporting the need for symmetrical hydroxyl groups. Symmetrical addition of methyl (Inh 20), chloro (Inh 21), or bromo groups (Inh 22) at the 2 and 2' positions to Inh 17 resulted in less, equal, or more potent inhibition of Nape-pld, respectively, than for Inh 17, suggesting that addition of strong electron-accepting groups at this position enhances inhibitory activity. The addition of a trichloromethane group to the carbon bridging the two aromatic rings of Inh 17 (Inh 23) increased potency ~10-fold. Inh 24, in which the 1/1' hydroxy groups of Inh 17 are at the 3/3' positions instead and there is dimethyl substitution of the carbon bridging the two aromatic rings, is ~3-fold less potent than Inh 17. Inh 25 (triclosan) also showed ~3-fold less potency than Inh 17, whereas Inh 26 (triclocarban) showed no inhibitory activity. Together, these results suggest that symmetrical halide substitution of the two phenolic groups is critical for potent inhibition. Although Inh 22 and Inh 23 showed similar potency of inhibition of recombinant Nape-pld as Inh 1 and Inh 2, both of them showed significantly greater cytotoxicity in HEK293 cells (data not shown), so we pursued further mechanistic testing only with Inh 1 and 2.

Inhibition of recombinant Nape-pld with Inh 1 and Inh 2 altered V_{max} but not K_{m}, consistent with a noncompetitive mechanism of inhibition. Apparent K_{i} for both Inh 1 and Inh 2 was ~2 μM (Fig. 4, A and B). Inhibition by Inh 1 was completely lost after 100-fold dilution of the enzyme–inhibitor complex, indicating a noncovalent, highly reversible interaction (Fig. 4C). In contrast, inhibition by Inh 2 persisted even after 100-fold dilution of the complex, indicating tight binding or potentially a covalent interaction. Thus, despite their chemical similarities, these two inhibitors appear to interact with Nape-pld by somewhat different mechanisms.

Selectivity of the two inhibitors for Nape-pld versus other lipases was first examined using *Streptomyces chromofuscus* phospholipase D (ScPld), which is a broad-spectrum phospholipase D that catalyzes the hydrolysis of the headgroup of a variety of lipid substrates including phosphatidylycholine, PE, NAPE, and other N-modified phospholipids (21, 22, 42). As with recombinant Nape-pld, incubation of ScPld with PED-A1 resulted in a rapid increase in fluorescence intensity. Both Inh 1 and Inh 2 inhibited ScPld activity, but only at concentrations ~100-fold higher (IC_{50} values of 149 and >200 μM, respectively) than required to inhibit Nape-pld (Fig. 5A). Potential inhibition of serum lipases was examined using 1-arachidonoylthioylglycerol (1-AT) as a general lipase substrate, with released thioylglycerol quantified by fluorescence resulting from reaction with 7-diethylamino–3-(4'-maleimidylphenyl)–4-methylcoumarin. The IC_{50} values for serum lipase activity were 110 and 46 μM for Inh 1 and Inh 2, respectively (Fig. 5B). Because Nape-pld is a member of the zinc-dependent metalloenzyme family, we determined whether Inh 1 and Inh 2 also inhibited other zinc metalloenzymes using carbonic anhydrase as a model enzyme. p-Nitrophenylacetate was used as substrate to measure carbonic anhydrase activity. The IC_{50} values for carbonic anhydrase activity were 107 and 178 μM for Inh 1 and Inh 2, respectively (Fig. 5C). Thus, both Inh 1 and Inh 2 showed relatively selective inhibition of Nape-pld when compared with their effects on other lipases or zinc-dependent metalloenzymes.

HEK293 endogenously express NAPE-PLD (37, 43) and were therefore used to assess the ability of these inhibitors to inhibit the NAPE-PLD activity of intact cells. To determine the maximum concentration of Inh 1 and Inh 2 that could be used for cellular inhibition studies without cytotoxicity, HEK293 were treated with 0–100 μM inhibitor, and cytotoxicity was mea-
Table 1
Compounds identified from HTS screen of spectrum collection

