Chapter 20

Assay of Endocannabinoid Uptake

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Abstract

Endocannabinoids at physiological concentrations are crossing cellular membranes by facilitated diffusion, a process that can be studied by measuring transport kinetics. Here, we describe a radiosubstrate-based assay using arachidonoyl[1-^{3}H]ethanolamine or arachidonoyl[1,2,3-^{3}H]glycerol to measure the cellular endocannabinoid uptake in a three-phase assay with human U937 cells.

Key words Endocannabinoid uptake, Anandamide, 2-Arachidonoylglycerol

1 Introduction

Endocannabinoids (ECs) are arachidonic acid-derived lipids that interact either with extracellular targets, such as type 1 and type 2 cannabinoid receptors (CB_1R and CB_2R, respectively), or with intracellular targets like ion channels, peroxisome proliferator-activated receptors (PPARs), intracellularly located CB_1 receptors (e.g., in mitochondria), cytoplasmic binding proteins (e.g., albumin, heat-shock protein-70, FABP5), and metabolic enzymes. While the EC family includes at least four lipid mediators, the most abundant and best characterized molecules are 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (anandamide, AEA). The biological effects of AEA and 2-AG are regulated by their cellular biosynthesis, extracellular release, reuptake, trafficking, and enzymatic cleavage. In contrast to the relatively clear knowledge about the EC biosynthetic and metabolic pathways, their cellular uptake remains poorly understood. Different models of EC uptake have been proposed and cell type-dependent mechanisms of EC cellular transport exist that have been recently reviewed in detail [1]. However, the measurement of AEA and 2-AG uptake is hampered by numerous confounding factors [2–5]. One of the principal issues in elucidating EC membrane transport is the tight interplay between the movement of ECs across plasma membranes and their rapid and almost complete cellular cleavage by the fatty acid amide hydrolase (FAAH) and the monoacylglycerol

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lipase (MAGL) or alpha/beta hydrolase 6 (ABHD6), respectively. At physiological concentrations, ECs are transported across the plasma membrane by facilitated diffusion, as best characterized by differential Michaelis-Menten kinetics in different cell types , independent of enzyme concentration [1, 6]. The uptake of AEA is driven by its breakdown primarily by FAAH, which belongs to the family of serine hydrolases and is expressed at the endoplasmatic reticulum, Golgi apparatus, and mitochondria. Although the cellular uptake of 2-AG has been poorly investigated, several studies suggest that AEA and 2-AG may share the same mechanism of membrane uptake despite their distinct intracellular fates [1, 7]. In addition, all ECs seem to compete for the same uptake process [7]. Importantly, the cellular uptake of AEA and 2-AG can be selectively and potently inhibited by small molecules that might be inhibitors of a putative endocannabinoid membrane transporter (EMT), and therefore constitute an independent class of modulators of the ECS [1, 20]. In order to identify and characterize novel EMT inhibitors, reproducible and sensitive assays are indispensable. Here, we employ the tritiated radiosubstrates [^{3}H]AEA and [^{3}H]2-AG, which allow robust measurements of EC cellular uptake by using differential extraction and radiometric-based assays. Radiometric assays show advantages over fluorescent and colorimetric assays, as chemically modified substrates might interfere with the membrane lipid environment leading to amplification or quenching of the signal. Moreover, the addition of a fluorophore to the EC core structure leads to modifications of its physicochemical properties, thus potentially altering cellular uptake kinetics. The quantitative measurements of EC intracellular levels by GC-MS/MS and LC-MS/MS are more costly and time consuming, and they do not seem optimal to screen for EMT inhibitors. Therefore, radiometric assays are most frequently used to assess EC uptake inhibition in combination with assays that measure inhibition of EC hydrolysis.

2 Materials

Prepare buffers using deionized H_2O and analytical grade reagents. Follow waste disposal regulations when disposing cells and radioactive waste material. For all radioactive substances, the half-life of radioactive isotopes must be taken into account. Store stock solutions at −20 °C, and perform regular analytical analyses to ensure product stability.

2.1 Components and Reagents

1. 2-Arachidonoylglycerol (2-AG).
2. Anandamide (AEA).
3. Arachidonoyl[1-^{3}H]ethanolamine (AEA).
4. Arachidonoyl[1,2,3-^{3}H]glycerol (2-AG) (*see* **Note 1**).
5. Bovine serum albumin (BSA), essentially fatty acid free (*see* **Note 2**).
6. Refrigerated centrifuge for 24 samples (e.g., Eppendorf centrifuge 5415 R).

