Chemical Proteomics Reveals Off-Targets of the Anandamide Reuptake Inhibitor WOBE437

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ABSTRACT: Anandamide or N-arachidonoylethanolamine (AEA) is a signaling lipid that modulates neurotransmitter release via activation of the type 1 cannabinoid receptor (CB1R) in the brain. Termination of anandamide signaling is thought to be mediated via a facilitated cellular reuptake process that utilizes a purported transporter protein. Recently, WOBE437 has been reported as a novel, natural product-based inhibitor of AEA reuptake that is active in cellular and in vivo models. To profile its target interaction landscape, we synthesized pac-WOBE, a photoactivatable probe derivative of WOBE437, and performed chemical proteomics in mouse neuroblastoma Neuro-2a cells. Surprisingly WOBE437, unlike the widely used selective inhibitor of AEA uptake OMDM-1, was found to increase AEA uptake in Neuro-2a cells. In line with this, WOBE437 reduced the cellular levels of AEA and related N-acyl ethanolamines (NAEs). Using pac-WOBE, we identified saccharopine dehydrogenase-like oxidoreductase (SCCPDH), vesicle amine transport 1 (VAT1), and ferrochelatase (FECH) as WOBE437-interacting proteins in Neuro-2a cells. Further genetic studies indicated that SCCPDH and VAT1 were not responsible for the WOBE437-induced reduction in NAE levels. Regardless of the precise mechanism of action of WOB437 in AEA transport, we have identified SCCPDH, VAT1, and FECH as unprecedented off-targets of this molecule which should be taken into account when interpreting its cellular and in vivo effects.

INTRODUCTION

Anandamide (AEA) is a lipid signaling molecule that belongs to the endocannabinoid system (ECS). It modulates neurotransmitter release via activation of the type 1 cannabinoid receptor (CB1R).1 AEA is produced by hydrolysis of phospholipids, mainly by N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), after which it is released to activate CB1R. AEA-induced CB1R signaling is terminated by a two-step process, that is, cellular uptake followed by hydrolysis of the amide bond by fatty acid amide hydrolase (FAAH). The ECS is responsible for the regulation of a large number of pathophysiological processes, including energy balance, pain, inflammation, and neurotransmission.2 Consequently, modulation of ECS signaling may have therapeutic benefits for a number of diseases, including neurodegenerative,3,4 inflammatory,5 and cardiovascular diseases,6−8 pain,9,10 psychiatric disorders,11,12 and others.13 Activation of CB1R signaling has been achieved by direct and indirect methods, that is, through the application of CB1R agonists and through altering endocannabinoid metabolism, respectively.13 The latter strategy may lead to fewer side effects that are usually associated with direct CB1R activation.14

Elevation of AEA levels can be achieved by inhibiting its reuptake across the plasma membrane or by inhibiting its hydrolysis through FAAH. While inhibition of FAAH is well-characterized and selective inhibitors are currently tested in phase 2 clinical trials, the mechanism of AEA reuptake remains unclear. Small lipophilic molecules may diffuse freely through the lipid bilayer, but AEA reuptake can be saturated.15 This indicates that a protein facilitator for transport across the membrane may exist. Numerous candidates for a purported endocannabinoid membrane transporter and their inhibitors (AM404,16 VDM11,17 UCM707,18 and OMDM-1/219) have been reported, but the existence of such a transporter remains as yet a subject of intense scientific debate.20−25 One of the difficulties to solve the issue is the technical challenge of reliably measuring AEA uptake in short timeframes.24 Moreover, FAAH inhibition results in accumulation of intracellular...
Figure 1. Structures of WOBE437 and its probe derivatives.

WOBE437 (1)
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Gertsch et al.
RX-055 (2)

pac-WOBE (3)

