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A novel live cell assay to measure diacylglycerol lipase α activity

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Synopsis

Diacylglycerol lipase α (DAGL α) hydrolyses DAG to generate the principal endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG) in the central nervous system. DAGL α dependent cannabinoid (CB) signalling has been implicated in numerous processes including axonal growth and guidance, adult neurogenesis and retrograde signalling at the synapse. Recent studies have implicated DAGL α as an emerging drug target for several conditions including pain and obesity. Activity assays are critical to the drug discovery process; however, measurement of diacylglycerol lipase (DAGL) activity using its native substrate generally involves low-throughput MS techniques. Some relatively high-throughput membrane based assays utilizing surrogate substrates have been reported, but these do not take into account the rate-limiting effects often associated with the ability of a drug to cross the cell membrane. In the present study, we report the development of a live cell assay to measure DAGL α activity. Two previously reported DAGL α surrogate substrates, *p*-nitrophenyl butyrate (PNPB) and 6,8-difluoro-4-methylumbelliferyl octanoate (DiFMUO), were evaluated for their ability to detect DAGL α activity in live cell assays using a human cell line stably expressing the human DAGL α transgene. Following optimization, the small molecule chromogenic substrate PNPB proved to be superior by providing lower background activity along with a larger signal window between transfected and parental cells when compared with the fluorogenic substrate DiFMUO. The assay was further validated using established DAGL inhibitors. In summary, the live cell DAGL α assay reported here offers an economical and convenient format to screen for novel inhibitors as part of drug discovery programmes and compliments previously reported high-throughput membrane based DAGL assays.

Key words: diacylglycerol lipase α (DAGL α), 6,8-difluoro-4-methylumbelliferyl octanoate (DiFMUO), live cell assay, *p*-nitrophenyl butyrate (PNPB).

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INTRODUCTION

The diacylglycerol lipases (DAGLs), diacylglycerol lipase α (DAGL α) and diacylglycerol lipase β (DAGL β), hydrolyse DAG to generate 2-arachidonoylglycerol (2-AG) for endocannabinoid (eCB) signalling which has been implicated in numerous important processes including axonal growth and guidance during development [1–6], synaptic plasticity in the form of retrograde synaptic transmission [7,8] and adult neurogenesis in the hippocampus and subventricular zone [1,7,9,10]. 2-AG generated by the DAGLs also acts as a substrate for monoacylglycerol lipase to maintain arachidonic acid (AA) levels in various tissues [7,11,12].

The DAGLs are emerging therapeutic targets for a range of conditions including fragile X syndrome, pain, inflammatory diseases and obesity [13–16]. Furthermore, drugs modulating other steps of eCB signalling have shown beneficial effects in obesity [17–20], pain [21,22], Alzheimer's disease [23] and Parkinson's disease [24]. DAGL α in particular is responsible for almost all of the 2-AG in the central nervous system and for retrograde signalling at the synapses via the cannabinoid (CB) receptors [7,8]. Selectively targeting DAGL α may therefore prove fruitful for the treatment of certain conditions as it has the potential to avoid the adverse psychiatric effects observed when targeting the cannabinoid receptor 1 (CB1) receptor [25].

As evidenced by previous and recent publications, there are several ongoing drug discovery efforts related to the DAGLs

Abbreviations: AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; CB, cannabinoid; CB1, cannabinoid receptor 1; DAG, diacylglycerol; DAGL, diacylglycerol lipase; DAGL α , diacylglycerol lipase α ; DAGL α , DAGL α with a C-terminal V5 tag; DAGL β , diacylglycerol lipase β ; DiFMUO, 6,8-difluoro-4-methylumbelliferyl octanoate; eCB, endocannabinoid; FAC, final assay concentration; OD400, optical density at 400 nm; PBST, phosphate buffered saline 0.1% Tween; PNPB, *p*-nitrophenyl butyrate; RT-PCR, reverse transcription polymerase chain reaction; THL, tetrahydrolipstatin.