7. Centrifuge, suited for Falcon tubes (e.g., Eppendorf centrifuge 5804 R).
8. Chloroform ($CHCl_3$).
9. Dimethyl sulfoxide (DMSO).
10. Ethanol (EtOH), ≥99 % pure.
11. Incubator for cell culture, at 37 °C and 5 % CO_2.
12. Liquid scintillation counter (e.g., Tri-Carb 2100TR).
13. Methanol (MeOH).
14. Micropipettes (10, 100, 200, and 1000 μl).
15. Mini-scintillation vials (6 ml).
16. Inverted microscope.
17. Neubauer "improved" cell counting chamber.
18. 0.01 M Phosphate-buffered saline (PBS), pH 7.4.
19. Reference inhibitors OMDM-2 and UCM707, and guineensine (available from J. Gertsch, University of Bern).
20. SafeSeal microtubes (2 ml, PP).
21. Screw-cap microtubes (1.5 ml, PP) (*see* **Note 3**).
22. Silanization fluid (AquaSil™), to be used according to instructions (Thermo Fisher Scientific).
23. ThermoMixer (1.5 ml).
24. Ultima Gold™ scintillation cocktail.
25. Vortex.
26. Water bath or thermoblock at 37 °C.

2.2 Cell Culture Material

- 150 cm^2 Tissue culture flasks.
- Amphotericin B.
- Cell line: Human monocytic lymphoma cells U937.
- Cell culture hood, biological safety cabinet (SterileGARD, Class 2A/B3).
- Falcon tubes (15/50 ml, PP).
- Fetal bovine serum (FBS) (*see* **Note 4**).
- Penicillin/streptomycin.
- RPMI-1640 medium with L-glutamine.

2.3 Buffers and Stock Solutions

1. [^{3}H]AEA-mix: 1 nM Arachidonoyl[1-^{3}H]ethanolamine, 99 nM AEA (prepare 100× stock solution in EtOH; 5 μl of [^{3}H]AEA mix will be used per sample):
   - 3 μl (167 nM) of arachidonoyl[1-^{3}H]ethanolamine in EtOH per sample.
   - 1.72 μl (28.8 μM) of AEA in EtOH per sample.
   - 0.28 μl of EtOH per sample.

2. [^{3}H]2-AG mix: 1 nM Arachidonoyl[1,2,3-^{3}H]glycerol (*see* **Note 1** and ref. 7), 999 nM 2-AG (prepare 100× stock solution in EtOH).
   - 2.5 µl (200 nM) of arachidonoyl[1,2,3-^{3}H]glycerol in EtOH per sample.
   - 2.5 µl (199.8 µM) of 2-AG in EtOH per sample.
3. 1 % (w/v) BSA in PBS, pH 7.4, at 4 °C.
   - Add 2 g BSA into 200 ml of PBS.

3 Methods

3.1 Cell Culture Maintenance

Grow human U937 cells in suspension at a density of approximately 1×10^6/ml in 150 cm^2 tissue culture flasks An endotoxin-free enviornment is essential. Use RPMI-1640 medium supplemented with 10 % FBS, 1 µg/ml amphotericin B, and 1 % (100 µg/ml) penicillin/streptomycin. Incubate cells at 37 °C with 5 % CO_2 for optimal growth (*see* **Note 5**).

3.2 Three-Phase AEA Uptake Assay

The workflow of this assay is illustrated in Fig. 1 (for details *see* **Note 6**).

1. Prepare inhibitor dilutions and sufficient [^{3}H]AEA mix. Heat PBS and RPMI-1640 in the water bath to 37 °C.
2. Count the cells using the "Neubauer improved" chamber (*see* **Note 7**), take the necessary number of U937 cells, and centrifuge them at 180 × *g* for 5 min at room temperature; wash with 10 ml of PBS (37 °C), centrifuge again at 180 × *g* for 5 min at room temperature, and resuspend in pre-warmed RPMI-1640 medium (without any additive) or PBS, to a density of 2×10^6 cells/ml.
3. Use 490 µl of cell suspension (~10^6 cells) per sample in silanized flip-cap microtubes.
4. Add 5 µl of OMDM-2 or UCM707 inhibitors (and of DMSO vehicle in positive controls); use 100× stock solutions (e.g., 1 mM solution to reach a final concentration of 10 µM), and vortex the tubes gently for 2 s (300 rpm in the ThermoMixer). Preincubate the cells for 30 min at 37 °C and 5 % CO_2. Run each sample in triplicate.
5. Add 100 nM [^{3}H]AEA mix (5 µl of 100× stock) to the cells. Vortex tubes carefully to distribute AEA homogenously. Incubate for 5 min at 37 °C.
6. Stop the uptake reaction by putting the tubes on ice, and rapidly centrifuge the samples at 800 × *g* for 5 min at 4 °C. Meanwhile, prefill 1.5 ml screw-cap microtubes with 2 volumes of an ice-cold $CHCl_3$:CH_3OH (1:1) mixture (*see* **Note 3**).

