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Two-step activity-based protein profiling of diacylglycerol lipase†

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Diacylglycerol lipases (DAGL) produce the endocannabinoid 2-arachidonoylglycerol, a key modulator of neurotransmitter release. Chemical tools that visualize endogenous DAGL activity are desired. Here, we report the design, synthesis and application of a triazole urea probe for DAGL equipped with a norbornene as a biorthogonal handle. The activity and selectivity of the probe was assessed with activity-based protein profiling. This probe was potent against endogenous DAGLα (IC_{50} = 5 nM) and it was successfully applied as a two-step activity-based probe for labeling of DAGLα using an inverse electron-demand Diels–Alder ligation in living cells.

Introduction

Endocannabinoids are key regulators of neurotransmitter release in the central nervous system (CNS). They are involved in virtually every aspect of brain function, including modulation of synaptic plasticity and (patho)physiological processes, such as anxiety, fear and neuroinflammation.[1] 2-Arachidonoylglycerol (2-AG) is one of the most important endocannabinoids and activates the cannabinoid CB_1 and CB_2 receptors. 2-AG is synthesized by two diacylglycerol lipases (DAGLα (120 kDa) and DAGLβ (70 kDa)).[2] Both enzymes belong to the family of serine hydrolases, which share the same catalytic Ser-His-Asp triad to hydrolyse the *sn*-1 ester of 1-acyl-2-arachidonoylglycerides to generate 2-AG. A method to measure endogenous DAGL activity in biological samples is therefore important to understand endocannabinoid physiology.

Activity-based protein profiling (ABPP) is a powerful technique for monitoring enzyme activity in living systems using chemical probes.[3] These activity-based probes (ABPs) covalently and irreversibly bind to the active site of an enzyme and this interaction can be subsequently monitored using different techniques depending on the reporter group.[4] Several fluorescent ABPs have been reported to study the two isoforms of DAGLs. For example, HT-01 (Fig. 1), a DAGL probe based on 1,2,3-triazole urea inhibitors developed by the Cravatt laboratory, was used to study endogenous DAGLβ in (primary) macrophages.[5] In addition, DH379, based on the potent DAGL inhibitor DH376 (Fig. 1), was developed as a tailored fluorescent probe for DAGLα and DAGLβ.[6,7] However, reporter groups may affect the affinity and selectivity of the probes as well as cell permeability, metabolic stability, protein binding, oral bioavailability, brain penetration and toxicity. These issues are avoided by two-step probes in which ligation of the reporter group to the probe after covalent binding of the target. Bioorthogonal chemistry enables the design of chemical probes with a minimalist handle for the conjugation of a reporter group after the probe target has been bound.[8] These two-step bioorthogonal probes also provide flexibility, as different reporter groups can be attached to the same probe. Different pairs of bioorthogonal reactants are currently available.[9] For two-step activity-based probes, the most popular pair is the azide–alkyne couple. These handles are reacted

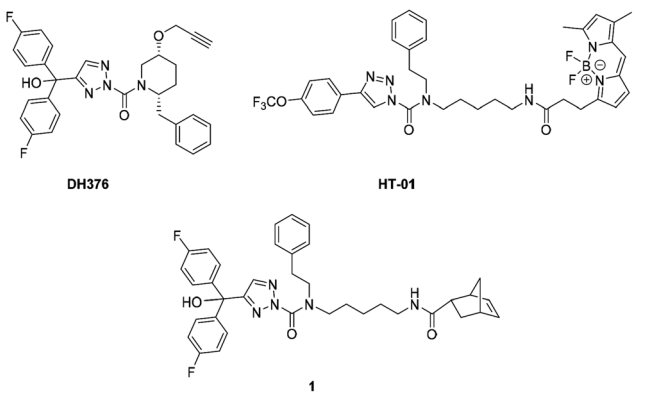

Fig. 1 Design of two-step labeling probe **1** based on HT-01 and DH376.

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using the copper-catalyzed azide–alkyne cycloaddition (CuAAC), often called "click" reaction. Both azides and alkynes are compact handles, chemically stable and synthetically accessible. An example of a two-step bioorthogonal probe for DAGL is DH376, which carries an alkyne handle.

The CuAAC is relatively slow and requires toxic Cu(I) as a catalyst, therefore the inverse electron-demand Diels–Alder (IEDDA) ligation is sometimes used as an alternative.[9] The reactants are an electron-rich dienophile, such as a norbornene, and electron poor diene, usually a tetrazine. Tetrazines attached to fluorophores can serve as both the bioorthogonal reactive group and the fluorescence quencher, creating fluorescence "turn-on" reporters ideal for imaging.[10,11] An additional advantage is that no catalyst is required for the IEDDA.

