



Universiteit
Leiden
The Netherlands

Discovery of selective diacylglycerol lipase β inhibitors

Zhu, N.

Citation

Zhu, N. (2024, May 22). *Discovery of selective diacylglycerol lipase β inhibitors*. Retrieved from <https://hdl.handle.net/1887/3754188>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3754188>

Note: To cite this publication please use the final published version (if applicable).

Chapter 5

Profiling DAGL inhibitors in the GRAB_{eCB2.0} assay reveals the primary role of DAGL α in ATP-stimulated 2-AG production in Neuro2A

5.1 Introduction

The endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA) are widely distributed throughout central and peripheral nervous systems, where they play physiological roles primarily by activating cannabinoid receptors type 1 and 2 (CB₁R and CB₂R).¹ Endocannabinoids, cannabinoid receptors and enzymes responsible for the biosynthesis and degradation of endocannabinoids constitute the endocannabinoid system (ECS). In the brain, endocannabinoids are produced and released from the postsynaptic terminal upon demand² to activate CB₁R on the presynaptic terminal. This process is distinct from classical neurotransmitters that are stored in synaptic vesicles and released from presynaptic compartments.^{3,4} In addition, glial cells⁵ and intracellular organelles such as mitochondria^{6,7} also contain components of the endocannabinoid system.

The biosynthesis of 2-AG is primarily mediated by *sn*1-specific diacylglycerol lipases (DAGLs), after which 2-AG is degraded by monoacylglycerol lipase (MAGL) and α/β -hydrolase domain-containing 6 and 12 (ABHD6/12) to form arachidonic acid and glycerol.⁸ The production and degradation of 2-AG are tightly regulated to maintain 2-AG levels. To qualitatively and quantitatively measure 2-AG levels, liquid chromatography coupled to mass spectrometry (LC/MS) is commonly employed.^{9,10} Despite being highly sensitive, this method requires sample preparation and lipid extraction, limiting its application in living cells and organisms and resulting in poor temporal and spatial resolution. In recent years, various genetically encoded fluorescent sensors^{11–13} based on G protein-coupled receptors (GPCRs) and circular-permuted fluorescent proteins (cpFPs) have been developed. These sensors enable the real-time detection of neurotransmitters and neuromodulators.¹⁴ This technology relies on the conformational change¹⁵ of the specific receptors induced by the binding of the ligands to elicit fluorescence of cpFP. To detect the dynamics of endocannabinoids, Yulong Li's lab recently developed a GPCR activation-based endocannabinoid sensor called GRAB_{eCB2.0} (Figure 5.1) based on human CB₁R and circular permuted enhanced green fluorescent protein (cpEGFP).¹⁶ The GRAB_{eCB2.0} sensor exhibits high specificity and rapid kinetics for 2-AG and AEA, and it has been successfully applied in cultured neurons, brain slices and living animals.^{16–19}

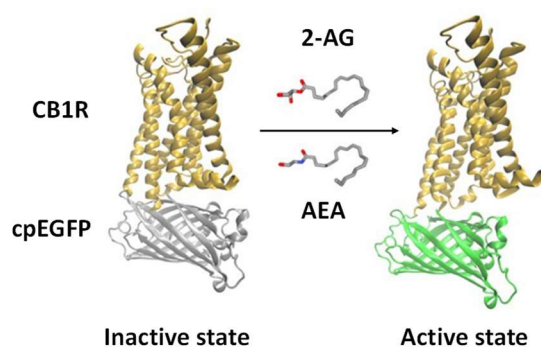


Figure 5.1 Schematic illustration of GRAB_{eCB2.0} activation upon the binding of 2-AG and AEA.¹⁶ The figure was adapted from Dong, A. *et al. Nat. Biotechnol.* **40**, 787-798 (2021).

In view of the successful applications of GRAB_{eCB2.0} in monitoring the 2-AG dynamics, we aimed to develop a fluorescence-based plate reader assay by using this sensor to evaluate the cellular activity of DAGL inhibitors. To achieve this, GRAB_{eCB2.0} was initially transiently transfected into mouse neuroblastoma cells (Neuro2A). The success of transient transfection and expression was confirmed by EVOS fluorescent imaging and kinetic fluorescent measurement in the plate reader. The sensor demonstrated direct activation upon the addition of 2-AG and AEA, as well as indirect activation by adding adenosine triphosphate (ATP) to the medium. Subsequently, a total of 23 DAGL inhibitors were profiled in the GRAB_{eCB2.0} assay using Neuro2A cells stably expressing the sensor. This profiling revealed the primary role of DAGL α in ATP-stimulated 2-AG production.