| Inhibitor # (common name) | Structure | Concentration Response Curve | IC₅₀ (µM) (95% CI) |
|---------------------------|-----------|-----------------------------|-------------------|
| Inh 1 (Hexachlorophene)   | ![Image](image1.png) | ![Graph1](graph1.png) | 1.6 (1.4-1.7) |
| Inh 2 (Bithionol)        | ![Image](image2.png) | ![Graph2](graph2.png) | 2.1 (1.8-2.4) |
| Inh 3 (Mangostin)        | ![Image](image3.png) | ![Graph3](graph3.png) | 2.8 (2.5-3.2) |
| Inh 4 (Ebselen)          | ![Image](image4.png) | ![Graph4](graph4.png) | 2.8 (2.4-3.4) |
| Inh 5 (Tripterine)       | ![Image](image5.png) | ![Graph5](graph5.png) | 3.0 (2.5-3.5) |
| Inh 6 (Gossypol)         | ![Image](image6.png) | ![Graph6](graph6.png) | 6.9 (5.9-8.0) |
| Inh 7 (Estradiol valerate)| ![Image](image7.png) | ![Graph7](graph7.png) | 7.8 (6.2-10.0) |
### Table 1—Continued

| Inhibitor # (common name) | Structure | Concentration Response Curve | IC<sub>50</sub> (µM) (95% CI) |
|--------------------------|-----------|------------------------------|---------------------------------|
| Inh 8 (Cetylpyridinium)  | ![Structure](image1) | ![Graph](image2) | 8.8 (6.8-10.0) |
| Inh 9 (Gambogenic acid)  | ![Structure](image3) | ![Graph](image4) | 9.9 (8.8-11.1) |
| Inh 10 (Chlorhexidine)   | ![Structure](image5) | ![Graph](image6) | 10.4 (9.1-11.9) |
| Inh 11 (Hyamine)         | ![Structure](image7) | ![Graph](image8) | 12.7 (11.4-14.2) |
| Inh 12 (Theaflavin monogallates) | ![Structure](image9) | ![Graph](image10) | 14.3 (11.6-17.7) |
| Inh 13 (Nordihydroguaiaretic acid) | ![Structure](image11) | ![Graph](image12) | 15.1 (11.8-19.3) |
| Inh 14 (Dioxybenzene)    | ![Structure](image13) | ![Graph](image14) | 19.1 (16.6-22.0) |
Novel NAPE-PLD inhibitors

Recombinant mouse NAPE-PLD or boiled nPE-PLD was diluted in assay buffer containing 50 mM Tris-HCl, pH 8.0, and 1% NOG to a final concentration of 1.0 µM and incubated with 10 µM PED-A1. Fluorescence (excitation/emission, 488/530 nm; fixed bandwidth, 16 nm) was measured at 1-min time points for 2 h in 96-well plate reader (Synergy H1 hybrid multimode reader, BioTek).

NAE-PLD activity assay optimization

*K*ₚ for PED-A1 hydrolysis by recombinant mouse NAPE-PLD was determined by enzyme kinetics. Briefly, NAPE-PLD was diluted in assay buffer to a final concentration of 4.5 µg ml⁻¹, and enzyme kinetics was performed in a 384-well plate using 0–20 µM PED-A1. Fluorescence (excitation, 482 ± 18 nm; emission, 536 ± 20 nm) was measured for 15 min using a Wavefront Panoptic kinetic imaging plate reader. The effects of varying concentrations of NOG (0–1%) were measured using final concentration of 4 µM PED-A1 and 4.5 µg ml⁻¹ NAPE-PLD. For the DMSO tolerance test, DMSO (35–900 nL) was transferred onto an empty 384-well plate using an Echo liquid handler. Then 30 µL of 4.5 µg ml⁻¹ enzyme diluted in assay buffer containing 0.4% NOG (w/v) was dispensed into the DMSO-containing plate using a Bravo liquid handler robot, followed by 5 µL of PED-A1 (final concentration, 4 µM), and fluorescence kinetics (excitation/emission, 482/536 nm) were measured for 15 min. The consistency of replicates across the 384-plate was determined using the final optimized concentrations of DMSO (1.6%, v/v), NOG (0.4%, w/v), and 4.5 µg ml⁻¹ NAPE-PLD, with replicates of the NAPE-PLD inhibitor, LCA, and dispersed in checkerboard pattern across the plate (‘Checkerboard Assay’), and then 5 µL of PED-A1 (final concentration, 4 µM) was added. The Z' factor for LCA inhibition across the plate was calculated as \( Z' = 1 - \frac{3(X' + Y')}{(Y' - Y)} \), where \( Y' \) and \( Y \) are the mean values of PED-A1 produced fluorescence in the absence and presence, respectively, of LCA as a control inhibitor at initial rate of reaction. \( X' \) and \( X \) are standard deviations of PED-A1–produced fluorescence.