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[13,16,26–35]. These studies have largely utilized assays employing isolated membranes (or lysates) overexpressing the DAGLs. At present, there are no simple assays that measure the activity of the human DAGLs in a live cell context. Such assays play an important role in drug discovery efforts and are particularly useful as they take into account the cell permeability of inhibitors and also present the target in a relatively native as opposed to isolated environment [36,37]. In the present study, we have generated a human U2OS cell line stably expressing an epitope tagged human DAGL α transgene. Two commercially available DAGL α surrogate substrates [26], namely *p*-nitrophenyl butyrate (PNPB) and 6,8-difluoro-4-methylumbelliferyl octanoate (DiFMUO) were evaluated in live cell assays using the cell line to determine if we could measure activity of the DAGL α transgene in intact cells. The small chromogenic molecule PNPB was found to be a suitable substrate, showing a larger difference in activity between transfected and parental cells along with lower background activity when compared with the fluorogenic substrate DiFMUO. The specificity of the response was confirmed using several established DAGL inhibitors. The live cell assay for DAGL α described here offers an economical and convenient format to screen for novel cell-permeant inhibitors as a complement to currently available membrane based assays.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma–Aldrich unless otherwise stated.

Generation of the V5 α 11 cell line

In order to express DAGL α with a V5 tag (C-terminal), full-length human DAGL α [2] was cloned into the pCDNA6.2/V5-DEST vector (Invitrogen). The construct was transfected (Lipofectamine 2000, Invitrogen) into a human U2OS osteosarcoma cell line (Tango™ CNR1-bla U2OS cells from Invitrogen) that for the purpose of future studies also expresses a CB1 reporter construct. Selection of stable transfected clones was performed using blasticidin (4 μ g/ml). Several clones were repeatedly screened by Western blotting and immunocytochemical analyses using a V5 antibody (mouse, Invitrogen) following which the cell line stably expressing DAGL α with a C-terminal V5 tag (DAGL α -V5) (V5 α 11) was established.

Membrane preparation

Membranes were prepared following the method described by Pedicord et al. [26]. Briefly, V5 α 11 or tango (parental) cells (~90% confluent) cultured in 10 cm dishes were washed with PBS following which the cells were scraped in 1 ml (per dish) of lysis buffer (20 mM HEPES, pH 7.0, 2 mM DTT, 0.25 M sucrose, 10 mM NaF, 1 mM Na₃VO₄ and 1 \times Roche ‘Complete™’

protease inhibitor). The cell suspensions were collected and homogenized on ice using a Polytron (PT 1200 E) homogenizer (three 7 s bursts at the maximum setting with 30 s interval in between). The homogenates were centrifuged at 100 000 *g* for 30 min at 4 °C following which the supernatants were discarded. The pellets (membrane preparations) were resuspended in 200 μ l of sucrose free lysis buffer using the Polytron homogenizer and stored at –80 °C.

Immunocytochemistry

Cells were first seeded on to polylysine coated coverslips at a density of 10000/well and cultured overnight following which they were fixed in 4% paraformaldehyde for 30 min. The fixed cells were washed with PBS and then permeabilized for 10 min using 0.2% Triton X-100-PBS. The permeabilized cells were washed with PBS and then blocked for 30 min using the block solution (1% BSA–PBS). The cells were then incubated with the V5 primary antibody (mouse, Invitrogen, diluted 1/1000 in block solution) for 1 h at room temperature, washed with PBS and then incubated for 1 h with the AlexaFluor 488 secondary antibody (mouse, Invitrogen, diluted 1/2000 in block solution) and the nuclear stain Hoechst 33258 (Invitrogen, diluted 1/10 000 in block solution). Finally, the cells were washed with PBS and the coverslips were mounted on to microscope slides. The Carl Zeiss LSM 710 microscope and the Carl Zeiss Zen software (version 1.0.1.0) were used to capture images of the immunostained cells.

Western blotting

V5 α 11 or tango membranes were diluted using 5 \times SDS protein loading buffer and water to a concentration of 1 μ g/ μ l. Diluted samples were denatured by boiling for 5 min. Ten micrograms of the denatured samples were loaded on to Tris-glycine gels (4% stacking and 10% resolving) and resolved at a setting of 100 V for ~2 h. Western blotting was performed using nitrocellulose membranes (GE healthcare) and a wet transfer method (1 h at 100 V and 4 °C). Membranes were blocked in PBS 5% milk (1 h at room temperature) and then incubated with the primary V5 antibody [diluted in PBS 0.1% Tween (PBST) 2% milk] overnight at 4 °C. The membranes were then washed in PBST and incubated with the mouse IR-Dye 680 secondary antibody (LI-COR, diluted 1/5000 in PBST 2% milk) for 1 h at room temperature. Finally, the membranes were washed in PBST (4 \times) and then PBS (1 \times). The Odyssey imaging system (LI-COR) was used to visualize the membranes. β -Actin was also detected as a loading control.