5.2 Results and discussion

5.2.1 Transient expression and activation of the GRAB_{eCB2.0} in Neuro2A

To express the GRAB_{eCB2.0} sensor, Neuro2A cells were transfected with eCB2.0 plasmid. At the same time, inactive eCBmut, green fluorescent protein (GFP) and an empty vector were transfected as well to serve as controls. Strong green fluorescence was observed for Neuro2A cells transfected with GFP (Figure 5.2), indicating the success of transfection and protein expression. Green fluorescence was also detectable in cells transfected with eCB2.0, which proved that the sensor was successfully expressed in the cells and the basal level of 2-AG was able to partially activate the sensor. In contrast, the basal fluorescent signal in cells expressing inactive eCBmut was low, and no fluorescence was detected in mock transfected and untransfected Neuro2A cells. Addition of a synthetic agonist of the CB₁R, CP55,940 (1 μ M), increased fluorescence in the eCB2.0 transfected but not in the eCBmut transfected cells (Figure 5.2, bottom row). The fluorescent signal was different in each Neuro2A cell because of the heterogeneity of transient expression.

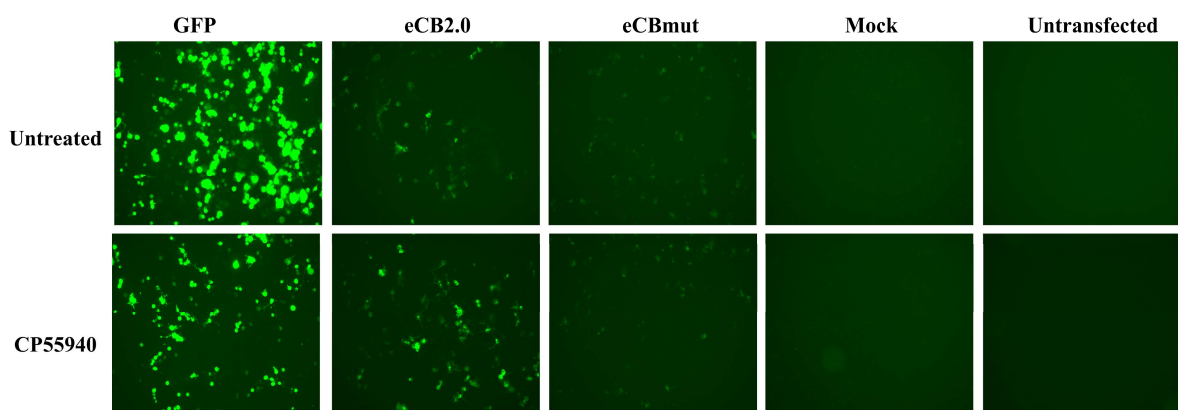


Figure 5.2 EVOS microscopy images of Neuro2A cells with different transient transfections before and after adding CB₁R agonist CP55,940.

To increase the throughput, the Neuro2A GRAB_{eCB2.0} assay was adapted for a 96-well plate format. The procedure involved measuring the baseline fluorescent signal of transiently expressed GRAB_{eCB2.0} in Neuro2A cells. Following this, agents were added and kinetic fluorescence measurement started immediately. Endocannabinoids 2-AG and AEA induced a concentration-dependent activation of GRAB_{eCB2.0} as shown in Figure 5.3A and B. The fluorescent signal peaked within 2 min, followed by a decline and stabilization at a plateau response. 2-AG, a full agonist of CB₁R, increased the fluorescence by a maximum of 3-fold, which was significantly higher than the partial agonist AEA (1.5-fold). The area under the curves (AUC, Figure 5.3C, D) indicated concentration-dependent activation of GRAB_{eCB2.0} in Neuro2A, with negative logarithms of half-maximal effective concentrations (pEC₅₀) of 6.37 ± 0.29 and 6.16 ± 0.19 for 2-AG and AEA, respectively (Figure 5.3E). Stimulation of calcium-permeable ionotropic receptors by ATP (1 mM) resulted in a pronounced increase in the fluorescence by 0.9-fold at maximum within 10 min, followed by a continuous decline (Figure 5.3F). This effect was completely blocked by CB₁R antagonist rimonabant (1 μ M). Together, these results demonstrate that changes in GRAB_{eCB2.0} fluorescence could be reliably measured in a 96-well plate reader to detect stimulus-induced 2-AG production.