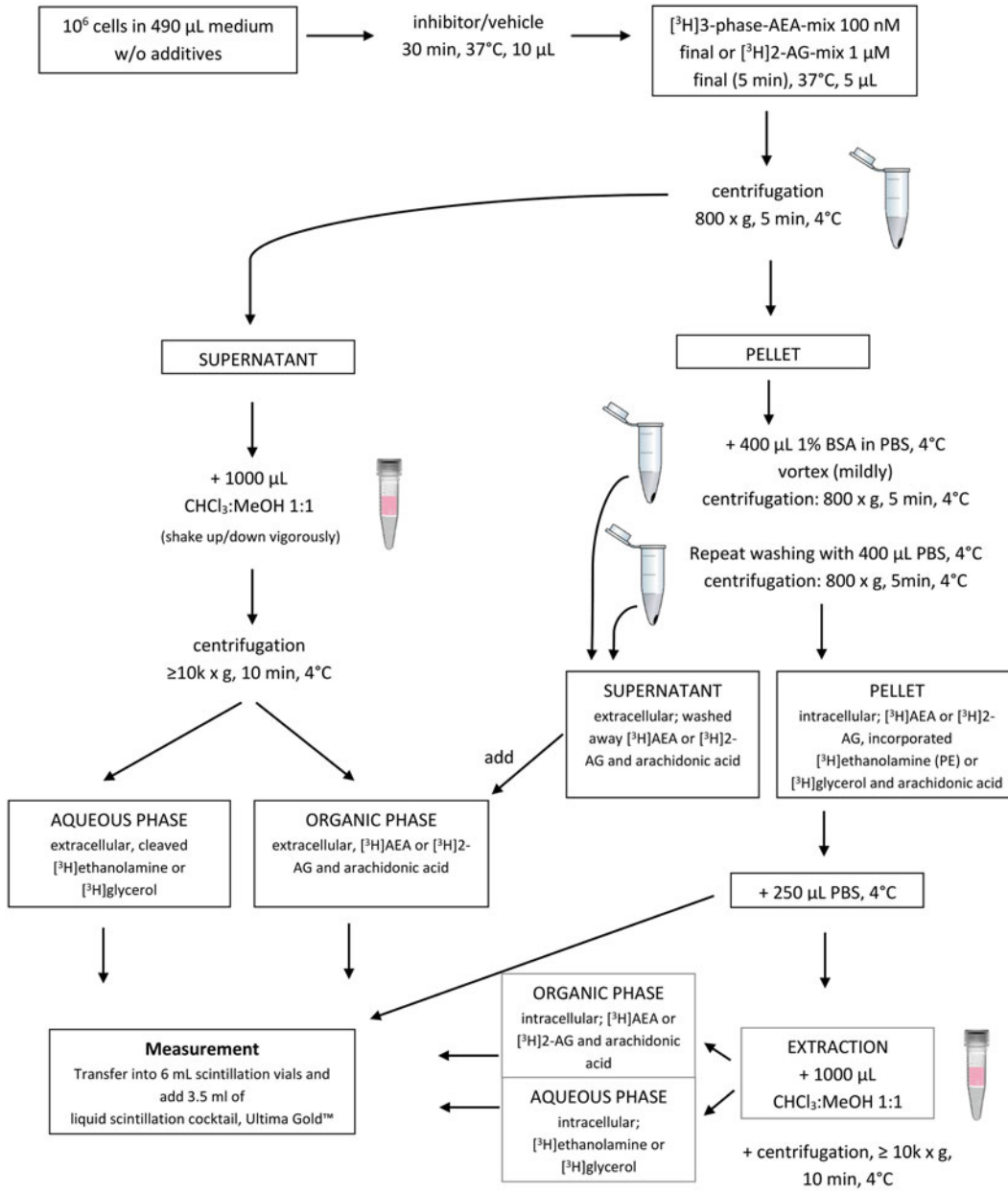

Fig. 1 Workflow of the three-phase AEA/2-AG uptake assay, according to the protocol

7. Transfer sample supernatants to the prefilled screw-cap microtubes including $CHCl_3$:CH_3OH, and put cell pellets on ice (pellet processing continues in **step 10**, also *see* **Note 8**).
8. Vortex or shake sample supernatants vigorously for extraction.
9. Centrifuge for 10 min at ≥10,000 × *g* at 4 °C, to separate aqueous and organic phases. Transfer the aqueous (upper) phase and organic (lower) phase separately into scintillation tubes for radioactive measurement (*see* **Note 9**).

