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Systems pharmacology of the endocannabinoid system

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Chapter 7

Systems pharmacology of an endocannabinoid system modulator in zebrafish larva

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Abstract

The zebrafish is increasingly being used as an alternative pre-clinical *in vivo* model in drug research, but the pharmacological aspects of this model still need to be established. Recently we quantified the pharmacokinetics of paracetamol in zebrafish larvae. Here, we used Fatty acid amide hydrolase (FAAH) inhibitor PF-04457845 as a model drug compound to study the dynamic effects of the drug on 5 day post fertilization (dpf) zebrafish larvae. PF-04457845 treatment showed a concentration-dependent increase of endogenous anandamide (AEA) levels, a substrate of FAAH which belongs to the class of N-acylethanolamines (NAEs). The increase in AEA was observed at a dose of 0.1 μ M PF-04457845 added in the swimming medium. Next, the serine hydrolase interaction profile of PF-04457845 was evaluated using MS-based activity-based protein profiling (ABPP). Through chemoproteomics, 55 serine hydrolases were identified in vehicle treated zebrafish larvae. We observed that *in vivo* exposure to PF-04457845 completely inhibited FAAH2 activity without cross-reacting with the other detected serine hydrolases. In addition, the effect of the drug on the lipid network in zebrafish was evaluated. Using LC-MS based lipidomics, 235 lipids from 15 different classes were identified in vehicle treated zebrafish. Upon PF-04457845 treatment, NAE levels were increased, consistent with drug engagement with FAAH. The levels of other lipid species were not altered, demonstrating the absence of off-target effects with regards to lipid metabolism. Overall these results indicate that zebrafish is an attractive pre-clinical vertebrate model to study the pharmacology of drugs. We could prove target engagement of the drug and the absence of off-target effects with regards to a part of the protein landscape and lipid metabolism in zebrafish larvae.

Introduction

Zebrafish (*Danio rerio*) is increasingly used as an alternative pre-clinical vertebrate model during drug discovery and development ^{1,2}. Due to their high genetic homology with higher vertebrates and their advantages of their transparent bodies in early development, their cost-effective usage, the possibilities in genetic manipulation, and their fast development make them as an attractive model in translational science ^{3,4}. Although this model is extensively used in toxicological studies ^{5,6}, the pharmacological aspects of this model needs more attention in order to draw accurate conclusions. Recently, we showed that internal drug exposure is an important parameter to consider when studying drug pharmacology in zebrafish larvae and we characterized the pharmacokinetic processes in zebrafish larvae using paracetamol as a model compound ⁷. Another important parameter is to generate drug concentration-effect relationship (Pharmacodynamics, PD) in zebrafish larvae. So far, PD properties in this model organism was not well established. To improve the translational potential, determining Pharmacokinetics/Pharmacodynamics (PK/PD) relationship in this species can help to understand how drug exposure drives the drug effect and then use that to predict PK/PD in humans to determine optimal dose and clinical outcome. Here, we used an endocannabinoid system modulator as an example drug compound to demonstrate that zebrafish can be an attractive model to study the drug effects.

PF-04457845, a clinically proven irreversible inhibitor for FAAH (Fatty acid amide hydrolase) which has entered phase II clinical trials ⁸, was used as an example drug compound. Pharmacological blockade of FAAH is an attractive strategy to maintain increased endocannabinoid (ECB) tone and to retain the beneficial effects of cannabinoid receptors activation (decrease stress-associated behavioral responses and inflammatory pain), while avoiding the undesirable effects of direct CB1 agonists ^{9,10}. FAAH, a membrane-bound serine hydrolase is primarily responsible for the hydrolysis of anandamide (AEA) to arachidonic acid and ethanolamine. Another form of FAAH, FAAH-2 is also identified in mammals responsible for the hydrolyses AEA but with weak hydrolytic activity and it contribute to the degradation of monounsaturated lipid amides *in vivo* ¹¹.

AEA is one of two well-studied endocannabinoids identified in mammals that activates the cannabinoid receptors CB1 and CB2. 2-AG is another well-studied endocannabinoid and studies showed FAAH can also metabolizes 2-AG ¹². AEA belongs to a family of bioactive N-acylethanolamines (NAEs), which include N-linoleoylethanolamine (LEA), N-palmitoylethanolamine (PEA), N-oleoylethanolamine (OEA) and N-stearoylethanolamine

(SEA), N-docosatetraenylethanolamide (DHEA). FAAH also hydrolyzes PEA, OEA, LEA, SEA and DHEA. The receptors, ligands (endocannabinoids) and the enzymes responsible for synthesis and hydrolysis of the endocannabinoids together constitutes the endocannabinoid system. The endocannabinoid system is used as an promising therapeutic drug target due to its regulatory and functional role in plethora of physiological and pathological conditions^{13,14}.

It was shown in previous studies that the endocannabinoid system, including receptors and enzymes including homologues of FAAH2, are highly conserved between zebrafish and humans, which leverages zebrafish to be used as an attractive pre-clinical model to study endocannabinoid system functionalities^{11,15–17}. Recently, it was shown FAAH2a attenuates locomotor responses and is an important mediator of stress repose in zebrafish larvae¹⁸. Furthermore, genetic functional studies have confirmed the endocannabinoid systems in zebrafish and mammals have similar functions. Several studies have already been performed to study the role of the endocannabinoid system in many pathophysiological conditions using zebrafish as a model organism: functional vision and locomotion¹⁹, learning and memory²⁰, addiction²¹, anxiety²², development²³, lipid metabolism studies²⁴.