We here report the synthesis and characterization of two-step ABP **1** equipped with a norbornene (Fig. 1) to study its *in situ* labelling activity in comparison with direct probe HT-01.[12,13]

Results and discussion

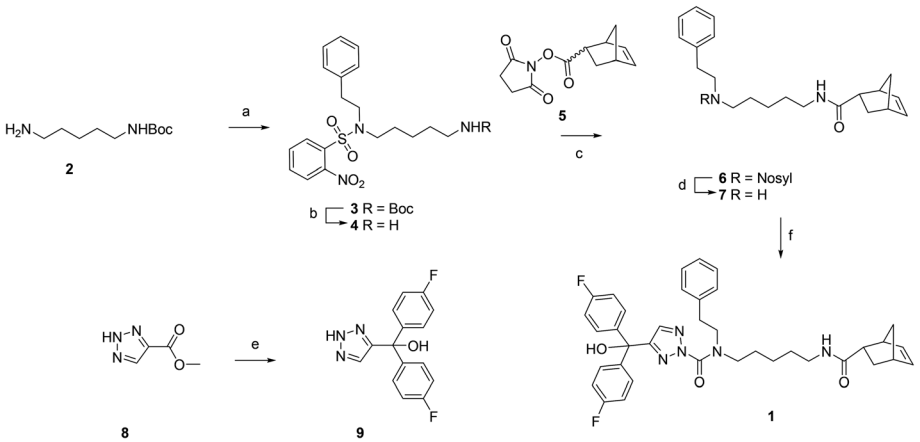
Synthesis

N-Boc-cadaverine (**2**) was first nosylated and reacted with phenethyl bromide to yield **3** (Scheme 1). The Boc-group of **3** was removed with acid to yield **4**, which was coupled to the activated norbornene ester **5** to give the amide **6** as the *endo*-isomer. Removal of the nosyl group yielded amine **7**. A Grignard reaction on ester **8** yielded triazole **9**. With the triazole **9** and amine **7** in hand, the final compound **1** was obtained using a triphosgene coupling.[14] Generally, this yields two regioisomers: N1 and N2. To assign the separate compounds as either N1-regioisomer or N2-regioisomer, it was anticipated that the NMR chemical shift of the triazole carbon could be used (ESI: Tables 1 and 2†). To this end, theoretical chemical shifts were computed with density functional theory (DFT) for simplified structures of the triazole urea scaffold (ESI: Table 1†). The chemical shift in DMSO was calculated for the lowest energy conformer of either the N1 or N2 regioisomer. This resulted in theoretical chemical shift differences of approximately 10 ppm between the triazole carbon of the N1 regiosiomer (±125 ppm) and N2 regioisomer (±135 ppm). In addition, the triazole proton is highly characteristic (broad, downfield peak) and HSQC experiments were used to confidently assign the triazole carbon peak in the ^{13}C aromatic region. Therefore, we synthesized and analyzed reference compounds (**11–13**, **22–24**, ESI: Fig. 1, Schemes 1 and 2, Tables 1 and 2†). The assignment of the reference isomers was in agreement with earlier reported triazole ureas as determined with a crystal structure or NMR measurements. Thus, this analysis allowed us to assign ABP **1** as a N2 regioisomer.

Biochemical analysis

The potency and selectivity of probe **1** was profiled in mouse brain proteome using ABPP with MB064, FP-TAMRA and DH379 as chemical probes (Fig. 2). Probe **1** showed a dose-dependent inhibition of DAGLα and DAGLβ with a pIC_{50} of 8.3 ± 0.3 and 8.6 ± 0.1, respectively (Fig. 2b and d) In addition, *in situ* experiments were performed with probe **1** (Fig. 2e) using the human cell line U2OS transiently transfected with recombinant human DAGLα. Live cells were treated with **1** and post-lysis labeled with MB064. Probe **1** was able to cross the cell membrane and label human DAGLα, albeit with a ten-fold lower potency compared to *in vitro* mouse brain proteome (Fig. 2f). Of note, probe **1** also inhibited the post-lysis labeling of endogenous α,β-hydrolase domain containing enzyme (ABHD6) (Fig. 2e, the band around 35 kDa) with a pIC_{50} of 8.5 ± 0.3 (Fig. 2f). This discrepancy between *in situ* and *in vitro* potency has been previously observed for other covalent, irreversible serine hydrolase inhibitors.[6,15]

To test if our norbornene probe **1** reacted with tetrazine **10** (See ESI†) *via* the expected IEDDA mechanism, the reaction



Scheme 1 Synthesis of probe **1**. Reagents and conditions: (a) i. NsCl, Et_3N, THF; ii. $Ph(CH_2)_2Br$, Cs_2CO_3, CH_3CN, 80 °C, 92%; (b) TFA/DCM 1 : 9, 100%; (c) DIPEA, DMF, 33%. (d) PhSH, Cs_2CO_3, CH_3CN, 41%; (e) 4-fluorophenylmagnesium bromide, THF, 74%; (f) i. triphosgene, DIPEA, THF, 0 °C; ii. **9**, DIPEA, DMAP, THF, 60 °C, 37%.

