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Chapter 19

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Elliot D. Mock, Wouter P. F. Driever, and Mario van der Stelt

Abstract

N-Acylphosphatidylethanolamine phospholipase D (NAPE-PLD) is regarded as the principal enzyme that generates *N*-acylethanolamines (NAEs), a family of signaling lipids that includes the endocannabinoid anandamide. To investigate the biological function and biosynthesis of NAEs, we sought to develop potent NAPE-PLD inhibitors. To this aim, we utilized a high-throughput screening-compatible NAPE-PLD activity assay, which uses the fluorescence-quenched substrate PED6. This assay conveniently uses membrane fractions of NAPE-PLD overexpressing HEK293T cell lysates, thus avoiding the need for protein purification. Here, we give a detailed description of the NAPE-PLD PED6 fluorescence activity assay, which has increased throughput compared to previous radioactivity- or mass-spectrometry-based assays.

Key words *N*-Acylphosphatidylethanolamine phospholipase D, NAPE-PLD, Fluorescence-based activity assay, PED6, Anandamide, *N*-Acylethanolamines, Inhibitor screening

1 Introduction

The enzyme NAPE-PLD uses two zinc ions in its active site to catalyze the hydrolysis of *N*-acylphosphatidylethanolamine (NAPE) to form NAEs and phosphatidic acid [1, 2]. Thus far, it is the only known human lipase having a metallo-β-lactamase fold [3]. Phosphatidylethanolamine (PE), a major component of cellular membranes, was found to enhance NAPE-PLD activity; therefore, the enzyme is believed to be constitutively active [4]. Other activators include specific bile acids and polyamines [5].

Multiple biosynthetic pathways have been described for NAEs, but NAPE-PLD is regarded to be the primary actor in the brain [6]. Previously, one NAPE-PLD knockout animal model failed to show reductions of brain anandamide, one of the most studied NAEs and an agonist for the CB_1 cannabinoid receptor [7]. It was proposed that compensatory mechanisms are involved that may arise in long-term genetic knockout models. Notably, two other mouse NAPE-PLD knockout models did show a brain anandamide decrease [8, 9]. We hypothesized that acute inhibition of

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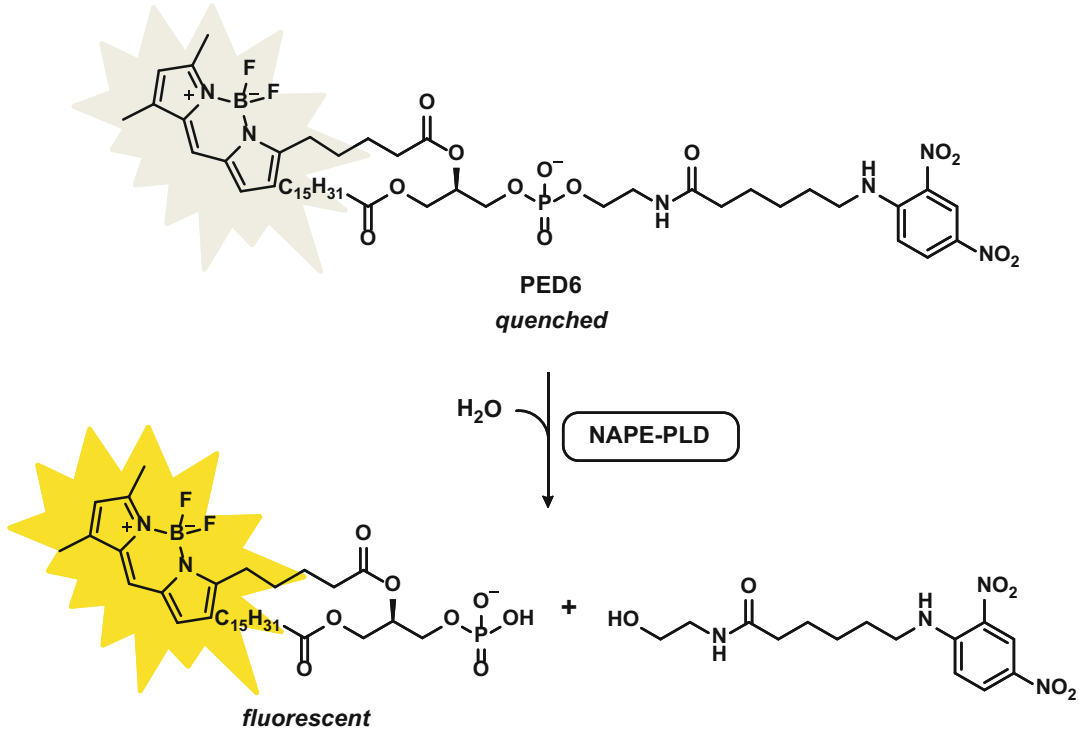