Figure 2. Synthesis and characterization of WOBE437. (A) Reagents and conditions: (a) ethyl 2-(diethoxyphosphoryl)acetate, NaH, 0 °C, then (E)-dec-2-enal, −78 °C to rt, 63%; (b) NaOH, 60 °C, quant.; (c) 2-(3,4-dimethoxyphenyl)ethan-1-amine, HOAt, EDC, rt, 78%. (B) Endocannabinoid uptake was assayed in Neuro-2a cells, which were preincubated with OMDM-1 (40 μM) as a positive control or different concentrations of WOBE437 for 10 min. [3H]-AEA was added, and cells were incubated for an additional 15 min, washed, and harvested to measure radioactivity. Control experiments were also carried out under the same conditions at 4 °C in order to subtract passive diffusion from active uptake. Data are expressed as means ± SEM of three independent experiments, each performed in triplicate. *p < 0.05; **p < 0.01 in comparison to vehicle-treated control (dotted line) using one-way ANOVA with Dunnett’s multiple comparisons correction.
ethyl 2-(diethoxyphosphoryl)acetate resulted in ester 5, which was saponified to afford carboxylic acid 6. A subsequent peptide coupling with 2-(3,4-dimethoxyphenyl)ethan-1-amine gave WOBE437 in 49% yield over three steps. The compound was characterized in a [3H]-AEA uptake assay in Neuro-2a cells according to a previously published method.39 In brief, Neuro-2a cells were treated with vehicle, WOBE437, or OMDM-1 as a positive control for 10 min in serum-free medium, after which AEA (400 nM) spiked with [3H]AEA was added. After 15 min, the cells were thoroughly washed and resuspended in aq. NaOH for measurement in a scintillation counter. Passive uptake at 4 °C was subtracted, and uptake of OMDM-1-treated cells was set as the baseline. In contrast to previous findings,31 WOBE437 resulted in a concentration-dependent increase in the uptake of anandamide when compared to the positive control OMDM-1 (Figure 2B).

To investigate the cellular effects of WOBE437 in more detail, N-acylethanolamines (NAEs), free fatty acids, and monoacylglycerols were measured using a LC-MS-based assay (Table S2). Neuro-2a cells were incubated for different time periods with either WOBE437 or vehicle and washed, and lipids were extracted. Compared to vehicle-treated Neuro-2a cells, WOBE437 induced a time-dependent decrease in all NAE levels, except stearoylethanolamide (SEA), pentadecanoyl ethanolamide (PDEA), and docosahexaenoyl ethanolamide (DHEA) (Figures 3, S1). The largest decrease was observed after 30 min. No effect on free fatty acids or monoacylglycerols was found (Figure S2). Previously, the inhibition of AEA uptake by WOBE437 was shown to be dependent on the passage number of Neuro-2a cells,31 but no effect of passage number was found in the current study (Figure S2A,B).

The decrease in NAE levels is consistent with increased AEA uptake as the transport is driven by the concentration gradient across the plasma membrane.21 To investigate whether the reduction of NAEs was due to inhibition of NAPE-PLD, WOBE437 was tested in a surrogate substrate-based fluorescence assay using purified enzyme (Figure S3A). WOBE437 did not inhibit NAPE-PLD activity nor any other serine hydrolase, as indicated by activity-based protein profiling (Figure S3B).40

**Synthesis and Characterization of pac-WOBE (3).** To profile the protein interaction landscape of WOBE437, a photoaffinity probe (3) was designed, guided by the reported structure–activity relationship.31 A minimalist diazirine and alkyne-containing moiety41 was introduced on the phenyl ring of WOBE437 by peptide coupling 5-(2-aminoethyl)-2-methoxyphenol with 6 after which an SN2 substitution on 3-...
(but-3-yn-1-yl)-3-(2-iodoethyl)-3H-diazirine afforded pac-WOBE (3) in 12% yield over two steps (Figure 4A).

To visualize the protein targets of WOBE437 by gel-based AfBPP, Neuro-2a cells were incubated with 3 and irradiated with UV light (350 nm, 10 min, “UV”) or exposed to ambient light (“no UV”). The cells were harvested and lysed, and the probe-bound proteins were conjugated to Cy5-N3 under CuAAC conditions. The protein samples were resolved by SDS-PAGE and visualized by in-gel fluorescence scanning (Figure 4B). This showed that 3 could UV-dependently label several proteins. Pretreatment of the cells with WOBE437 resulted in a reduced labeling intensity of two bands around 50 kDa, which suggested that these proteins specifically interact with WOBE437.

Identification and Characterization of WOBE437 Targets. Next, a label-free chemical proteomics experiment was performed to identify the WOBE437-interacting proteins. Neuro-2a cells were pretreated with WOBE437 (10 μM) or vehicle, after which they were incubated with 0.1 or 1.0 μM pac-WOBE (3) with or without UV exposure. Cells were lysed and treated with biotin-N3 under CuAAC conditions. Probe-bound proteins were enriched using avidin-coated agarose beads, digested by trypsin, and analyzed by LC−MS/MS. Proteins displaying >2-fold UV enrichment with a p-value <0.05 were designated as pac-WOBE-interacting targets. This afforded 8 and 39 significantly UV-enriched targets for the two probe concentrations, respectively (Figures 5A, S4A), of which none could be outcompeted by AEA reuptake inhibitor VDM11 or FAAH inhibitor URB597. Three of these probe targets [saccharopine dehydrogenase-like oxidoreductase (SCCPDH), vesicle amine transport 1 (VAT1) and ferrochelatase (FECH)] could be outcompeted by preincubation with WOBE437 (Figure 5B, S4A).