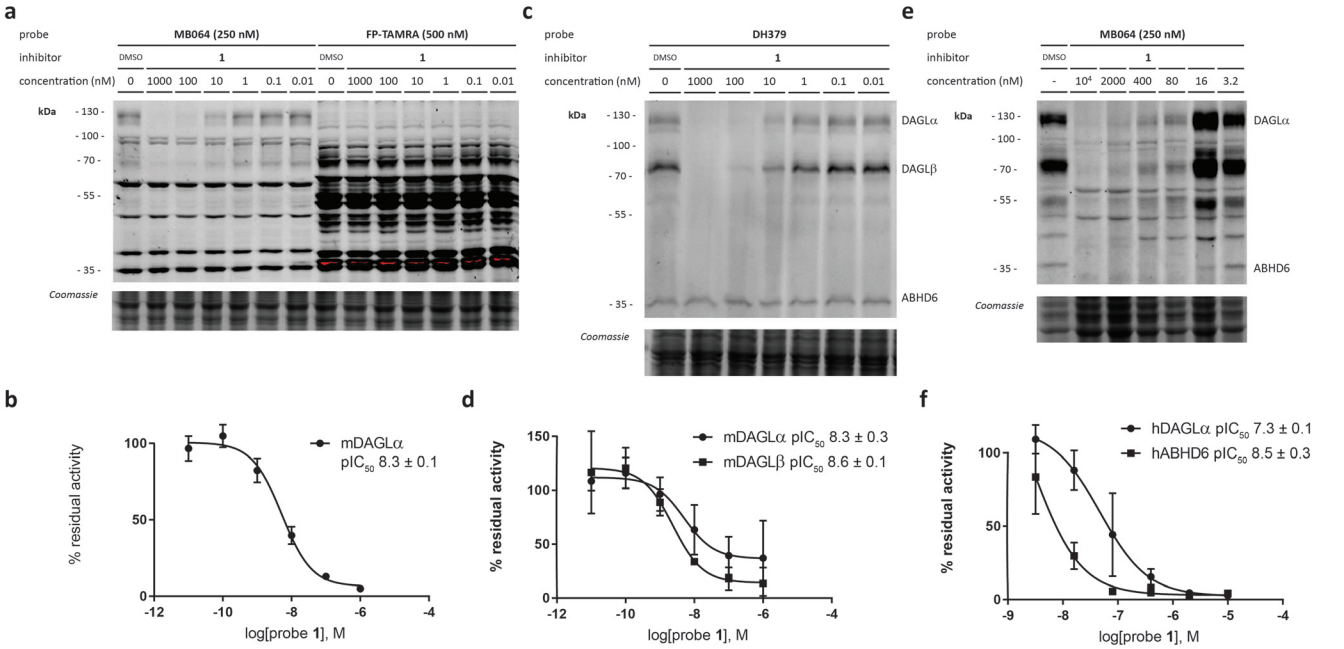


Fig. 2 Activity-based protein profiling of probe **1** in mouse brain membrane proteome against (a) MB064 and FP-TAMRA and (c) DH379. (b) Quantification of residual DAGLα activity as measured with MB064 in mouse brain. (d) Quantification of residual DAGLα and DAGLβ activity as measured with DH379 in mouse brain. (e) *In situ* treatment with **1** of U2OS cells transfected with DAGLα. (f) Quantification of residual DAGLα and ABHD6 activity as measured with MB064 in U2OS-DAGLα cells.

products were analyzed by LC-MS. The mass of the proton adduct of the expected reaction products was observed (ESI: Fig. 3†).

Next, the toxicity of probe **1** to living cells was evaluated and compared to existing probes HT-01 and DH376. U2OS cells were treated with each probe separately and a live cell count was performed (Fig. 3). Under the conditions tested, none of the probes were toxic to cells. To evaluate the toxicity

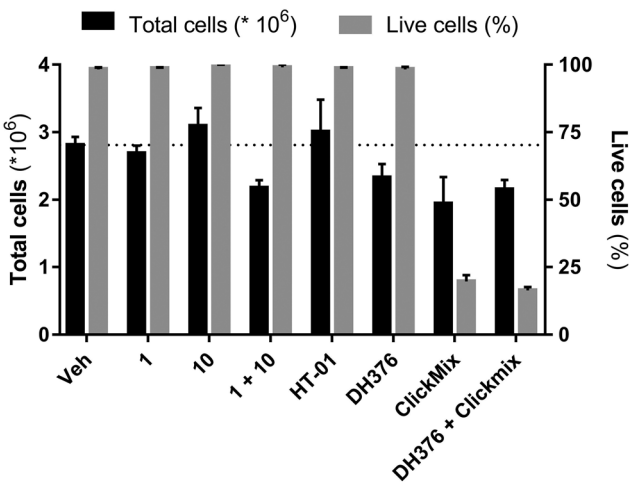


Fig. 3 Cell survival of wildtype U2OS, treated for 1 h at 37 °C with: probe **1** (5 μM), BODIPY-tetrazine **10** (10 μM), probe HT-01 (1 μM), DH376 (1 μM), or ClickMix.