Membrane assays

All membrane assays were carried out in 96-well clear polypropylene plates following a previously published method with some modifications [26]. Membranes were first diluted in assay buffer (4 \times FAC, i.e. final assay concentration) following which 50 μ l/well was dispensed. Fifty microlitres of assay buffer or 50 μ l of inhibitor (diluted to 4 \times FAC using assay buffer) was then added to the membranes. Membranes were typically

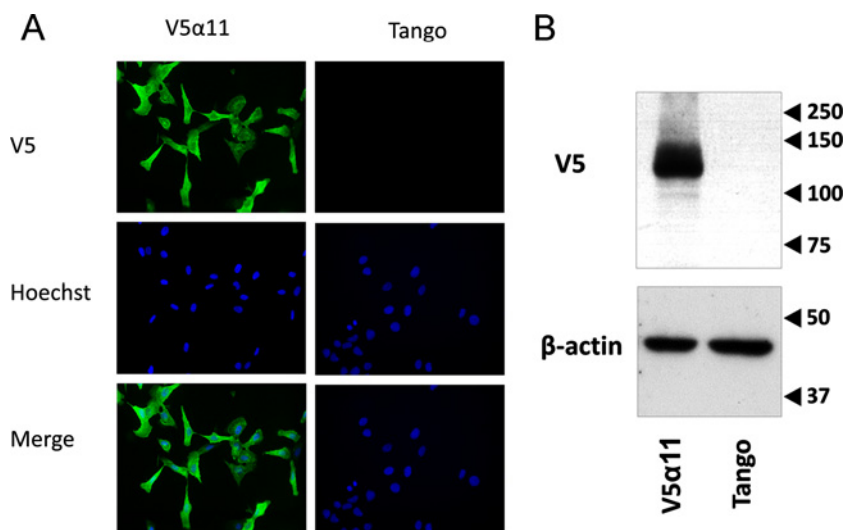


Figure 1 Stable expression of human DAGL α -V5 in the V5 α 11 cell line

DAGL α -V5 expression in the V5 α 11 cell line was periodically monitored by Western and immunocytochemical analyses using a V5 antibody. A representative image demonstrating the stable and clonal expression of DAGL α -V5 in fixed V5 α 11 cells immunostained with a V5 antibody (green) and the nuclear stain Hoechst (blue) is presented above along with parental cells as a negative control (A). Western analyses using V5 α 11 membranes (and parental membranes as a negative control) and a V5 antibody revealed DAGL α -V5 expression at a size close to the predicted molecular mass (120 kDa) (B).

incubated with the inhibitors in the plates for 5 min at room temperature. Substrate was first diluted in DMSO to 40 \times FAC and then to 2 \times FAC using the assay buffer without DMSO. Hundred microlitres/well of the substrate solution was dispensed and the plates were read immediately. For the PNPB assay, 50 mM HEPES pH 7.5 and 5% DMSO was used as the assay buffer and reactions were monitored by measuring the optical density at 400 nm (OD400) every 12 s for 30 min using a Spectramax plate reader (Molecular Devices). For the DiFMUO assay, 50 mM MES pH 6.5 and 5% DMSO was used as the assay buffer and the reactions were monitored by measuring the fluorescence (excitation 360 nm, emission 450 nm) every 30 s for 30 min using the FlexStation (Molecular Devices). Typically, FACs were 12.5 μ g/ml membranes, 250 μ M PNPB or 10 μ M DiFMUO, and 5% DMSO in a total assay volume of 200 μ l. Activity was determined by calculating the reaction rates over the first 10 min (linear) using three replicate wells.

Live cell assay

Cells were seeded at a density of 40000/well (96-well plates) in FreeStyle media (Invitrogen) and maintained overnight. Prior to assaying, the media were discarded and the cells were washed with the assay buffer (50 mM HEPES, pH 7.5 for the PNPB assay and 50 mM MES, pH 6.5 for the DiFMUO assay). One hundred microlitres of inhibitor (2 \times FAC) or assay buffer was then added to the wells following which the plate was incubated for 5 min. One hundred microlitres of substrate (diluted to 100 \times FAC in DMSO and then to 2 \times FAC in assay buffer) was then added to each well and the activity was measured as described for the corresponding membrane assay (six replicate wells). Typically,

the total assay volume was 200 μ l containing 1% DMSO and either 500 μ M PNPB or 10 μ M DiFMUO.