Fig. 1 NAPE-PLD PED6 activity assay principle

brain NAPE-PLD would result in brain anandamide depletion. To test this hypothesis *in vivo*, active and brain-penetrant inhibitors were necessary, which were not available previously. We therefore resorted to high-throughput screening (HTS) for which a compatible NAPE-PLD activity assay was required. Earlier described NAPE-PLD activity assays are based on radioactivity or mass spectrometry-based readouts and are not suitable for HTS campaigns [10, 11].

The fluorescence-quenched substrate PED6 was initially reported as a chemical probe for PLA_2 activity [12]. Its suitability to detect NAPE-PLD activity was first described by workers from Sanofi-Aventis, in which purified NAPE-PLD protein was used [13]. Figure 1 depicts the assay principle, where the hydrolysis of the PED6 phosphodiester bond by NAPE-PLD results in the release of the dinitroaniline quencher and a subsequent increase in fluorescence. We adapted this assay to allow the use of membrane lysate of NAPE-PLD-overexpressing HEK293T cells, thereby avoiding laborious protein purification steps. The resulting assay is robust, inexpensive, and can be run in 96-, 384-, and 1536-multiwell plates, giving increased throughput compared to traditionally performed radioactivity- and mass-spectrometry-based assays. It was successfully used to screen a compound library of

300,000 compounds at the European Lead Factory (ELF), affording new NAPE-PLD inhibitor chemotypes [14]. Here, we describe the protocol for conducting this assay, which is routinely used in our lab in 96-well format.

2 Materials

2.1 Cell Culture

1. HEK293T cells (ATCC)
2. Culture medium: DMEM with 2 mM GlutaMax, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) newborn calf serum
3. Full length human cDNA of human *NAPE-PLD* gene, cloned into mammalian expression vector pcDNA3.1, containing a C-terminal FLAG-tag and genes for ampicillin and neomycin resistance
4. Polyethyleneimine (PEI, 1 mg/mL (w/w))
5. PBS (pH 7.4)

2.2 Lysate Preparation

6. Lysis buffer A: 20 mM HEPES (pH = 7.2), 2 mM DTT, 0.25 M sucrose, 1 mM $MgCl_2$, 2.5 U/mL Benzonase
7. Storage buffer B: 20 mM HEPES (pH = 7.2), 2 mM DTT
8. Heidolph SilentCrusher S homogenizer, or equivalent
9. Beckman Coulter ultracentrifuge with Ti70.1 rotor or equivalent
10. Protein concentration assay: Quick Start Bradford, Qubit, or equivalent

2.3 In Vitro Activity Assay

11. PED6 (*N*-((6-(2,4-Dinitrophenyl)amino)hexanoyl)-2-(4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-*s*-Indacene-3-Pentanoyl)-1-Hexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine, Triethylammonium Salt)
12. Assay buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02% Triton X-100
13. Inhibitor of interest
14. 96-well plate (Greiner, black, flat bottom, 655076)
15. Multichannel pipette
16. CLARIOstar plate reader (or equivalent) with excitation at 477 ± 16 nm and emission at 525 ± 30 nm