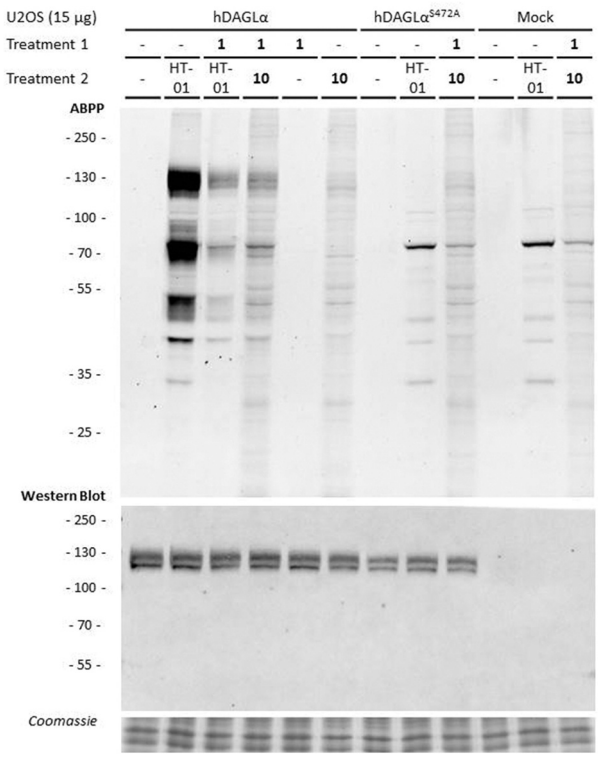


Fig. 4 *In situ* labeling of recombinant DAGLα expressed in U2OS with direct probe HT-01 (1 μM), probe **1** (5 μM) with BODIPY-tetrazine **10** (10 μM), and competition between probe **1** (5 μM) and HT-01 (1 μM). All treatments: 1 h *in situ* at 37 °C. Western blot (anti-FLAG) is shown as a protein expression control, Coomassie staining is shown as a loading control.

of two-step labeling in living cells, tetrazine **10** was also tested and appeared to be non-toxic. Cells also survived the combination of probe **1** and tetrazine **10** treatment. For the CuAAC reaction conditions however (ClickMix alone or with DH376), almost 75% of the treated cells died within 1 h. This result illustrates the advantages of IEDDA chemistry compared to CuAAC for live cell labeling experiments.

Finally, *in situ* two-step labeling was performed with fluorogenic BODIPY-tetrazine **10** (Fig. 4).[11] U2OS cells were transfected with either human DAGLα, catalytically inactive DAGLα^{S472A} or a mock control. The two-step labeling was compared to direct labeling with probe HT-01, which resulted in a strong fluorescent band just below 130 kDa, which was absent in the DAGLα^{S472A} and mock controls. Pre-treatment of the cells with probe **1** partially blocked the labeling with HT-01, suggesting probe **1** does not fully inhibit DAGLα. Treatment with norbornene probe **1**, followed by *in situ* treatment with tetrazine **10** resulted in partial labeling of DAGLα, which was absent in the DAGLα^{S472A} and mock controls. Treatment of cells expressing DAGLα with tetrazine **10** showed some background labeling. The background labeling pattern in the DAGLα^{S472A} and mock controls was similar. In conclusion, norbornene probe **1** reacted to the catalytic serine of DAGLα in live cells and can be labeled with a tetrazine fluorophore, but its fluorescent signal is weaker compared to labeling with direct probe HT-01, which might be due to a difference in the fluorescent reporter group and/or the efficiency of labeling.

Conclusions

Norbornene probe **1** was successfully synthesized as a two-step ABP for labeling of DAGLα using an IEDDA ligation in living cells and compared to a click reaction and direct probe HT-01. The IEDDA reaction is complementary to the CuAAC reaction for labeling DAGLα, but is preferred for *in situ* imaging of enzyme activity (due to its low cellular toxicity).[16] However, the labeling efficiency of direct probe HT-01 is higher than the two-step probe **1**. Therefore, additional probes with improved activity and bioorthogonal handles with faster reaction rates should be made to study endogenous DAGL activity. For live cell imaging, fluorogenic tetrazines with longer wavelengths than **10** are required.[17] It is envisioned that live cell imaging of DAGL activity will enable the study of this endocannabinoid enzyme's localization and processing during differentiation and other cellular processes.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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