RESULTS

The U2OS cell line stably expressing DAGL α -V5 (V5 α 11) was established to support the development of a DAGL α live cell assay. Stable expression of DAGL α -V5 was confirmed beyond 30 passages in this cell line by both Western and immunocytochemistry analyses using a V5 antibody (Figure 1). In order to test the activity of DAGL α -V5 expressed in the V5 α 11 cell line, activity of membranes derived from parental and V5 α 11 cells was measured using two previously reported DAGL α surrogate substrates (DiFMUO and PNPB) [26]. A relatively modest but significant 1.6-fold difference in activity was detected between the V5 α 11 and parental membranes using the larger fluorogenic DiFMUO as the substrate (Figure 2A). This 'window' was achieved following optimization of various parameters including assay buffer constituents and the substrate and membrane concentrations. Notably, this difference in activity was inhibited by the DAGL inhibitor tetrahydrolipstatin (THL) which provided a slightly improved 1.8-fold difference in THL sensitive activity of the two membranes.

On the other hand, the V5 α 11 cell line showed 6-fold greater PNPB hydrolysis activity than parental membranes; this difference was enhanced to 11-fold when considering the THL sensitive activity (Figure 2B). These results indicate that PNPB is a superior substrate for the measurement of DAGL α activity

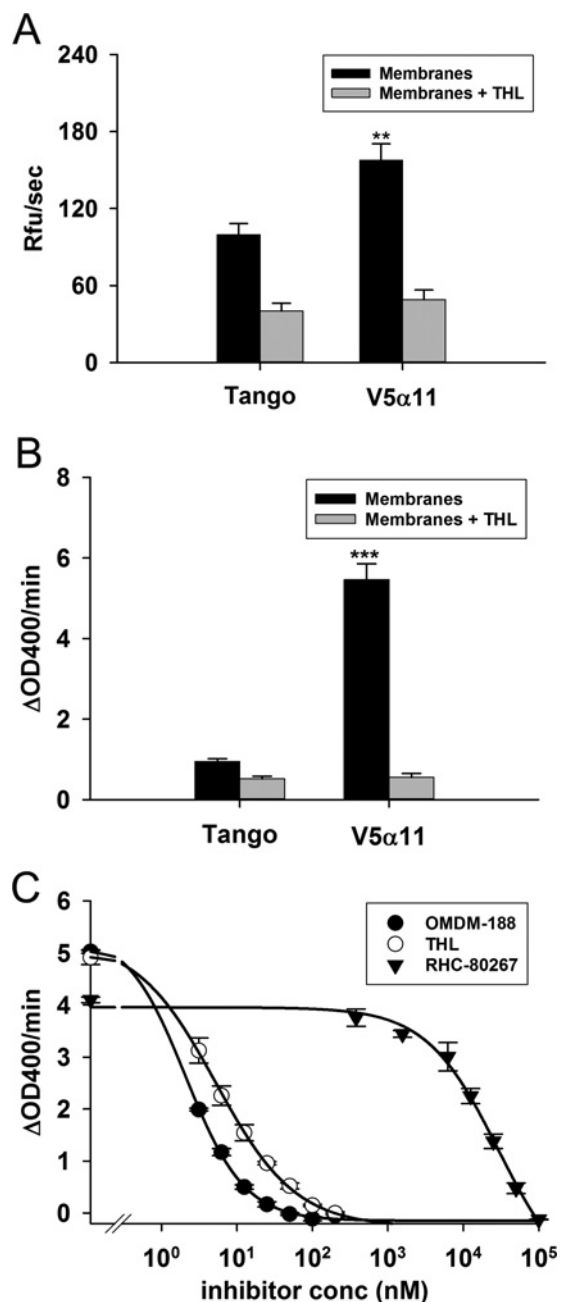


Figure 2 Characterizing DAGL α activity in the V5 α 11 membranes using DiFMUO and PNPB

Activities of V5 α 11 and parental membranes were measured using surrogate substrates. Membranes (12.5 μ g/ml) were first incubated in the presence or absence of THL (1 μ M) for 5 min following which the activity was measured using 10 μ M DiFMUO (mean \pm S.E.M., $n=7$) (A) or 250 μ M PNPB (mean \pm S.E.M.; V5 α 11, $n=10$; parental, $n=13$) (B). The difference in PNPB hydrolytic activity between the membranes was inhibited by three different DAGL inhibitors (OMDM-188, THL and RHC-80267) (mean of three wells \pm S.E.M.) (C). The reaction rates were calculated over the first 10 min; ** $P < 0.01$, *** $P < 0.001$ (two-tailed t test).