Activity based protein profiling (ABPP) is a chemoproteomics method that uses active site-directed chemical probes (e.g., fluorophosphonate (FP), or b-lactones for serine hydrolases) to assess the functional state of entire enzyme classes directly in native biological systems^{25,26}. ABPP is unique in its ability to rapidly identify inhibitor interactions and selectivity within large enzyme families in complex proteome samples either by analysis on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using fluorescent probes or by mass spectrometry (MS)–based analysis for target identification using biotinylated probes. As the enzymes of the endocannabinoid system in zebrafish larvae are well conserved²⁷, ABPP could serve as a powerful profiling technique to investigate the protein interaction landscape of newly developed drugs. It has been recently shown that most of the essential lipid classes in humans are present in zebrafish larvae²⁸, which allows us to study the interaction profile of the drug molecules on the lipid metabolism in zebrafish larvae. Taken together, ABPP and lipidomics are powerful phenotypic tools to assess the target engagement and off-target effects of small molecule drugs developed during early pre-clinical phases. For example, using ABPP and lipidomics, the off-target effect of BIA 10-2474 drug, which failed in phase I clinical trial, was elucidated in iPSC-derived cortical neurons²⁹.

Here, we quantified the pharmacodynamic processes in zebrafish larvae using FAAH inhibitor PF-04457845 as a model drug compound. We also studied the effect of FAAH inhibitor on the zebrafish behavior. In addition, we investigated the on-target engagement effects and off-target effects of PF-04457845 drug on part of protein landscape and lipid networks in zebrafish larvae.

Materials and methods

Zebrafish husbandry

Zebrafish larvae were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Embryos were collected from family crosses of AB/TL wild-type strain and grown at 28°C in embryo medium (60 µg/mL Instant Ocean sea salts; Sera Marin) in the dark. All embryos were raised in an incubator at 28.5°C with a 14/10-hour light/dark cycle until they were 5-day old post fertilization (dpf). At 5-dpf, 20 larvae were collected in a 1.5ml safe-lock Eppendorf tube to quantify endogenous endocannabinoid levels.

Drug solutions

PF-04457845 was purchased from Sigma-Aldrich, the Netherlands. Stocks are prepared in DMSO. For zebrafish experiments, the drug stocks were diluted using egg medium and prepared in way that the end solution has 0.05% of DMSO.

Time-response curve

5dpf zebrafish larvae were treated with drug PF-04457845 at 1µM for 30min, 1h, 2h, 4h, 8h and 24h. At each time, three replicates of 20 larvae each were collected and washed three times with 80/20 (V/V) egg medium/methanol, after which excessive medium was removed. The samples were subsequently frozen in liquid N₂ and extracted endocannabinoids as described below.

Dose in medium to response curve

5dpf larvae were treated with drug PF-04457845 at different concentrations (DMSO, 0.1, 0.5, 1, 5, 10 µM) for 8hrs. At each time, three replicates of 20 larvae each were collected and washed three times with 80/20 (V/V) egg medium/methanol, after which excessive medium was removed. The samples were subsequently frozen in liquid N₂ and extracted endocannabinoids as described below.

Endocannabinoid analysis

Sample extraction

Endocannabinoids and their congeners were quantified using LC-MS/MS analysis. The sample extraction was performed on ice. In brief, 20 zebrafish larvae at 5dpf were transferred to 1.5 mL Eppendorf tubes, spiked with 10 μ L of deuterated labeled internal standard mix for endocannabinoids (N-arachidonoyl ethanolamine (AEA)-d8, N-arachidonoyldopamine (NADA)-d8, N docosahexaenoylethanolamide (DHEA)-d4, 2-arachidonoylglycerol (2-AG)-d8, N-stearoylethanolamine (SEA)-d3, N-palmitoylethanolamine(PEA)-d4, N-linoleoylethanolamine (LEA)-d3 and N oleoylethanolamine (OEA)-d4, followed by the addition of ammonium acetate buffer (100 μ L, 0.1 M, pH 4). After extraction with methyl tert-butyl ether (800 μ L), the tubes were thoroughly sonicated until the sample is homogenous. The samples were mixed for 6 min using a bullet blender at medium speed (Next Advance, Inc., Averill park, NY, USA), followed by a centrifugation step (5000 g, 12 min, 4°C). Then 750 μ L of the upper layer methyl tert-butyl ether was transferred into clean 1.5 mL Eppendorf tubes. Samples were dried in a speed-vac followed by reconstitution in acetonitrile/water (30 μ L, 90/10, v/v). The reconstituted samples were centrifuged (14000 g, 3 min, 4°C) before transferring into LC-MS vials. 5 μ L of sample was injected for LC-MS analysis.

LC-MS/MS analysis

A targeted analysis of 21 endocannabinoids and related NAEs (*N*-acylethanolamines) were measured using an Acquity UPLC I class Binary solvent manager pump (Waters, Milford, USA) in conjugation with AB SCIEX 6500 quadrupole-ion trap (QTRAP) (AB Sciex, Massachusetts, USA). Separation was performed with Acquity HSS T3 column (1.2 x 100 mm, 1.8 μ m) maintained at 45 °C. The aqueous mobile phase A consisted of 2 mM ammonium formate and 10 mM formic acid, and the organic mobile phase B was acetonitrile. The flow rate was set to 0.4 ml/min; initial gradient conditions were 55% B held for 2 min and linearly ramped to 100% B over 6 minutes and held for 2min; after 10 secs, the system returned to initial conditions and held 2 min before next injection. Electrospray ionization-MS was operated in positive mode for measurement of 21 endocannabinoids and NAEs, and a selective Multiple Reaction Mode (sMRM) was used for quantification. Peak area integration was performed with MultiQuant (AB Sciex, Version 3.0.2) data analysis software. The obtained peak areas of targets were corrected by appropriate internal standards. Calculated response

ratios, determined as the peak area ratios of the target analyte to the respective internal standard, were used to obtain absolute concentrations from their respective calibration curves.

The validation parameters and the absolute concentrations for endocannabinoids in 5dpf zebrafish larvae are shown in **Table. S1**.