10. Wash cell pellets from **step 6** with 400 µl of an ice-cold 1 % BSA in PBS, and shake carefully. Centrifuge at 800 × *g*, for 5 min and 4 °C. Add the supernatant to the scintillation tube containing the organic phase (from **step 9**), leave 5–10 µl of liquid to make sure that cell pellet remains intact, and cells are not removed with the supernatant.
11. Wash the pellets again with 400 µl of ice-cold PBS to remove any residual amounts of washing solution, and shake carefully (*see* **Note 10**).
12. Centrifuge for 5 min at 800 × *g* and 4 °C, and then transfer the supernatants to the scintillation tube containing the supernatant organic phase (from **step 9**).
13. Resuspend cell pellets in 250 µl of PBS, add 2 volumes (0.5 ml) of ice-cold $CHCl_3$:CH_3OH (1:1), and vortex vigorously.
14. Sonicate cell solutions for 5 min on ice, and then centrifuge for 10 min at ≥10,000 × *g* at 4 °C. Transfer the upper (aqueous) phase to the scintillation tube containing the aqueous phase derived from the extracted supernatant (*see* **step 9**) while collecting the cellular organic phase in a separate scintillation tube.
15. Perform liquid scintillation counting after addition of 3.5 ml of Ultima Gold™ scintillation liquid to all scintillation vials, followed by 1-h shaking. Also measure total radioactive signal of the substrate by adding 5 µl of [^{3}H]AEA mix directly to new scintillation vials.

Examples of the reference inhibitors OMDM-2 and UCM707 are shown in Fig. 2. This assay allows to characterize differential EC cellular uptake inhibition in U937 cells [7], to investigate the profiles of FAAH and MAGL inhibitors [7]], suitable and to characterize new selective AEA uptake inhibitors like guineensine [20], or highly potent inhibitors of FAAH [4].

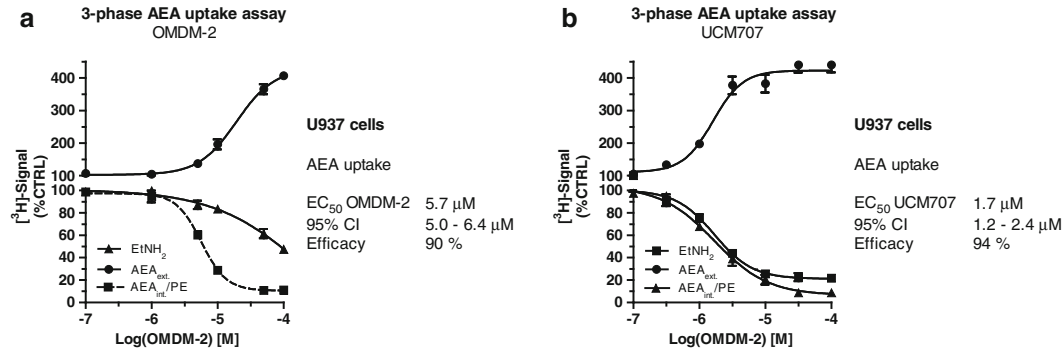

Fig. 2 Concentration-dependent effects of the reference inhibitors (**a**) OMDM-2 and (**b**) UCM707, determined in the three-phase AEA uptake assay. $AEA_{int./PE}$ describes the intracellular free AEA and the AEA-derived ethanolamine which was incorporated into phosphatidylethanolamine (PE). $EtNH_2$ is the free ethanolamine cleaved but not incorporated, and AEA_{ext} (extracellular) expresses the amount of free AEA which did not penetrate into the cell. 1×10^6 U937 cells were used per sample. Experiments were performed in triplicate with $n = 3$ ($N = 9$); means ± SEM are reported