when using the V5 α 11 membranes. The relatively low PNPB hydrolysis activity in the parental membranes (compared with V5 α 11) validated the V5 α 11 membranes as a tool to screen for DAGL α inhibitors. IC₅₀s of three DAGL inhibitors, OMDM-188 (2.1 \pm 0.15 nM), THL (5.3 \pm 0.23 nM) and RHC-80267 (28.8 \pm 11.1 μ M) were determined in the assay (Figure 2C). The results are in agreement with previous studies; OMDM-188 has been reported to be the more potent of the three DAGL inhibitors whereas RHC-80267 has been reported to be the least potent [26,38,39].

Cell based assays in conjunction with biochemical assays are a critical part of drug discovery programmes. Having identified significant differences between the activity of the V5 α 11 membranes using PNPB and DiFMUO, we next investigated whether these surrogate substrates could also be used as part of DAGL α live cell assays. In order to assess whether background activity due to cytoplasmic enzymes could prove to be problematic, we first tested the activity of crude cell homogenates prepared from V5 α 11 and parental cells using the substrates. Unsurprisingly, only a 1.5-fold difference was observed when comparing activity of the homogenates using DiFMUO as the substrate (Supplementary Figure S1A) and despite optimization of various parameters, only a modest (but significant) 1.3-fold (1.4-fold THL sensitive) difference was observed when measuring the activity in intact cells (Figure 3A).

On the other hand, encouragingly, 4-fold greater activity was observed in V5 α 11 crude homogenates when compared with parental homogenates using PNPB indicating that potential background hydrolysis by cytoplasmic enzymes was relatively low compared with DAGL α -V5 activity when using this substrate (Supplementary Figure S1B). Following optimization of assay buffer constituents (Supplementary Figures S1C and S1D), substrate concentration (Supplementary Figure S1E) and cell numbers (Supplementary Figure S1F), 2.1-fold greater PNPB hydrolysis activity was observed in the V5 α 11 cells compared with the parental cells when the substrate was added to intact cells. This difference in activity was inhibited by THL (Figure 3B). IC₅₀s of THL and RHC-80267 (IC₅₀s 31.8 \pm 4.1 nM and 5.8 \pm 0.2 μ M respectively) were also determined using this assay (Figure 3C). When subtracting the background activity seen in the presence of THL, the difference in activity between the two cell lines using PNPB was much higher at 3.5-fold. Thus, we conclude that PNPB is a more suitable substrate to measure DAGL α activity in both membrane and live cell assays.

DISCUSSION

The DAGLs have been implicated in a range of diseases and therefore have previously and recently been the focus of several studies aiming to discover novel inhibitors of these enzymes [13–16,27,30,31]. In support of such studies, numerous assays that can measure DAGL activity in isolated membranes and/or lysates have been reported. These include