Activity based protein profiling (ABPP)

Gel based ABPP

Zebrafish larvae (5dpf) were treated with inhibitor PF-0445785 (1 μ M) or vehicle (DMSO) for 8 hours. The treated larvae were pelleted and stored at -80 °C (n=4, ~60 larvae per pellet). Each pellet was resuspended in lysis buffer (250 mM Sucrose, 20 mM Hepes pH 7.2, 2 mM DTT, 1 mM MgCl₂, 10 U/mL Benzonase) and homogenized with a pestle. Clear lysate was obtained after two slow speed centrifugation steps (5 min, 1000 g, 4 °C). The total protein concentration of the clear lysate was determined with a Bradford Assay and the samples were diluted to a final concentration of 1.25 mg/mL in lysis buffer. ABPP was performed by incubating the samples with probes synthesized in-house, MB064 (2 μ M, 20 min, 28.5°C) or FP-TAMRA (500 nM, 20 min, 28.5°C). The reaction was quenched with Laemlli sample buffer (15 min, 50 °C) and 12.5 μ g sample was resolved by SDS-PAGE (10% acrylamide gel, 75 min, 180 V). In-gel fluorescence was measured in the Cy3 and Cy5 channel on a Chemidoc Imaging system (BioRad) and was analysed using Image Lab software (BioRad). Coomassie staining was used as a protein loading control. Probe labelling is expressed as the percentage fluorescence as compared to the fluorescence in the corresponding band of the vehicle treated sample.

Label free proteomics

Zebrafish larvae (5dpf) were treated with inhibitor PF-0445785 (1 μ M, final concentration) or vehicle (DMSO) for 8 hours. The treated larvae were pelleted and stored at -80 °C (n=3, ~60 larvae per pellet). Each pellet was resuspended in lysis buffer (250 mM Sucrose, 20 mM Hepes pH 7.2, 2 mM DTT, 1 mM MgCl₂, 10 U/mL Benzonase) and homogenized with a pestle. Clear lysate was obtained after two slow speed centrifugation steps (5 min, 1000 g, 4 °C). The total protein concentration of the clear lysate was determined with a Bradford Assay and the samples were diluted to a final concentration of 2.0 mg/mL in buffer (20 mM Hepes pH 7.2, 2 mM DTT). ABPP was performed by incubating the samples (150 μ L, 300 μ g protein) with probe cocktail (MB108 and FP-Biotin, 10 μ M each, 30 minutes, 28°C, 300 rpm). Heat-inactivated

samples (1% SDS, 100 °C, 5 min) of vehicle, BIA 10-2474 and PZ04457845 were used as control (n=1, each). Pull-down and label-free proteomics proceeded according to the protocol described by Van Rooden et al.³⁰, with adjustments that beads were washed twice with PBS/SDS and samples were reconstituted in 25 μ L LC-MS solution (10 min, RT).

Lipidomics analysis

Sample extraction

Zebrafish larvae (5dpf) were treated with inhibitor PF-0445785 (1 μ M) or vehicle (DMSO) for 8 hours. Briefly, 20 zebrafish larvae as were used and the endocannabinoid extraction protocol was followed. In addition to the above internal standards, 10 μ L non-endogenous lipid internal standards were added (lysophosphatidylcholine (LPC)17:0, phosphatidylethanolamine (PE)17:0/17:0, phosphatidylcholine (PC)17:0/17:0, sphingomyelin (SM) d18:1/17:0, triglyceride (TG) 17:0/17:0/17:0, ceramide (Cer) d18:1/17:0) and fatty acid (FA)17:0-d33). Each sample was injected on three different lipidomics platforms: endocannabinoids (5 μ L), positive lipids (2 μ L) and negative lipids (8 μ L).

LC-MS/MS analysis

The endocannabinoid analysis was followed as described above. Both lipidomics methods (positive and negative lipids) are adapted and modified from previously published work²⁹. Briefly, these methods are measured on an Acquity UPLC Binary solvent manager pump (Waters) coupled to an Synapt G2 electrospray ionization quadrupole time-of-flight (ESI-Q-TOF, Waters, Wilmslow, United Kingdom) high resolution mass spectrometer using reference mass correction. The chromatographic separation was achieved on an Acquity HSS T3 column (1.2 x 100 mm, 1.8 μ m) maintained at 40°C for both methods. The positive lipid run includes targets from different lipid classes including (lyso)phosphatidylcholines, triglycerides, ceramides. (lyso)phosphatidylethanolamines and sphingomyelins were separated using a flow of 0.4 mL/min over a 16 min gradient. In positive mode, the mobile phase A consisted of 60:40 (v/v) acetonitrile: H₂O with 10 mM ammonium formate, and the mobile phase B consisted of 10:90 (v/v) acetonitrile: isopropanol with 10 mM Ammonium formate.

The negative lipids constitute mainly free fatty acids and (lyso)phosphatidylcholines were separated with a flow of 0.4 mL/min over 15 min gradient. In negative mode, the aqueous mobile phase A consisted of 5:95 (v/v) acetonitrile: H₂O with 10 mM ammonium formate, and

the organic mobile phase B consisted of 99% (v/v) methanol with 10 mM Ammonium formate. The targets in both lipid methods were detected full scan (100-1000 m/z) in their respective ion charge mode.

PF-04457845 quantification

Sample extraction

Zebrafish larvae (5dpf) were treated with inhibitor PF-0445785 at 1 μ M for 8 hours. PF - 04457845 molecule was quantified using LC-MS/MS analysis in blood samples pooled from 20-25 zebrafish larvae at 5dpf, roughly 83 nL. The blood sample was collected from anatomical locations of the larval circulation using a pulled needle (borosilicate glass capillary, original diameter: 0.5 mm. Sutter Instruments) in a micromanipulator, connected to a manual CellTram pump (Eppendorf) under 20x microscopic magnification (Leica). For determination of sample volume, an image was taken of each sample's blood content within the needle. To prevent coagulation, different strategies were tested, including heparin coating of needle and collection tube. The sample extraction was performed on ice. In brief, 200 μ L of acetonitrile was added to the blood samples. The samples were vortexed and then centrifuged at 13000 xg for 10min. 150 μ L of supernatant was evaporated to dryness using speedvac and reconstituted in 10 μ L of acetonitrile.