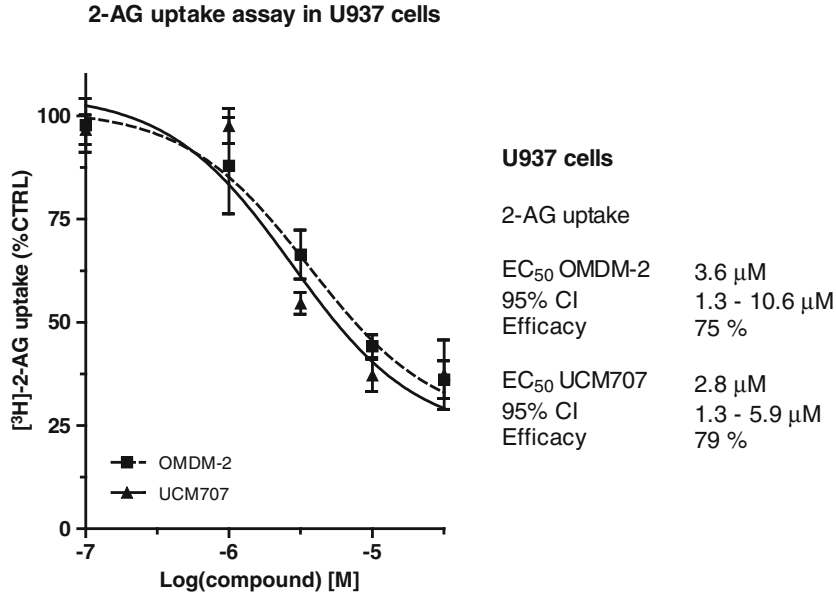

Fig. 3 Concentration-dependent effects of the reference inhibitors OMDM-2 and UCM707 in the three-phase 2-AG uptake assay. 1×10^6 U937 cells were used per sample. The cellular fraction is shown. For further details and graphs *see* [7]. Experiments were performed in triplicate with $n = 3$ ($N = 9$); means ± SEM are reported

3.3 Three-Phase 2-AG Uptake Assay

Perform as described in the three-phase assay for AEA uptake but apply the following adaptions:

Use 5 µl of 1 µM [^{3}H]2-AG mix per sample instead of 100 nM [^{3}H]AEA mix. Incubate [^{3}H]2-AG mix for 2–5 min.

Examples of reference inhibitors OMDM-2 and UCM707 are shown in Fig. 3.

4 Notes

1. 2-AG uptake is more difficult to measure than AEA uptake. The radioactive label in arachidonoyl[1-^{3}H]ethanolamine is in the ethanolamine moiety, which is quickly cleaved and then incorporated into phospholipids [7–9]. Therefore, the radioactive signal associated to the organic phase of the cell pellets is derived from free [^{3}H]AEA (minimal contribution) and [^{3}H]phosphatidylethanolamine (higher abundance). From the experimental point of view, this leads to higher radioactivity counts, which result in a robust signal-to-noise ratio. In case of arachidonoyl[1,2,3-^{3}H]glycerol uptake, the cleaved [1,2,3-^{3}H]glycerol freely and rapidly moves out of the cell *via* aquaporins, leading to a "loss" of intracellular radioactive signal [10]. The remaining signal is therefore only dependent on intracellular arachidonoyl[1,2,3-^{3}H]glycerol [7]. Furthermore, while the most relevant AEA hydrolytic enzyme (FAAH) is located predominantly in intracellular mem-

branes such as ER, Golgi apparatus, and mitochondria [8, 11, 12], 2-AG is degraded by at least three different enzymes, which are all located at the plasma membrane [12, 13]. Therefore, once taken up by the cell 2-AG is quickly cleaved, mainly by the membrane-associated enzyme MAGL (~85 % of the total 2-AG hydrolysis) and by the membrane-bound enzymes ABHD6 and ABHD12 (~15 %). Interestingly, the predicted structure of the latter two hydrolases suggests that they face the catalytic site towards the intracellular lumen and the extracellular milieu, respectively [13]. Direct cleavage of 2-AG at the outer membrane by ABHD12 might represent an important confounding factor when measuring 2-AG uptake, especially in specific cell lines lacking MAGL but not ABHDs (e.g., some macrophages and microglial cells) [14]. Nonetheless, it must be taken into account that inhibitors of arachidonic acid incorporation into phospholipids might reduce 2-AG uptake [15]. Moreover, short incubation times (≤5 min) are crucial to measure 2-AG uptake, since the arachidonoyl[1,2,3-^{3}H]glycerol is cleaved quickly after being taken up by the cells.

2. Serum albumin (fatty acid free) is an endogenous carrier of endocannabinoids [16, 17] and has been shown to affect endocannabinoid uptake [4]. Indeed, BSA (1 % in PBS) is the optimal washing solution to remove endocannabinoids associated to the outer leaflet of the plasma membrane.
3. Screw-cap microtubes are well suited for Folch's extraction, because they do not leak the MeOH/$CHCl_3$ mixture upon vigorous vortexing.
4. FBS is a nutritional supplement used for many cell cultures. Different batches were shown to contain variable amounts of endocannabinoids [18]. FBS low in endocannabinoid content should be selected. Uptake experiments should always be performed without FBS, in order to avoid biases.
5. Cell culture maintenance is one of the most crucial factors for the success of the EC uptake assay. When cells are only once malnourished, overgrown, activated by cell debris/endotoxins, or at unsuitable pH levels (i.e., yellow medium), they will acquire different phenotypes. U937 cells should be well-round shaped (Fig. 4a) and devoid of any dendritic structures which would indicate differentiation (Fig. 4b). Under suboptimal conditions, incorporation of ethanolamine into phospholipids (PE) is decreased (Fig. 4d), compared to healthy cells (Fig. 4c). Furthermore, endotoxin activated cells show reduced AEA uptake efficacy and FAAH enzymatic activity (Fig. 5a, b). Although the cells might recover and proliferate steadily, they will not be suitable for EC uptake any longer, and should be replaced. The RPMI-1640 medium contains the pH indicator phenol red, and turns from purple (pH > 8.2) to yellow (pH < 6.8) depending on the cell metabolic activity. Usually,