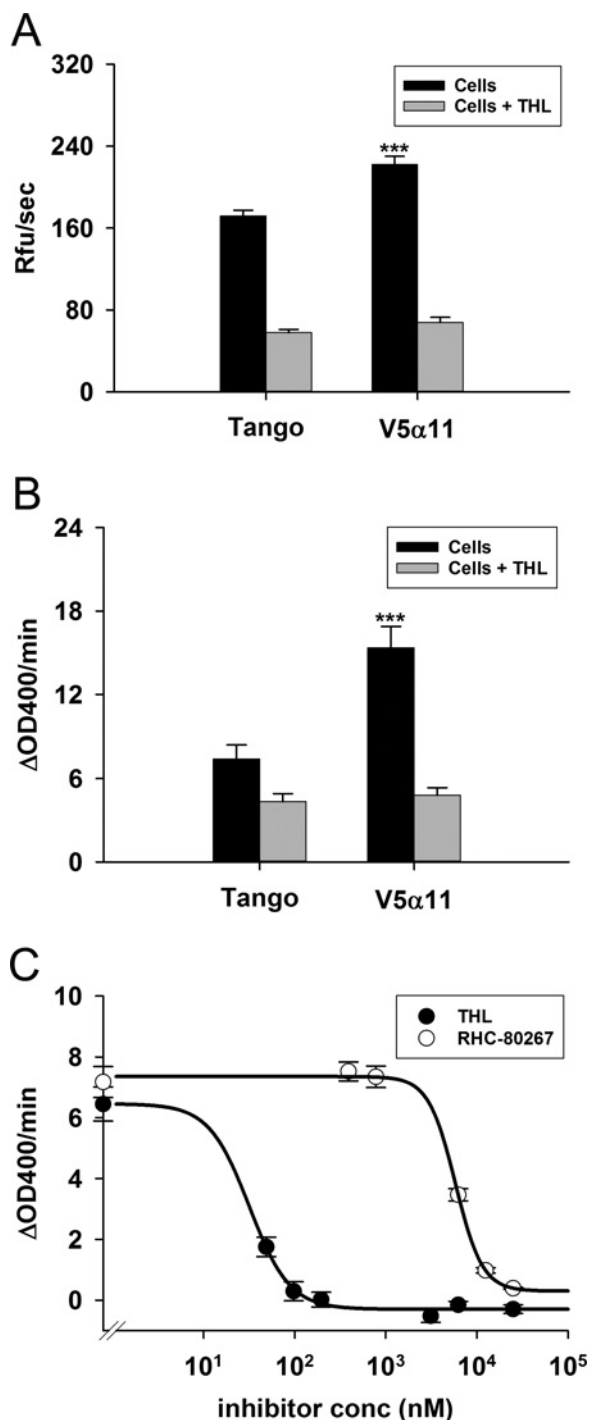


Figure 3 Live cell based assays to measure DAGL α activity

V5 α 11 or parental cells (40000 cells/well, seeded and maintained overnight in 96-well plates) were washed and then incubated in the presence or absence of THL (25 μ M) for 5 min. The activity was then measured using 10 μ M DiFMUO (mean \pm S.E.M.; V5 α 11, $n=16$; parental, $n=22$) (A) or 500 μ M PNPB (mean \pm S.E.M., $n=9$) (B). The difference in PNPB hydrolytic activity between the two cell lines was inhibited by the DAGL inhibitors RHC-80267 and THL (six replicate wells \pm S.E.M.) (C). The reaction rates were calculated over the first 10 min; *** $P < 0.001$ (two-tailed t test).

low-throughput MS based native substrate assays that employ synthetic DAG as a substrate coupled with the measurement of 2-AG as the product [40,41]. Low-throughput assays that utilize radiolabelled substrates or activity based probes have also been described [2,13,29–31,41]. Previously, relatively higher throughput surrogate substrate (fluorogenic or chromogenic), FRET (using ether lipid reporters) and native substrate (coupled enzyme) assays of particular utility for drug discovery programmes have been reported [26,28,29]. However, at present there are no simple assays that measure the activity of the DAGLs in a live cell context. Live cell assays are clearly of considerable value as they will act as a filter for drugs that have limited access to the cytoplasm in cells, and efficacy measurements will take into account this important variable. Furthermore, they present the target in a relatively native environment.

As a complement to the above mentioned assays, we report here the development of a simple live cell assay to measure DAGL α activity. This live cell assay does not involve a cell lysis step and employs the relatively economical chromogenic DAGL surrogate substrate PNPB [26]. Firstly, by establishing a U2OS cell line that stably and robustly expresses the DAGL α transgene, we have developed a tool to directly study DAGL α catalytic activity (using membranes or cells). Notably, the parental human cell line has also been previously modified to express a CB1 reporter construct (TangoTM CNR1-bla U2OS cells from Invitrogen) and therefore the V5 α 11 cells also have the potential to measure DAGL-dependent CB1 activation. In the present study, the difference in activity between the V5 α 11 and parental cells (or membranes) was higher when using the smaller chromogenic PNPB substrate (2.1- to 3.5-fold in the cellular assay, 6- to 11-fold in the membrane assay) in comparison with the larger fluorogenic substrate DiFMUO (≤ 1.8 -fold). Higher background enzymatic activity in the parental cells and/or generally lower DAGL α activity against DiFMUO when compared with PNPB may account for these differences. Pedicord et al. [26] suggested that DiFMUO may access DAGL α through the membrane whereas PNPB may access this enzyme through the aqueous phase in which case DiFMUO membrane diffusion rates could prove to be rate limiting and explain the differences observed in activity. DiFMUO (338 Da) is also a larger molecule when compared with PNPB (209 Da) and this may also influence DAGL α activity against this substrates as the active site of lipases are regulated by a displaceable lid like structure [42,43].