LC-MS/MS analysis

PF-04457845 was measured using an Acquity UPLC I class Binary solvent manager pump (Waters, Milford, USA) in conjugation with AB SCIEX 6500 quadrupole-ion trap (QTRAP) (AB Sciex, Massachusetts, USA). Separation was performed with Acquity HSS T3 column (1.2 x 100 mm, 1.8 μ m) maintained at 45 °C. The aqueous mobile phase A consisted of 2 mM ammonium formate and 10 mM formic acid, and the organic mobile phase B was acetonitrile. The flow rate was set to 0.5 ml/min; initial gradient conditions were 60% B held for 0.5 min and linearly ramped to 100% B over 2.5 minutes and held for 0.5 min; after 10 sec the system returned to initial conditions and held 1.5 min before next injection. Electrospray ionization-MS was operated in positive mode for measurement of PF-04457845 and a selective Multiple Reaction Mode (sMRM) was used for quantification. The ion masses monitored were Q1/Q3 456/335 (m/z). Peak area integration was performed with MultiQuant (AB Sciex, Version

3.0.2) data analysis software. The limits of detection (LOD) was 1pM and the linear range used was from 1 to 1000 pM.

Behavioral study

Embryos (0 day) were put individually in a 96 well plate (Costar 3599, Corning Inc., NY, USA) with 250 μ L egg water and left until 5 dpf. All analyses were performed between 10:00 and 19:00. After addition of the drug compounds PF-04457845 at 1 μ M and DMSO for controls, the 96 well plate was immediately transferred to the recording apparatus (Daniovision, Noldus, the Netherlands). After starting of the recording, the fish were first allowed to acclimatize for 4 min with the lights on. This was followed by an alternating 4 min dark and 2 h light cycle and continued to record for 8h. These 4 min dark pulses induce anxiety-like behavior, which allows for analyzing potential anxiolytic effects of our compounds. Basal behavior could be derived from the 2h light periods. Recordings were analyzed using EthoVision 12 (Noldus, the Netherlands). Each experiment was performed three times with a different clutch of eggs for each experiment. Data shown are means of all larvae \pm standard error of the mean (SEM).

Results

Pharmacological response of PF-04457845

a. Time-response profiles

We investigated if endogenous endocannabinoid system of zebrafish larvae can be modulated by an exogenous drug molecule. When treated with FAAH inhibitor PF-04457845 at $1\mu\text{M}$ in zebrafish swimming medium, anandamide levels followed an increasing trend with continuous exposure (**Fig. 1a**). Of note, no effect of FAAH inhibitor was observed on 2-AG levels (**Fig. 1b**). Next, we assessed the drug incubation time required to achieve maximum inhibition. Although we observed decrease in FAAH activity at 1hr of drug incubation, after from 5hrs FAAH activity is completely inhibited and reached steady state condition.

b. Dose in the medium to response profiles

Next, we assessed the amount of dose required to achieve maximal inhibition of FAAH activity. Anandamide levels increase started at $0.1\mu\text{M}$ (swimming medium) of PF-04457845 and followed an increasing trend with increase in drug dose (**Fig. 1c**). Of note, no effect of FAAH inhibitor was observed on 2-AG levels even at high concentrations (**Fig. 1d**).

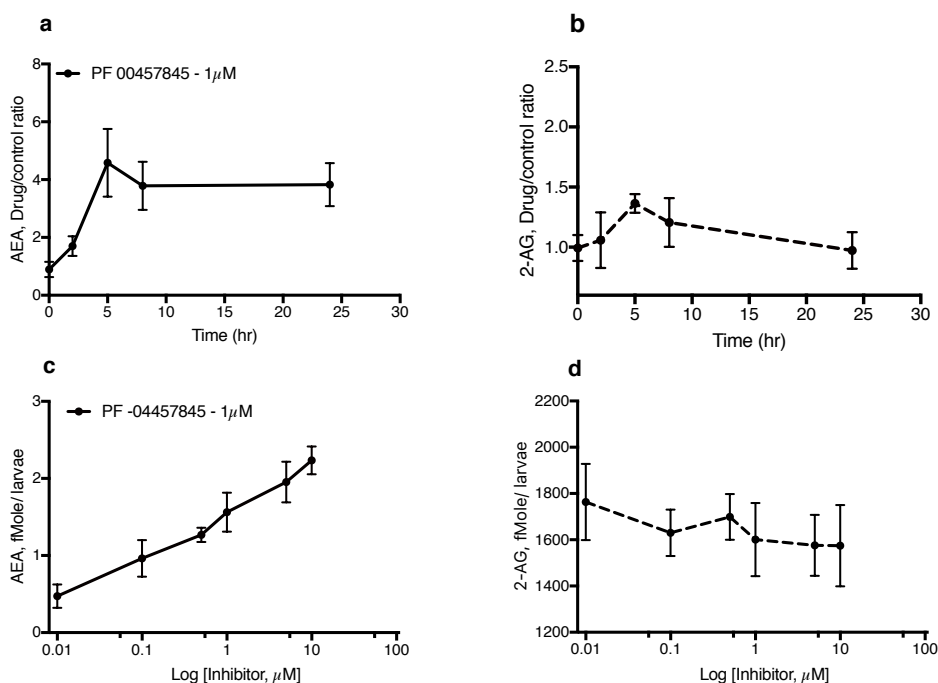


Figure 1: Pharmacological response of PF-04457845. Time-response curve and dose in medium to response curves of PF-04457845 for AEA (solid line **a**, **c**) and 2-AG (dashed line **b**, **d**) levels in zebrafish larvae.

c. Drug concentration in blood samples of zebrafish larvae

Blood samples from drug treated zebrafish larvae were drawn to assess if the drug actually entered the circulatory system of zebrafish larvae. Using LC-MS/MS analysis, PF-04457845 was quantified to be at 0.5 μ M after 8h (n=1) of drug incubation.