Mouse and human orthologues of these three targets were recombinantly expressed in HEK-293-T cells, and target engagement with AEA was investigated using gel-based AfBPP (Figure 5C). AEA engaged in a dose-dependent manner with SCCPDH and VAT1 (Figure S5A,B). AEA did not compete with pac-WOBE (3) labeling of FECH, a mitochondrial enzyme extensively studied for its role in heme biosynthesis, and a common off-target of kinase inhibitors43 and lipid probes.44 In view of these results, further experiments were conducted with mouse SCCPDH and mouse VAT1. The latter has previously been shown to be involved in lipid binding and transport.45,46 To investigate whether SCCPDH and VAT1 were selective for WOBE437 and AEA, competitive AfBPP was performed with a selection of other closely related lipids, such as 2-arachidonoylglycerol (2-AG), N-palmitoylethanolamine (PEA), arachidonic acid (AA), and the FAAH inhibitor URB597 (Figure 5D−G). WOBE437 was the most potent competitor of mSCCPDH labeling followed by AEA > URB597 > AA > 2-AG > PEA. Mouse VAT1 was much more selective since its labeling was only significantly inhibited by WOBE437, AEA, and PEA (Figure 5D−G).

Next, a genetic approach was used to investigate whether SCCPDH or VAT1 is responsible for WOBE437-induced decrease in NAE levels in Neuro-2a cells since no selective SCCPDH or VAT1 inhibitors are available. Notably, single cell heterogeneity prevented the unequivocal analysis of single cell clone knockouts.47 Therefore, disruption of SCCPDH and VAT1 genes was achieved by three consecutive rounds of transfection of Cas9 and single-guide RNAs in Neuro-2a cell
populations. SCCPDH and VAT1 expressions in these cell populations were significantly, albeit not completely, decreased as determined by gel-based AfBPP and Western blot for VAT1 (Figure 6A,C). The residual expressions of SCCPDH and VAT1 can be explained by an imperfect transfection efficiency and by insertion or deletion of a full codon upon Cas9-mediated DNA modification, preventing the frameshift that generally results in an early STOP-codon. Next, the cellular NAE levels of these genetically modified Neuro-2a populations were determined using LC−MS and serine hydrolase activity by ABPP. No change in NAE levels or serine hydrolase activity was observed for these knockdown populations compared to wild-type (WT) cells (Figures 6B,D and S6,S7). Notably, WOBE437 was still able to significantly reduce NAE levels in these genetically modified cells (Figure 6E). This indicated that targets other than SCCPDH or VAT1 are responsible for the WOBE437-mediated reduction in NAE levels.

**CONCLUSIONS**

WOBE437 has been reported as an AEA-uptake inhibitor that shows various in vivo effects that are consistent with direct CB1R activation by elevated extracellular AEA levels. However, the molecular target of WOBE437 remains unknown. In this study, a photoaffinity-based approach was employed to identify the protein targets of WOBE437 in Neuro-2a cells. Surprisingly, WOBE437 increased AEA uptake and decreased endogenous NAE levels. Although the WOBE437-induced time-dependent decrease in endogenous AEA levels appears in keeping with the increased AEA uptake in Neuro-2a cells, it is unclear why the current results are in contrast to the previous findings. Differences in the experimental protocol of AEA-uptake experiments and/or heterogeneity of Neuro-2a cells may be contributing factors as washing steps, addition of lipid carrier BSA, or including control experiments at reduced temperatures have previously been shown to affect results of such assays. However, it should be noted that the positive control OMDM-1 did reduce AEA uptake in Neuro-2a cells under the same conditions. At the very least, our findings suggest that OMDM-1 and WOBE437 have different molecular modes of action, although both compounds are reported to act primarily as AEA uptake inhibitors.

AfBPP using a WOBE437-based photoaffinity probe identified SCCPDH, VAT1, and FECH as WOBE437-interacting proteins in Neuro-2a cells. Competitive gel-based...
were transfected by addition of the PEI/DNA mixture to the cells. When compared to WT, cells treated with vehicle. Data represent means ± SEM (n = 4). One-way ANOVA with Dunnett’s multiple comparisons correction: not significant when compared to WT.