The DAGL α -V5 PNPB live cell assay described here provided a THL sensitive signal window of ~ 3.4 -fold but the difference in activity when comparing the two cell lines was considerably lower (2-fold) when including the THL insensitive activity. It is unlikely that endogenous DAGL α (or DAGL β) are major contributors towards the PNPB hydrolytic activity observed in the parental cells for the following reason. As the DAGLs are membrane proteins and both are inhibited by THL, any endogenous DAGL activity would have contributed towards the THL sensitive activity measured in the parental membranes which was however much lower than the activity attributed to the overexpressed DAGL α -V5 (10-fold greater). This is further

corroborated by the fact that DAGL α -V5 expression is 10- to 15-fold greater than endogenous DAGL α or DAGL β [reverse transcription polymerase chain reaction (RT-PCR) analysis; results not shown] and this along with the assay data indicate that cytoplasmic lipases/hydrolases are likely contributors towards the higher PNPB hydrolytic activity observed in the parental cells when compared with membranes. Candidate 'interfering' lipases include the hormone sensitive lipase and lipoprotein lipase which have reported PNPB hydrolytic activity [44,45]. In this context, as PNPB is a surrogate DAGL substrate and is also hydrolysed by some other lipases/hydrolases, it is unlikely that PNPB can be used to measure endogenous DAGL α activity in primary cells although it will still be tempting to measure PNPB hydrolytic activity in primary cells that are 'high' expressers of DAGL α (e.g. neurons) in conjunction with recently developed specific DAGL inhibitors in order to determine the contribution of DAGL α towards the measured activity [2,13,27,30]. In general though, PNPB is more suitable for measuring DAGL α activity in assays employing overexpressing cell lines as described here where the activity of the recombinant enzyme is several fold higher than the endogenous and/or background activities. Although the use of whole cells (or membranes as opposed to purified protein) preclude K_{cat} calculations, as is the case with other published DAGL assays [2,13,26,28,29], the assay reported here is continuous and employs a chromogenic substrate and so can be used to monitor inhibition kinetics (time course; different inhibitor and substrate concentrations) in a cellular environment [26]. Improving the signal window of the assay would be beneficial for such studies and would entail three approaches – (1) Increasing DAGL α -V5 activity which could be achieved by generating cell lines expressing higher amounts of DAGL α -V5. (2) Reducing non-DAGL α enzymatic activity which could be achieved by employing pharmacological tools to identify candidate 'interfering' enzymes and then genetic tools to disrupt their expression/activity. (3) Reducing non-enzymatic activity by further optimization of assay conditions/reagents, e.g. culture conditions, choice of plates, buffer components [46].

The DAGL α -V5 PNPB live cell assay described here could be applied as a primary or secondary assay for DAGL α drug discovery programmes or as a selectivity assay for DAGL β drug discovery programmes. In this context, DAGL α is responsible for almost all of the 2-AG in the brain and for CB1 receptor mediated retrograde signalling at the synapses [7,8]. Selectively targeting DAGL α may therefore prove fruitful for the treatment of certain conditions (e.g. obesity) as it has the potential to avoid the adverse psychiatric effects observed when targeting the CB1 receptor [16,25]. On the other hand, there is growing interest in DAGL β as a target for inflammatory diseases and maintaining selectivity of DAGL β inhibitors over DAGL α may help avoid unwanted modulation of synaptic signalling [13]. Similarly, the assay described here could also be applied as a selectivity assay for modulators targeting other members of the eCB system or closely related lipases.

In conclusion, the DAGL α overexpressing V5 α 11 cells and the chromogenic surrogate substrate, PNPB, provide the basis for relatively simple assays that can characterize the ability of

drugs to inhibit the activity of this enzyme in membranes and in a live cell context with utility as primary, secondary and selectivity assays for drug discovery programmes.

AUTHOR CONTRIBUTION

Patrick Doherty and Gareth Williams conceived and supervised the study; Patrick Doherty, Gareth Williams, Praveen Singh, Fiona Howell and Rachel Markwick designed experiments; Praveen Singh, Rachel Markwick and Fiona Howell performed experiments; Praveen Singh and Rachel Markwick analysed data; Praveen Singh and Patrick Doherty wrote the manuscript; Gareth Williams and Fiona Howell made manuscript revisions.

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