Gel and proteomics based ABPP revealed selective FAAH2 inhibition by PF-04457845

To investigate the selectivity of PF-04457845 in zebrafish larvae we performed competitive ABPP using broad spectrum probe TAMRA-FP which targets serine hydrolases and a tailored lipase probe MB064 which targets serine hydrolases part of endocannabinoid system, as well as their biotinylated counterparts FP-Biotin and MB108 respectively. First, we used gel based ABPP to assess the PF-04457845 protein interaction profile in 5 dpf zebrafish larvae. The results revealed a clean profile under *in vivo* conditions (**Fig. 2a-c**).

It is worth mentioning that FAAH was not labelled in gel-based ABPP due to lack of sensitivity in this assay. To identify the serine hydrolases, including FAAH and PF-04457845 targets in these zebrafish larvae, we performed chemoproteomics, an MS-based approach of ABPP. We identified 55 different serine hydrolases (**Fig. 2d**). Here, we confirmed that PF-04457845 at 1 μ M fully inhibited FAAH2. The proteomics data demonstrates the drug is very selective towards FAAH and do not cross-reacts with other serine hydrolases (**Fig. 2a**). Of note, we investigated whether longer incubation (24h) of PF-04457845 effects the zebrafish protein interaction landscape. The results showed a similar protein profiles even with longer incubation times (data not shown).

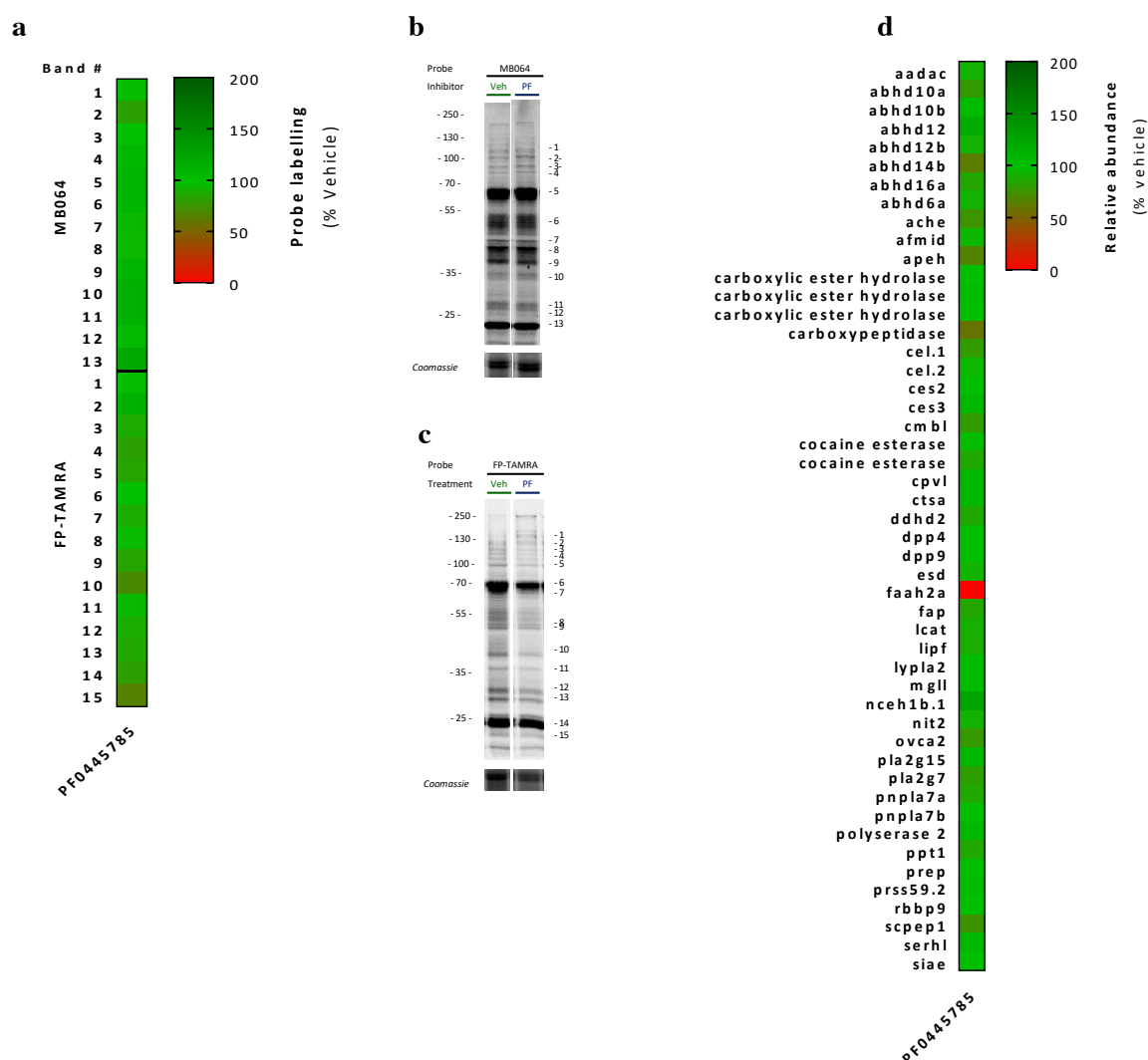


Figure 2: Activity based profiling of serine hydrolase targets of FAAH inhibitors in Zebrafish larvae (5dpf). **a**) Heat-map summary of gel-based ABPP, probe labelling (% vehicle) for MB064 (targeting serine hydrolases part of ECS) and FP-TAMRA (targeting boarder serine hydrolases) (n=4). Color scale indicates inhibition by inhibitor (red = full inhibition, bright green = no inhibition, blank = not detected in all samples, including vehicle). Numbers correspond to those indicated in panel (b-c). **b-c**) Representative probe labelling pattern of treated zebrafish (8h) labelled with MB064 (2 μ M, 20 min, 28.5°C) (c) or FP-TAMRA (500 nM, 20 min, 28.5°C) (d). **d**) Heat-map summary of activity based protein profiling data, showing relative abundance (% vehicle) for MB108 and THL-Biotin (n=3).