3 Methods

3.1 Cell Culture and Transfection

1. HEK293T cells are kept in culture under the following conditions: 37 °C and 7% CO_2 in culture medium. Cells are passaged twice a week to appropriate confluence by thorough pipetting up and down.
2. One day before transfection, plate 10^7 cells in a 15 cm dish with 20 mL culture medium.
3. Refresh the medium 2 h before the transfection (13 mL).
4. Prepare two 15 mL tubes containing:
   A. 20 μg plasmid DNA (encoding NAPE-PLD) in 1 mL DMEM (no serum nor antibiotics).
   B. 60 μg polyethylenimine (PEI, 1:3 DNA/PEI ratio (m/m), 1 mg/mL) in 1 mL DMEM (no serum or antibiotics).
5. Combine tubes A and B and incubate for 15–30 min at room temperature.
6. Add the mixture dropwise to the 15 cm dish with the HEK293T cells and mix by gently swirling the plate. Place the dish back in the incubator.
7. Refresh the medium after 24 h (25 mL).
8. After 72 h, aspirate the media and suspend the cells in PBS (10 mL). Transfer the suspension to a 50 mL tube, rinse the plate with additional PBS (10 mL), and combine in the 50 mL tube.
9. Pellet the cells (10 min at 200 × g, 4 °C) and aspirate the supernatant.
10. Flash freeze the cell pellet and store at −80 °C.

3.2 Membrane Lysate Preparation

11. Prepare two buffers:
    Lysis buffer A: 20 mM HEPES (pH = 7.2), 2 mM DTT, 0.25 M sucrose, 1 mM $MgCl_2$, 2.5 U/mL Benzonase (*see* **Note 1**)
    Storage buffer B: 20 mM HEPES (pH = 7.2), 2 mM DTT
12. Thaw the cell pellet on ice and suspend in ice cold lysis buffer A (2 mL).
13. Transfer the cells to a glass vial and homogenize on ice with a Heidolph SilentCrusher S (5F, 20,000 rpm, 3 × 7 s)
14. Incubate the lysate for 30 min on ice.
15. Transfer the lysate to an ultracentrifuge tube while keeping the suspension ice-cold (*see* **Note 2**).
16. Centrifuge for 30 min at 32,000 rpm, 100,000 g at 4 °C using a Beckman Coulter, Type Ti70.1 rotor (*see* **Note 3**).

17. Collect the supernatant in a separate tube, flash freeze, and store at −80 °C.
18. Suspend the membrane pellet in storage buffer B (1 mL) and transfer to a glass vial. Homogenize on ice with a Heidolph SilentCrusher S (5F, 20,000 rpm, 3 × 7 s).
19. Determine the protein concentration using a Quick Start Bradford (Bio-Rad) or Qubit (Invitrogen) protein assay (*see* **Notes 4** and **5**).
20. Flash freeze the cell lysate and store at −80 °C.

3.3 NAPE-PLD PED6 Activity Assay

21. Prepare the assay buffer containing: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02% Triton X-100 (*see* **Note 6**).
22. Prepare a PED6 10 mM stock in DMSO (*see* **Note 7**).
23. Dilute the membrane protein lysate to 0.4 mg/mL in assay buffer to make a 10× working stock.
24. Make a dilution series of the to be tested compound(s) in DMSO to make 100× working stocks.
25. To a 96-well plate (black, flat bottom), add per well the following: 79 μL assay buffer, 1 μL compound (*see* **Note 8**) or vehicle (DMSO), and lastly 10 μL membrane protein lysate. Always include four control samples of mock-transfected lysate with DMSO and four NAPE-PLD transfected with DMSO (*see* **Note 9**).
26. Incubate for 30 min at 37 °C in the dark.
27. Start up the plate reader 10 min before the end of the incubation time and set at 37 °C.
28. Dilute the PED6 stock 10× in DMSO to make a 1 mM solution. Then dilute further 100× in assay buffer to make a 10 μM solution.
29. Add 10 μL of the PED6 10 μM working stock to each well using a multichannel pipette (*see* **Note 7**). Final concentrations: 0.04 mg/mL membrane protein lysate and PED6 1 μM.
30. Immediately start the experiment measuring every 2 min for 60 min at 37 °C on a CLARIOstar (or equivalent) with excitation at 477 ± 16 nm, emission at 525 ± 30 nm, gain = 1000, 30 flashes per well per timepoint, spiral average.