Figure 6. Partial SCCPDH and VAT1 knockouts were generated by CRISPR-Cas9. (A) SCCPDH and (C) VAT1 KO Neuro-2a lines were generated and checked by gel-based AfBPP using 0.1 μM pac-WOBE (3) for residual expression. VAT1 protein was tested by VAT1 Western blot. Coomassie served as a protein loading control. Lipid levels were tested by lipidomics on (B) SCCPDH KO and (D) VAT1 KO cells and compared to WT cells. Further characterization and the complete list of ratios are depicted in Figures S6 and S7. (E) Neuro-2a cells were treated with 10 μM WOBE437 or vehicle for 30 min and harvested to be analyzed by MS-based lipidomics. Lipid levels are displayed as ratio against the same type of cells treated with vehicle. Data represent means ± SEM (n = 4). One-way ANOVA with Dunnett’s multiple comparisons correction: not significant when compared to WT.

Materials and Methods

Cell Culture. General Cell Culture. Neuro-2a and HEK-293-T cells were maintained at 37 °C under 7% CO2 atmosphere in DMEM (Sigma-Aldrich, HEK-293-T: D6546, Neuro-2a: D1145) containing stable glutamine, 10% (v/v) Newborn Calf Serum (Thermo Fisher), and penicillin and streptomycin (200 μg/mL of each, Duchefa). Cells were passaged two times per week at 80–90% confluence by resuspending them in fresh medium. Cell lines were purchased from ATCC and were tested regularly for mycoplasma contamination. Cultures were maintained for 2–3 months before being disposed. SCCPDH/VAT1/FECH-overexpressing Neuro-2a or HEK-293-T cells were produced by seeding resuspended cells on 12-well plates (4.0 × 104 cells/cm2) 24 h prior to transfection. The culture medium was then aspirated and replaced with 400 μL of fresh medium. A mixture of polyethylenimine [PEI, Neuro-2a: 5:1 (m/m), HEK-293-T: 3:1 (m/m)] and plasmid DNA (0.625 μg/well) was diluted in serum-free medium (100 μL) and incubated for 15 min at rt. Cells were transfected by addition of the PEI/DNA mixture to the cells.

After 24 h, the medium was aspirated, and fresh, complete medium was added. Cells were used 48 h post transfection.

Photoaffinity-Based Protein Profiling. Gel-Based AfBPP. For gel-based profiling, transfected HEK-293-T or Neuro-2a (WT or KO) on 12-well plates were treated with the probe as follows: Growth medium was aspirated, a solution of indicated competitor (2X, 10 μM final) or vehicle in serum-free DMEM supplemented with 0.1% (w/v) delipidated BSA (0.5 mL) was added, and the cells were incubated for 30 min at 37 °C. Then, a solution of pac-WOBE (3, 2X, 100 nM final) in serum-free DMEM supplemented with 0.1% (w/v) delipidated BSA (0.5 mL) was added, and the cells were incubated for 30 min at 37 °C. The medium was aspirated and replaced with 1 mL of ice-cold DPBS, and the cells were irradiated using a Caprobox (10 min, 4 °C, 350 nm, “UV”) or exposed to ambient light (10 min, 4 °C, “No UV”). The cells were harvested by pipetting and pelleted by centrifugation (1000g, 10 min, 4 °C). The supernatant was removed, and the cells were lysed by resuspension in lysis buffer (250 mM sucrose, 20 mM HEPES pH 7.5, 1 mM MgCl2, 1X protease inhibitor cocktail (Roche), 25 U/mL benzonase) and sonication in a bath sonicator (0 °C, 5 min). Protein concentration was measured by Qubit assay (Invitrogen), and the samples were adjusted to 1.5 mg/mL and a volume of 100 μL, after which the samples were treated with 10.4 μL of click mix (5.5 μL of aq 25 mM CaSO4, 3.25 μL of aq 250 mM NaAsc, 1.1 μL of 25 mM THPTA in DMSO, and 0.55 μL of 0.9 mM Cy5-N3 in DMSO) and left at rt for 1 h. Samples were then quenched by addition of 4X Laemmli buffer, boiled (5 min, 95 °C), and resolved by SDS-PAGE (10% acrylamide gel, ±80 min, 180 V) along with a protein marker (PageRuler Plus, Thermo Fisher). In-gel fluorescence was measured in the Cy3- and Cy5-channels (Chemidoc MP, Bio-Rad), and the gels were subsequently stained with Coomassie and imaged as a loading control for normalization of fluorescence intensity. Band intensities were quantified using Image Lab 6.0.1 (BioRad).
The uptake of AEA was measured in Neuro-2a cells (seeded in triplicate in 12-well plates) according to the literature protocol with minor modifications.24,35 Neuro-2a cells were preincubated in serum-free medium with OMDM-1 (40 μM) for 15 min or different concentrations of WOBE437 (0.1, 1, and 10 μM) or vehicle for 10 min by adding the substance directly to the incubation medium. Then, the cells were incubated with AEA (400 nM) supplemented with [arachidonoyl-5,6,8,9,11,12,14,15-3H]AEA (30,000 cpm, ARC, St. Louis, MO, USA) at 37 °C for 15 min. The medium was then aspirated, and the cells were washed three times with PBS supplemented with 1% (w/v) BSA (1 mL) and resuspended in aq NaOH (0.5 M, 0.5 mL) and measured in a scintillation counter. Control experiments were also carried out at 4 °C in order to subtract passive diffusion from active uptake.