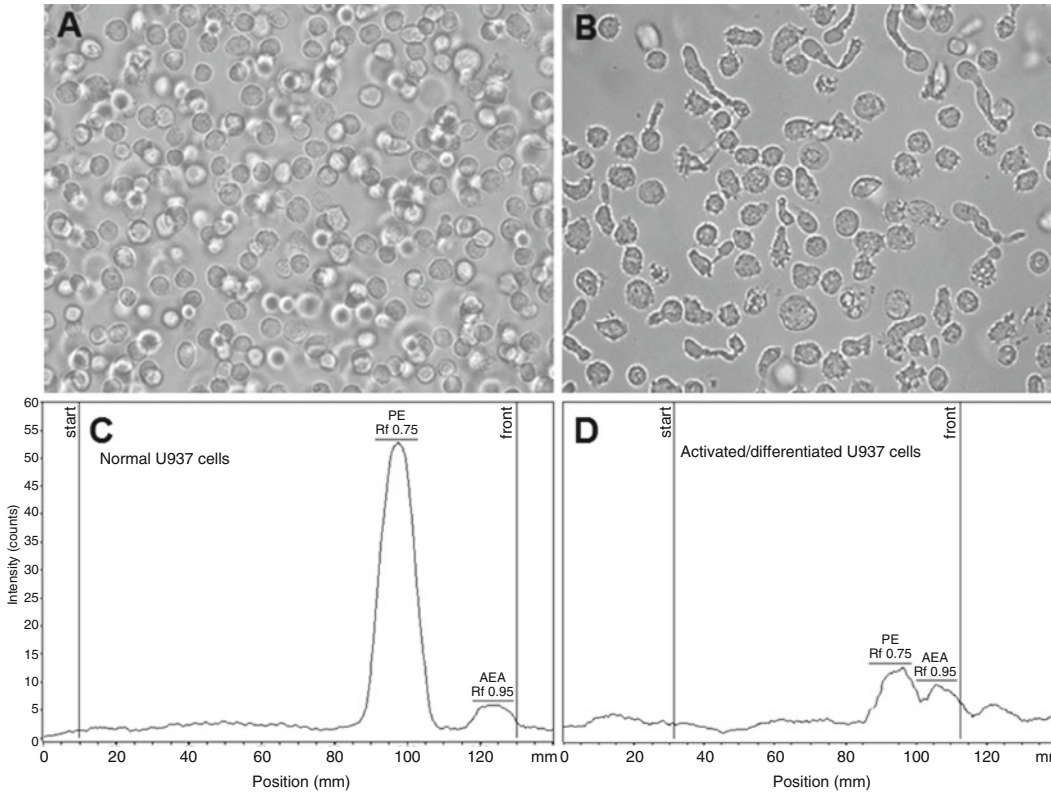

Fig. 4 (**a**) Normal U937 cells at 400× magnification. Cells are well rounded in suspension. (**b**) Activated and partially differentiated U937 cells at 400× magnification. Cells show deformations, dendritic structures, and partial adherence. (**c**) Incorporation of [^{3}H]ethanolamine into phosphatidylethanolamine (PE) and AEA on a TLC. (Assay was performed as described in [7].) (**d**) Activated/differentiated cells show a heavily reduced incorporation of [^{3}H]ethanolamine into phosphatidylethanolamine in comparison to normal U937 cells

when cells reach the density of about 1×10^6/ml, the medium turns into a yellowish color, implying a high metabolic activity. These cells should be used for EC uptake on the same day. When U937 cells are maintained under optimal culturing conditions, the generation time is roughly 24 h at cell densities $>0.8 \times 10^6$/ml. However, our experience is that under these exponential growth conditions the EC uptake assay works best.