High-throughput screen

Screening was performed on 2,388 compounds. 35 µL of 10 mM test compounds and 140 µL of 100% DMSO were combined in black-walled, clear-bottomed 384-well plates using an Echo Acoustic liquid handler, resulting in a final compound concentration of 10 µM for the primary screen. 175 µL of 20 mM LCA or...
Table 2
Structure–activity relationships for substituted chlorophene analogs

| Inhibitor # (common name) | Structure | Concentration Response Curve | IC₅₀ (µM) (95% CI) |
|---------------------------|-----------|------------------------------|-------------------|
| Inh 15 (CAS 2888-15-5)   | ![Structure](image1) | ![Response Curve](image2) | No effect observed |
| Inh 16 (Chlorophene)     | ![Structure](image3) | ![Response Curve](image4) | 132.9 (77.2-336.4) |
| Inh 17 (Dichlorophen)    | ![Structure](image5) | ![Response Curve](image6) | 23.2 (15.8-34.2)   |
| Inh 18 (Fenticlor)       | ![Structure](image7) | ![Response Curve](image8) | 26.8 (18.2-41.6)   |
| Inh 19 (CAS 7569-57-5)   | ![Structure](image9) | ![Response Curve](image10) | No effect observed |
| Inh 20 (CAS 57693-35-3)  | ![Structure](image11) | ![Response Curve](image12) | ~294.1 (198.0-512.4) |
| Inh 21 (CAS 1940-43-8)   | ![Structure](image13) | ![Response Curve](image14) | 26.5 (18.3-38.6)   |
175 nl of DMSO were added to control wells as inhibited and uninhibited signal controls, respectively. 30 μl of 4.5 μg ml⁻¹ Nape-pld enzyme in buffer solution with 0.4% NOG (w/v) was dispensed in each well using an Agilent Bravo robotic liquid handler. The plates were incubated at 37 °C for 1 h. The assay was initiated by the Panoptic instrument’s internal Bravo robotic liquid handler when it dispensed 5 μl of PED-A1 (final concentration, 4 μM) in buffer solution (1.6% DMSO final, v/v) to each well. Changes in fluorescence (excitation/emission, 482/536 nm) were measured for 15 min. Initial slope measurements used slope from \( t = 30–100 \) s.

**Concentration-response curve**

The IC₅₀ for each hit compound was determined using similar conditions as for screening except that the concentration of inhibitor was varied from 0 to 200 μM. The averaged initial slope measurements for NAPE-PLD activity in the absence of inhibitor were set as 100% activity, and the normalized percentage of activity was calculated by dividing the initial slope observed with each concentration of inhibitor by this value. Fitting of the response curve and calculation of the IC₅₀ and 95% confidence interval were performed using log(inhibitor) versus normalized response–variable slope analysis in GraphPad Prism version 7.04.

**Rapid dilution assay**

This assay was performed using the methods of Castellani et al. (37). Briefly, 81 μg ml⁻¹ recombinant Nape-pld was pre-incubated with Inh 1 or Inh 2 at 50 μM for 1 h, and then the samples were diluted 100-fold with 50 mM Tris-HCl (pH 8.0) immediately prior to addition of 4 μM PED-A1. Fluorescence was measured at 1-min intervals for 30 min.
Novel NAPE-PLD inhibitors

**Cytotoxicity**

Effect of compounds on viability of HEK293 cells were determined using MTT assay to measure changes in cellular redox state. Briefly, $10 \times 10^6$ HEK293 cells/well were seeded in 24-well plates for 24 h. The cells were treated with a concentration of each compound that represented 500% of the IC$_{50}$ concentration determined in the concentration-response curve assay ($5 \times IC_{50}$) prepared in opti-MEM reduced serum medium containing 1% HI-FBS for 24 h. After treatment, the medium was removed, and 300 µl of 0.5 mg ml$^{-1}$ MTT solution was added. After 3 h, the medium was removed, the purple crystals were dissolved in 100 µl of DMSO, 80 µl of dissolved crystal solution was transferred to 96-well plate, and absorbance was measured at 590 nm. The percentage of viability was normalized to cells treated with vehicle (DMSO) only.