3.4 Data Analysis

31. Transfer the data to an Excel spreadsheet. Remove the background signal by subtracting the averaged mock-transfected membrane lysate data points from each individual data point.
32. Determine the slope where the fluorescence increase is linear (usually between t = 2–6 min), which is a proxy for the enzyme activity rate in RFU/min.

33. Calculate the Z' using enzyme rates of the wells treated with DMSO for mock transfection and NAPE-PLD transfection, representing negative and positive controls, respectively (*see* **Note 10**).
34. Normalize the average of the DMSO-treated wells with NAPE-PLD-transfected lysate to 100%.
35. Transfer the normalized enzyme rates to GraphPad Prism v.9 (or equivalent) and use the log (inhibitor) vs. normalized response with variable slope setting to calculate the IC_{50}.
36. Calculate the K_i value using the Cheng-Prusoff equation $K_i = IC_{50}/(1 + ([S]/K_M))$, where $K_M = 0.59$ μM for human NAPE-PLD in case of reversible competitive inhibitors (*see* **Note 11**).

4 Notes

1. Benzonase should be added as the last component.
2. When using the ultracentrifuge, utmost care must be taken to ensure the rotor is properly balanced. Ultracentrifuge tubes should differ no more than 10 mg in weight.
3. It is important to make sure to pre-cool the rotor to 4 °C before using the ultracentrifuge.
4. Typically 3–6 mg of membrane protein fraction is obtained from one 15 cm dish, making a 3–6 mg/mL solution.
5. It is recommended to divide the lysate into 120 μL aliquots of membrane lysate at a concentration of 4 mg/mL, which allows the user to perform a single assay in one full 96-well plate.
6. Important: Freshly add Triton X-100 from a 10% in MilliQ (v/v) stock on the day of use to complete the assay buffer. The Triton X-100 stock can be stored at 4 °C.
7. For performing 96-well assays, dividing the PED6 10 mM stock into aliquots of 12 μL 1 mM is recommended.
8. All tested compounds are ideally dissolved in DMSO.
9. Typically, two technical replicates (n = 2) and two plate replicates (N = 2) are sufficient to obtain reliable IC_{50} curves.
10. A $Z' \geq 0.6$ can be considered as a robust assay. When $Z' < 0.6$, an extra replicate experiment is recommended.
11. The Michaelis–Menten constant K_M follows from the kinetics experiment where the substrate PED6 concentration is varied as depicted in Fig. 2.

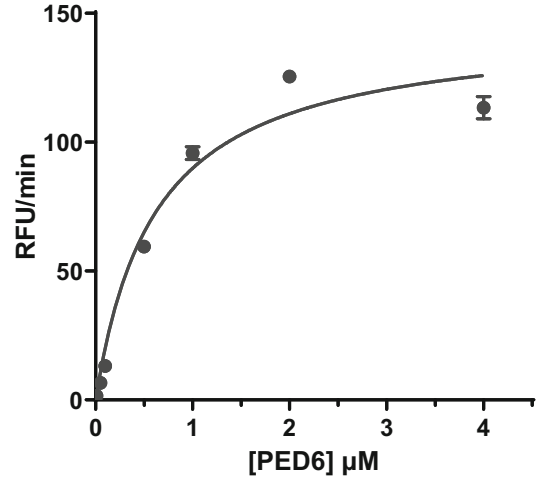

Fig. 2 Michaelis–Menten kinetics for PED6, K_M = 0.59 μM and V_{max} = 145 RFU/min. Data represent mean values ± SD (n = 4)

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