6. The three-phase assay is an advanced EC uptake assay providing at once multiple readouts, which lead to a characteristic profile of an EC uptake inhibitor (Fig. 2a, b; also *see* refs. 7, 20). The assay reveals the intracellular and extracellular distribution pattern of AEA or 2-AG and their metabolites in the cells. For instance, the extracellular aqueous phase contains cleaved glycerol from 2-AG or ethanolamine from AEA, revealing the activity of EC-degrading enzymes; the extracellular organic phase contains intact 2-AG or AEA, which was not

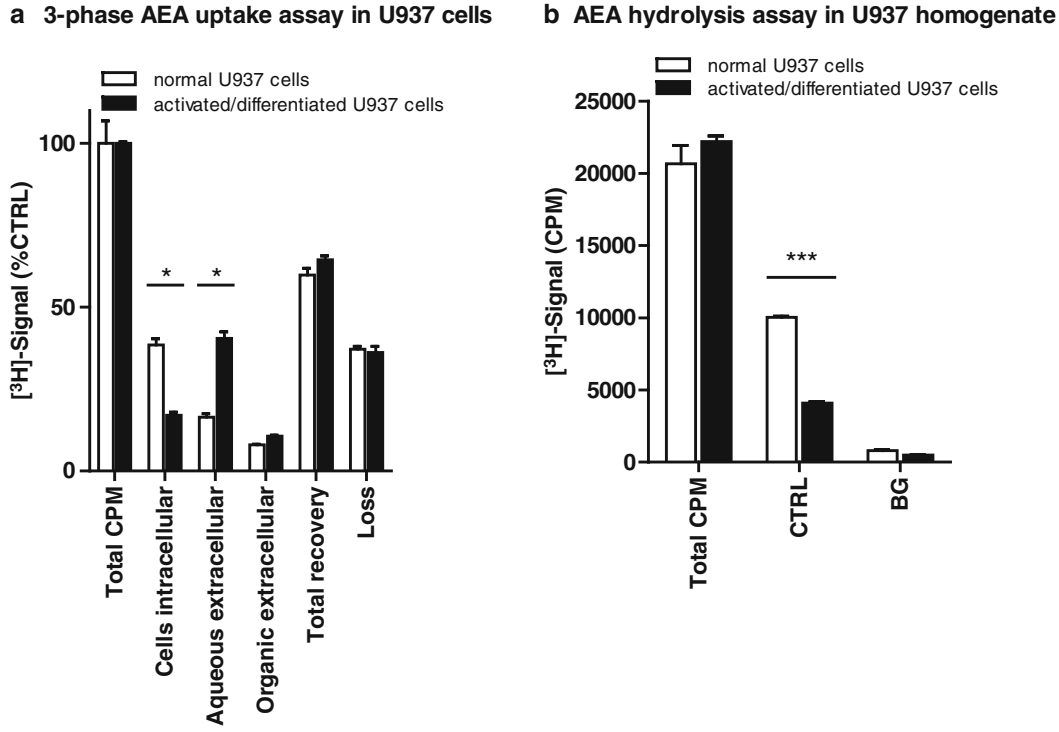

Fig. 5 (**a**) Three-phase AEA uptake assay of normal U937 cells versus "activated" U937 cells. The retention of radioactivity inside the activated/differentiated cells was significantly decreased and the [^{3}H]ethanolamine was found to be increased in the aqueous extracellular phase instead of being trapped in the cell by incorporation into PE. Overall, total signal of [^{3}H]3-phase-AEA mix, recovery, and remaining extracellular intact [^{3}H]AEA remained the same. Experiments were performed in triplicate with $n = 3$ ($N = 9$); means ± SEM and 95% CI are reported. (**b**) AEA hydrolysis assay in U937 cell homogenates, performed as previously described [4, 19]. The activated/differentiated cells showed significantly lower FAAH activity than normal U937 cells. Total [^{3}H]AEA added and background (BG) signals were equal. Experiments were performed in triplicate, $n = 3$ ($N = 9$); means ± SEM are reported

taken up by the cells. The three-phase assay enables collection of a complete set of information regarding the distribution and degradation of AEA or 2-AG as a concentration-dependent function of an EC uptake inhibitor. The assay is also suitable to characterize different cell types for AEA and 2-AG uptake, and to compare the pharmacological properties of new EMT inhibitors. AEA uptake in particular is heavily driven by the activity of FAAH that generates an inward concentration gradient. Thus, an inhibitor of FAAH will always inhibit EC uptake as well [2, 4]. However, each process can be inhibited independently, thus leading to $EtNH_2$ curve shifts that reveal an apparent selectivity for AEA cellular uptake (e.g., with OMDM-2 (Fig. 2a) or guineensine [20], but see also [4, 7] in comparison to FAAH inhibitors). After identification of a new AEA uptake inhibitor, further potential targets such as intracellular shuttles