**Cellular NAPE-PLD activity**

HEK293 cells in DMEM buffer containing 4.5 g liter$^{-1}$ glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, and phenol red and supplemented with 10% HI-FBS were seeded at ~20,000 cells/well in tissue culture-treated, clear-bottomed, black-walled, 96-well plates. After reaching ~95% confluency (48 h), the medium was removed and replaced with similar DMEM buffer (70 µl/well) except without phenol red or HI-FBS. 5 µl of inhibitor or vehicle solutions were then added to each well to generate final concentrations of inhibitors of 0.2–20 µM. 30 min after addition of inhibitors, 5 µl of 56 µM PED-A1 was added to each well (final concentration, 3.5 µM), and fluorescence signal (excitation/emission, 488/530 nm) was recorded every minute for 25 min in Synergy H1 plate reader. NAPE-PLD activity was measured as the increase in fluorescence from time = 1–25 min and normalized to average activity found in absence of inhibitor. For each concentration of inhibitor, three replicate wells on two separate days were measured ($n = 6$ wells total), and the means ± S.E. were determined.

**Counter-screens and specificity screens**

All assays were carried out in 96-well plates. To assess effect of compounds on BODIPY fluorescence, 4 µM of BODIPY-FL C5 was incubated with or without 10 µM of each compound in total 80 µl of 50 mM Tris-HCl (pH 8.0), and fluorescence (excitation/emission, 482/536 nm) was measured for 30 min. To assess the effects of compound on *S. chromofuscus* phospholipase D (ScPld) activity, 0.1 µM ScPld was incubated with 0–200 µM of Inh 1 or Inh 2 in 70 µl of 50 mM Tris-HCl (pH 8.0) for 1 h, PED-A1 was added to 4 µM, and fluorescence (excitation/emission, 488/530 nm) was measured by Synergy H1 plate reader for 15 min. For assays assessing the effect of compounds on serum enzymes, deidentified surplus human serum was obtained from the Vanderbilt Clinical Pharmacology Phlebotomy Core and diluted 1:9 (i.e. 10-fold dilution factor) using 50 mM Tris-HCl (pH 8.0) to generate a diluted serum stock solution. Pan-lipase activity was measured using a lipase assay kit (Cayman Chemical, 700640), which uses 1-AT as substrate (final concentration, 23 µM), and the released thiglycerol was detected using thiol detection agent (7-dethylamino-3-(4-formylphenyl)-4-methylcoumarin; final concentration, 50 µM). Recombinant mouse NAPE-pld (4.5 mg liter$^{-1}$) did not hydrolyze 1-AT to any appreciable extent, whereas the bovine milk lipoprotein lipase supplied in the assay kit (diluted according to manufacturer’s recommendation of 10 µl of supplied stock diluted with 140 µl of assay buffer) gave robust 1-AT hydrolysis (Fig. S6A). To determine the $K_m$ for 1-AT hydrolysis activity in diluted serum, 1-AT concentration was varied from 0 to 90 µM. This gave an estimated $K_m$ of 6.6 ± 3.4 µM (Fig. S6B). To measure effect of inhibitors on serum lipase activity, 5 µl of Inh 1, Inh 2, or vehicle (DMSO) (final inhibitor concentration, 0–200 µM) was added to each well containing 60 µl of 50 mM Tris-HCl (pH 8.0), and 10 µl of diluted serum stock solution

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**Figure 4. Mechanism of inhibition by Inh 1 and Inh 2.** A, recombinant mouse Nape-pld was incubated with 0–3 µM Inh 1, then 0–10 µM PED-A1 was added, and the initial velocity of PED-A1 hydrolysis was determined for each (means ± S.E., $n = 3$). B, recombinant Nape-pld was incubated with 0–3 µM Inh 2, then 0–10 µM PED-A1 was added, and the initial velocity of PED-A1 hydrolysis was determined for each (means ± S.E., $n = 3$). C, a rapid dilution assay for Inh 1 and Inh 2. Recombinant Nape-pld was incubated with DMSO (veh) or 50 µM Inh 1 or Inh 2 for 1 h, then samples were diluted 100-fold with buffer immediately prior to addition of 4 µM PED-A1, and the resulting fluorescence was measured as arbitrary fluorescence units (AFU) (means ± S.E., $n = 9$).
and was incubated together for 30 min. 5 μl of a solution containing 1-AT and thiol detection agent (7-dethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin; final concentrations, 23 and 50 μM, respectively) was then added, and the change in fluorescence (excitation/emission, 380/515 nm) was monitored using Synergy H1 plate reader for 30 min. 100% activity was set as the fluorescence detected for vehicle only. Serum carbonic anhydrase activity was measured using a carbonic anhydrase assay kit (Biovision Inc., K472), which uses anhydrase activity was measured using a carbonic anhydrase assay kit (Biovision Inc., K472), which uses

**Statistical analysis**

In all reactions, the IC₅₀ was calculated using log(inhibitor) versus normalized response–variable slope analysis in GraphPad Prism version 7.04.

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