**Lipidomics.** Neuro-2a (WT or KO) cells were suspended and counted, and 2.5 × 10⁶ cells were seeded in 6 cm dishes (Sarstedt) and allowed to recover for 48 h. Cells were then washed with DBPS (2 mL), and treatment was initiated by addition of 2 mL of serum-free DMEM with 0.1% (w/v) delipidated BSA with indicated concentration of WOBE437 or vehicle (0.1% EtOH) for indicated times at 37 °C and 7% CO₂. After incubation, the treatment medium was aspirated, and the cells were washed with DBPS (2 mL) and then harvested on ice with ice-cold DBPS, of which a portion was reserved for normalization using protein concentration after lysis using probe sonication (Branson Sonifier probe sonicator, 5 s, 30% amplitude). Cells for lipidomic analysis were spun down (100,000 g, 5 min, 4 °C), the supernatant was aspirated, and the cells were snap-frozen. Samples were thawed on ice and spiked with 10 μL of deuterium labeled internal standard mix (Table S2), vortexed, and incubated for 5 min on ice. Subsequently, NaCl (0.5% w/v), 100 μL and NH₄Ac (0.1 M, pH 4, 100 μL) were added. Ice-cold methyl tert-butyl ether (MTBE) (HPLC grade, 1 mL) was added, and the tubes were thoroughly mixed for 7 min using a bullet blender blue (Next advance Inc., Averill park, NY, USA) at speed 8, followed by a centrifugation step (16,000g, 11 min, 4 °C). Next, 925 μL of the upper MTBE layer was transferred into a clean 1.5 mL Safe-Lock Eppendorf tube. Samples were concentrated in a speedvac (Eppendorf Concentrator Plus 5301) and redissolved in LC–MS solution [30 μL per sample: 28.5 μL of Milli-Q, 2.85 μL of acetonitrile, 0.095 μL of formic acid, and 600 fmol yeast enolase peptide digest (Waters Corporation, 186002325)].
plastic spring, ME 060232, Screening devices). 5 μL of each sample was injected into the LC−MS system.

Targeted lipidomics was performed on a panel of 23 lipids consisting of endocannabinoids, related NAEs, and free fatty acids (Table S2). Lipidomics measurements were performed on an Acquity UPLC I class binary solvent manager pump linked to a tandem quadrupole mass spectrometer (Waters Corporation). Lipids were separated using an initial gradient of 55% B held for 2 min, which was linearly increased to 100% B over 6 min and held for 2 min. The column was equilibrated with 55% B held for 2 min before each run. For lipid quantification, electrospray ionization-mass spectroscopy and selective multiple reaction mode (MRM) were used. MRM transitions for each lipid were optimized using synthetic references and internal standards (Table S2). Peak area integration was performed with MassLynx 4.1 software (Waters Corporation). The quantified peak areas were divided over the peak areas of the corresponding internal standards to obtain response ratios, which were translated to absolute concentrations using their respective calibration curves. Concentrations were then normalized to the amount of protein in the sample as determined by the Bradford assay.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE50 partner repository with the dataset identifier PXD031488.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemicalbio.2c00122.

Methods and materials used and additional figures (PDF)

Complete list of identified proteins from photoaffinity experiments (XLSX)

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Notes

The authors declare no competing financial interest.

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