PF-04457845 altered FAAH substrate levels in zebrafish larvae

Next, we investigated whether PF-04457845 engages with FAAH and alters its substrates and other lipid network in zebrafish larvae. Initially, a semi-targeted lipidomics was performed to confirm the presence of 235 known lipids falling under 15 different lipid classes in 5dpf

zebrafish larvae (**Fig. 3a**). Zebrafish larvae were exposed to vehicle (DMSO) or PF-04457845 (1 μ M). Drug treatment did not alter the lipid species, except NAE's, the FAAH substrates (**Fig. 3b**). Of note, we investigated if longer incubation (24h) alters the zebrafish lipid metabolism. The results showed similar profiles even with longer incubation times (data not shown).

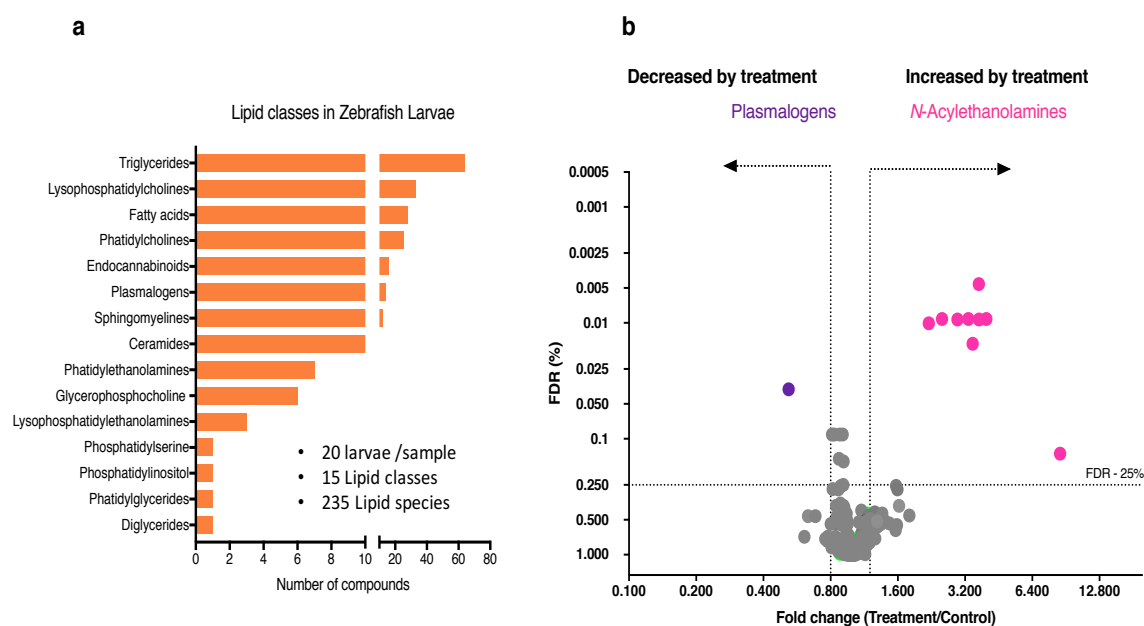


Figure 3: LC-MS/MS based lipidomics of FAAH inhibitor PF-04457845 in zebrafish larvae. **a)** Total lipid species detected in zebrafish larvae. **b)** Zebrafish larvae treated with DMSO or PF-04457845 (1 μ M) for 8 hours. The x-axis shows the fold change of lipid species in the inhibitor-treated versus DMSO-treated cells. Lipidomic data are presented as a volcano plot, and lipids with a fold-change threshold of ≥ 1.20 or ≤ 0.80 and a Benjamini-Hochberg false-discovery rate (FDR) $\leq 25\%$ are represented by colored circles indicating lipid class.

Behavioral assay

It was shown in a previous study that the CB1 receptor agonist has an effect on zebrafish behavior³¹. So, we investigated if FAAH inhibitor PF-04457845 at 1 μ M incubated for 8h, has an effect on zebrafish behavior. The activity of each larva was automatically recorded and analyzed in the ZebraBox recording apparatus equipped with VideoTrack software (both from Viewpoint S.A.). The experimental recording consists of two steps. First, larvae were acclimated to the behavioral setup with lights on for 4 min. This period was necessary and sufficient to ensure a low and stable behavioral activity. Once basal levels of the locomotor activity were stabilized following the acclimatizing period, the basal swimming activity was

recorded during 2 h with lights on. This period is referred to as the “basal phase.” Immediately following the basal phase recording, the lights were suddenly turned off for 4 min. The behavioral activity in the dark was also automatically recorded during this period. This period is referred to as the “dark challenge phase.”

We observed no change in the zebrafish behavior (**Fig. 4**). The variation of behavior in the first and second light (L) and dark (D) phase is due to the adaption of the zebrafish. Additionally, there is a decreasing trend observed in the distance moved in the light cycle (L), which is due to the circadian rhythm in zebrafish.