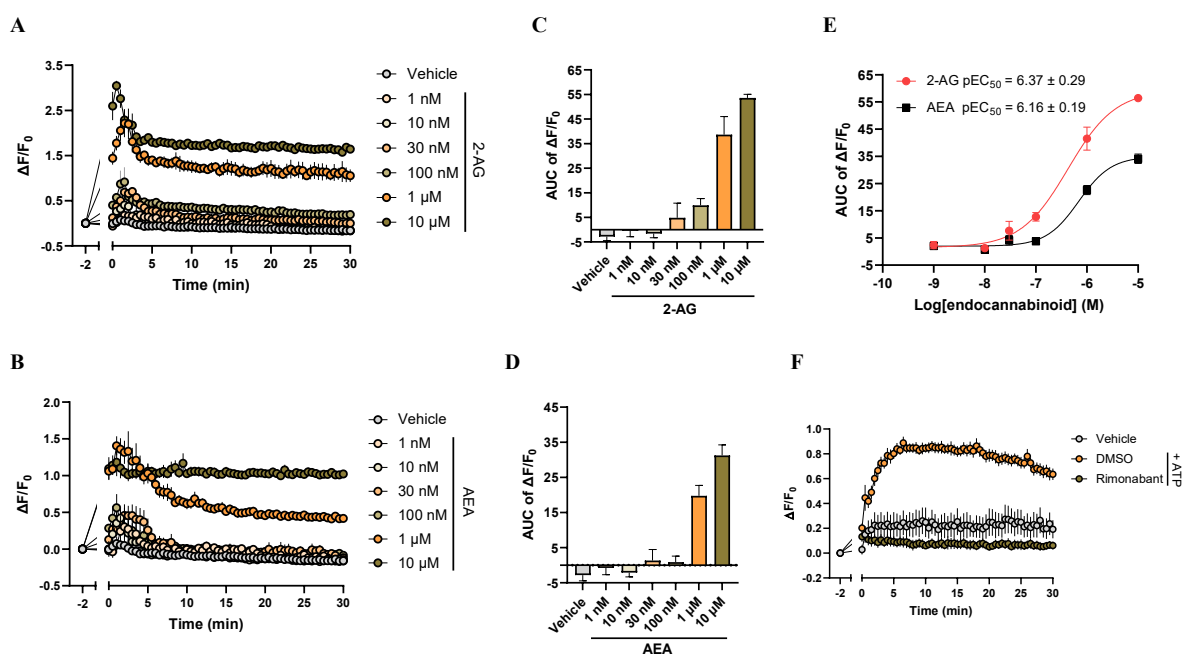


Figure 5.3 The fluorescent detection of GRAB_{eCB2.0} transiently expressed in Neuro2A by a 96-well plate reader upon different stimuli. (A, B) Time course of $\Delta F/F_0$ for the vehicle and different concentrations of 2-AG (A) and AEA (B). (C, D) Area under the curve (AUC) of $\Delta F/F_0$. (E) Concentration-response curves of 2-AG and AEA. (F) Time course of $\Delta F/F_0$ for the vehicle and ATP stimulation with and without CB₁R antagonist rimonabant. Data shown are mean $\pm$ SEM (n = 3-6).

5.2.2 Profiling DAGL inhibitors in the Neuro2A GRAB_{eCB2.0} assay

To evaluate the potential of the developed GRAB_{eCB2.0} assay for assessing the cellular activity of DAGL inhibitors, Neuro2A cells stably expressing GRAB_{eCB2.0} (Neuro2A GRAB_{eCB2.0}) were established and the inhibitory effect of DAGL inhibitors DH376 and LEI-106 on ATP-