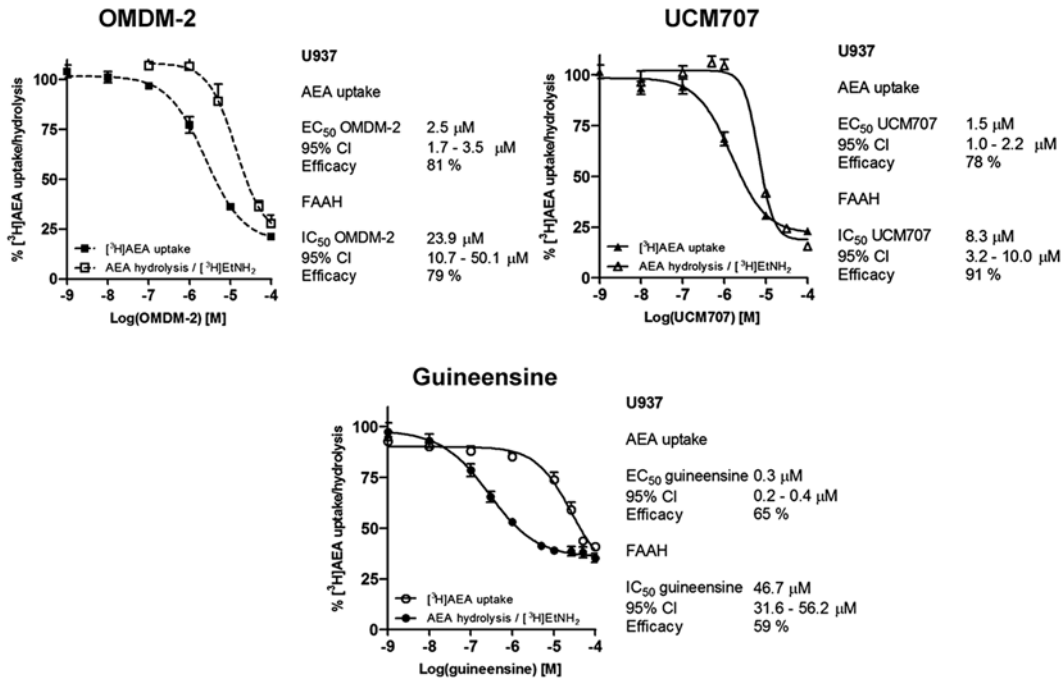

Fig. 6 Inhibition of [^{3}H]AEA uptake and [^{3}H]AEA hydrolysis by the reference inhibitors OMDM-2, UCM707, and guineensine in separate assays, as previously described [20]. 0.5×10^6 U937 cells or cell homogenates, respectively, were used per sample. Curves are shown without normalization. For AEA uptake, EC_{50} values (i.e., relative IC_{50} values) are shown because complete inhibition (i.e., efficacy of 100 %) cannot be reached due to non-inhibitable passive diffusion of the substrate, while absolute IC_{50} values are shown for FAAH activity. Reference FAAH inhibitors using the same assay protocol are described in [4]. Experiments were performed in triplicate with $n = 3$ ($N = 9$); means ± SEM and 95% CI are reported

[16, 21] and most importantly FAAH should be addressed as well (Fig. 6). To confirm an observed apparent selectivity (i.e., $EtNH_2$ right shift) of a new inhibitor of AEA uptake over FAAH inhibition, two independent assays can be performed as previously reported [4, 19, 20], as shown in Fig. 6.

7. We use the Neubauer improved cell counting chamber to quickly determine cell density, and to check at the same time their viability by trypan blue.
8. Consider that in EC uptake assays, the steps with the cell pellet are the most crucial and sensitive (the supernatant is transferred into $CHCl_3$:MeOH (1:1) and does not react any further). It is therefore advised to continue with **step 10** straight after **step 7** and do **steps 8** and **9** at the end.
9. Folch's extraction leads to a three-phasic separation. A bottom phase with $CHCl_3$ and lipophilic substances such as the arachidonic acid, phospholipids, 2-AG, or AEA; a precipitated phase of proteins in the middle; and an upper aqueous phase with MeOH, H_2O, and either free glycerol or ethanolamine, depending on the assay. After centrifugation at maximal speed

($\geq$10,000 $\times g$) the entire upper phase can be collected. Alternatively, a constant volume (e.g., 800 µl) per sample can be collected to reduce signal deviations.

10. In the presence of EMT inhibitors, most of [^{3}H]AEA and [^{3}H]2-AG which does not penetrate into the cells will stick to the membrane phospholipid bilayer, due to their high hydrophobic nature. Washing cell pellets with 1 % BSA solution ensures almost complete removal of this "extracellular fraction" of [^{3}H]AEA and [^{3}H]2-AG, which sticks on the external leaflet of the membrane. In our experience, this fraction usually contains a high radioactive signal; therefore further washing of cell pellets with PBS after the washing step with 1 % BSA is done to remove the residual extracellular signal from the cell pellet. Avoiding this additional washing step with PBS alone can lead to a high variability of the radioactive signal associated to the cell pellets.

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