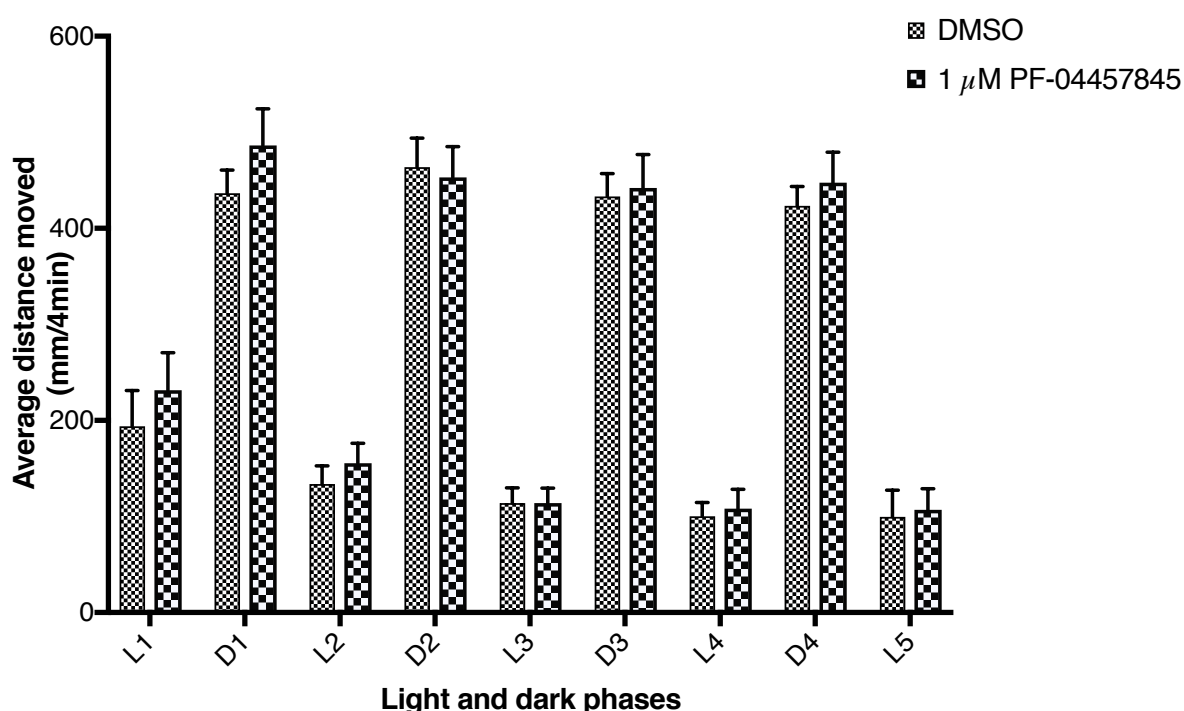


Figure 4: Behavioral study of zebrafish larvae treated with FAAH inhibitor PF-04457845 (1 μM) or DMSO and recorded for 8 hours. The x-axis represents the zebrafish behavior recorded in light and dark phases. After the fish were first allowed to acclimatize for 4 min with the lights on. This was followed by an alternating 4 min dark and 2 hours light cycle. The y-axis shows the average distance moved per 4 mins.

Discussion

The inter-species translation potential of the zebrafish model to study the drug effects during discovery and early drug development phases is gaining more interest. Recently, we have successfully quantified the PK processes in zebrafish larvae and showed that they can be translated to humans⁷. However, the pharmacodynamics aspects in zebrafish remain to be elucidated. In the present study, we aimed to quantify the pharmacodynamic (PD) processes in zebrafish larvae, which further strengthens the translational potential of this model during early drug development studies. In that context, we sought to expand the applicability of using zebrafish model to investigate the on-target engagement of newly synthesized drug molecules, including the drug effect on zebrafish behavior.

We assessed the effect of the exogenous FAAH inhibitor PF-04457845 on the endogenous endocannabinoid system in zebrafish larvae. The pharmacological characterization of cannabinoid-binding sites in zebrafish brain showed that the zebrafish cannabinoid system might not significantly differ from the cannabinoid system in higher vertebrates³². Our UPLC-MS/MS based endocannabinoid analysis in zebrafish larvae confirmed that PF-04457845 inhibited FAAH activity. PF-04457845 treatment showed a concentration-dependent increase of the endogenous anandamide levels similar to what has been observed in higher vertebrates^{8,33}. Furthermore, internal exposure of PF-04457845 was assessed by taking a blood sample of the zebrafish larvae and quantified using LC-MS/MS analysis.

As it has been recognized that the drug concentrations rather than the drug doses are driving drug effects in any pharmacological systems, quantification of full pharmacokinetic processes of the FAAH inhibitor PF-04457845 will be invaluable for drawing accurate conclusions. Although we have developed a methodology to quantify the pharmacokinetics studies in 3dpf zebrafish larvae⁷, we cannot apply this method to the current drug due to its lipophilic nature, hence strongly binding to the skin of the larvae and cannot be removed with washing (data not shown).

We next investigated the target engagement of the FAAH inhibitor PF-04457845 on the protein landscape of zebrafish larvae. To the best of our knowledge, for the first time, we identified 55 different serine hydrolase proteins in the zebrafish larvae using MS-based proteomics, depicting the power of this technique. Gel-based and MS-based ABPP revealed that the drug engages with FAAH2. Furthermore, lipidomics data demonstrated that the drug changes with FAAH activity as the levels of FAAH substrates *N*-acyl ethanolamines increased

with drug treatment. It should be noted that the PF-04457845 treatment did not cross-react with other serine hydrolases or did not alter lipid network. The results depict only the engagement of PF-04457845 towards the target and showed no interaction with other serine hydrolases measured in the current assay²⁹. Our drug target engagement of PF-04457845 results are in line with the studies performed in mice and humans where they showed target engagement with FAAH activity, depicting the translational potential of zebrafish larvae³³. It is worth mentioning that FAAH2 is obscured to some extent as rodent models have one gene encoding FAAH while many non-rodent vertebrates, including humans and zebrafish, have FAAH¹¹, which gives advantage of using zebrafish model. Previous reports investigated the role of FAAH2 in modulating anxiety in humans³⁴ stimulating the use of zebrafish model to study the role of FAAH2 in anxiety compared to rodent models which lack this enzyme.

Furthermore, we investigated the impact of FAAH inhibitor PF-04457845 on zebrafish behavior. It was previously observed that cannabinoids have behavioral effects in zebrafish larvae that are comparable to mammals. The authors reported that zebrafish larvae when treated CB1 agonist (D9-THC or WIN 55,212-2) show a biphasic response (increasing hyperactivity succeeded by suppression of activity with higher doses) while CB1 antagonist (AM2451) attenuates the increased locomotor activity induced by CB1 agonist³¹. We hypothesized that FAAH inhibition leads to increase in the levels of AEA, an endogenous agonist for CB1 receptor, which can potentially effect zebrafish behavior. Unfortunately, FAAH inhibitor did not affect zebrafish behavior at our experimental conditions. A possible explanation for not observing behavior effect could be that the anandamide level increase upon FAAH inhibitor treatment is not sufficient to cause a behavior effect.

The strength of this study is that we have investigated different pharmacological aspects in zebrafish larvae which allows us to reliably employ this model during drug discovery and early drug development phases. Taking into account the advantages of using zebrafish larvae⁶, this model fits in the well-established pre-clinical animal model testing applied in drug research³⁵. In modern drug discovery and development, there is an increase in the high attrition rate due to lack of proper balance between drug efficacy and potential adverse effects and only few compounds make the transition to clinical trial drug candidates³⁶. Furthermore, during clinical trials, some drugs fail due to undesirable off-target activity which cannot be easily screened out from the mode of action of the target. Therefore, we need proper *in vivo* models to prevent drugs with undesirable effects before entering or during clinical trials. Here we used zebrafish as an appropriate model to study the pharmacological properties, like PD to assess

the systemic drug effects. Additionally, we showed ABPP and lipidomics as powerful profiling platforms to investigate the target engagement of the drug developed in early pre-clinical stages.

A limitation of this study is that with the current experimental conditions, we cannot study the localized drug effect, for example in specific tissues, as it will be difficult to isolate tissue samples from a large number of larvae and to get sufficient signal with the current profiling platforms. This could be overcome by developing miniaturized analytical platforms to detect the low concentrations of drugs using limited number biological matrix. Secondly, the pharmacokinetic processes could not be quantified for the lyophilic drugs due to their sticking to the skin of the larvae. However, this problem could be addressed either by orally dosing the zebrafish larvae using nano-particles³⁷ rather than adding the drug in the swimming medium or by sampling blood samples from zebrafish larvae, which we measured for a single point in this study but require further optimization to make it practically feasible for collecting samples at more time points.

In summary, these results indicate that zebrafish is an attractive pre-clinical vertebrate model for pharmacology studies of drugs. We could prove target engagement of the drug, and the absence of off-target effects with regards to a part of the protein landscape and lipid metabolism in zebrafish larvae. Overall, this study expanded the applicability of zebrafish larvae as an attractive pre-clinical model in drug discovery and early drug development, also demonstrating the inter-species translational potential.

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Supplementary Table 1: Target list and validation parameters

S. No	ID	Metabolite Name	PE (%)	R ²	Precision (%RSD)	Mean \pm SEM fM/larvae
1	2&1AG (20:4)	Arachidonoylglycerol	78	0.998	5	2269.6 \pm 59.3
2	AEA (20:4)	Anandamide	85	0.999	12	0.5 \pm 0.02
3	DHEA (22:6)	<i>N</i> -Docosahexaenoyl ethanolamide	49	0.999	4	42.4 \pm 1.6
4	LEA (18:2)	<i>N</i> -Linoleoylethanolamide	79	0.999	3	0.38 \pm 0.11
5	OEA (18:1)	<i>N</i> -Oleoylethanolamide	84	0.999	6	1.2 \pm 0.3
6	PEA (16:0)	<i>N</i> -Palmitoylethanolamide	87	0.999	3	21.6 \pm 0.91
7	SEA (18:0)	<i>N</i> -Stearoylethanolamide	91	0.999	2	11.7 \pm 0.60
9	DEA (22:4)	<i>N</i> -Docosatetraenoyl ethanolamide	48	0.998	19	0.4 \pm 0.02
10	DGLEA (18:3)	Dihomo- γ -Linolenoyl Ethanolamide	65	0.999	35	0.1 \pm 0.01
11	2-LG (18:2)	2-Linoleoyl Glycerol	63	0.999	8	6153.6 \pm 493.7
12	1-LG (18:2)	1-Linoleoyl Glycerol	74	0.981	7	773.4 \pm 65.8
13	2-OG (18:1)	2-Oleoyl Glycerol	73	0.999	3	17999.4 \pm 1740.0
14	1-OG (18:1)	1-Oleoyl Glycerol	N/A	0.986	3	2563.7 \pm 191.45
15	EPEA (20:5)	<i>N</i> -Eicosapentaenoyl ethanolamide	65	0.999	5	7.3 \pm 0.44
16	POEA (16:1)	<i>N</i> -Palmitoleoylethanolamide	99	0.999	4	0.8 \pm 0.04
17	PDEA (15:0)	<i>N</i> -Pentadecanoylethanolamide	82	0.999	7	0.2 \pm 0.01
18	AA (20:4)	Arachidonic Acid	122	0.998	7	80167.4 \pm 5529.8

The target list includes endocannabinoids and *N*-acylethanolamines (NAEs). The compound ID is the abbreviation of metabolite name. R²= correlation coefficient. The method is validated in terms of linearity, sensitivity (LOQ), process efficiency, PE (matrix effect with recovery) and precision (RSD: Relative standard deviation). Endogenous concentrations are denoted as mean \pm SEM