stimulated 2-AG production was investigated. To this end, Neuro2A GRAB_{eCB2.0} were seeded in a clear-bottom 96-well plate a day before the treatment. The cells were then treated with different concentrations of inhibitors or DMSO for 1 h after which the baseline fluorescence was measured. Subsequently, ATP or MilliQ (vehicle) was added and the kinetic fluorescence measurement started immediately. ATP induced a rapid and transient fluorescent signal, which was blocked by DH376 and LEI-106 in a concentration-dependent manner (Figure 5.4A, B). At the highest tested concentrations, DH376 (1 μ M and 100 nM) and LEI-106 (10 μ M) showed lower $\Delta F/F_0$ and AUC values than the vehicle control (Figure 5.4C, D), which might be indicative of constitutive 2-AG production. Based on AUC values, the residual activity of DAGL was calculated and plotted to obtain the concentration-response curves (Figure 5.4E, F), which showed that DH376 and LEI-106 inhibited endogenous DAGL in Neuro2A with a pEC₅₀ of 7.51 ± 0.20 and 5.82 ± 0.12 , respectively. The activity of DH376 and LEI-106 in Neuro2A was significantly lower than their biochemical activity (Table 1), possibly due to the different affinity of these compounds to mouse and human DAGL as well as cellular permeability. Notably, the carboxyl group in LEI-106 may limit cellular permeability at physiological pH.

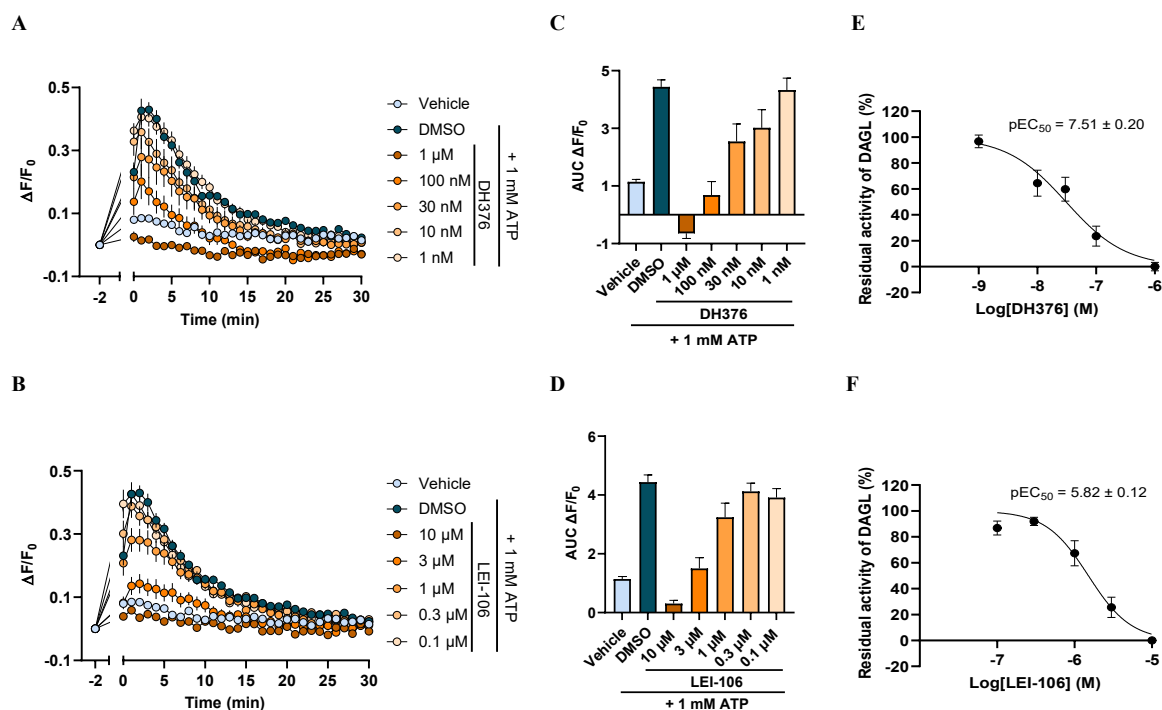


Figure 5.4 The evaluation of DAGL inhibitors DH376 and LEI-106 in the Neuro2A GRAB_{eCB2.0} assay. (A, B) Time course of $\Delta F/F_0$ for the vehicle, DMSO and different concentrations of DH376 (A) and LEI-106 (B) upon ATP stimulation. (C, D) Area under the curve (AUC) of $\Delta F/F_0$. (E, F) Concentration-response curves of DH376 (E) and LEI-106 (F) with their pEC₅₀ values. Data shown are mean $\pm$ SEM (n = 5, N = 2).

The structure-activity relationship of glycine sulfonamides as inhibitors of DAGL α and DAGL β was investigated in depth in Chapter 3 and 4, leading to the discovery of numerous inhibitors with varying biochemical activity and DAGL subtype selectivity. To assess their cellular inhibitory activity for DAGLs, a diverse set (chemical structures in Supplementary Figure S5.1) were tested in the Neuro2A GRAB_{eCB2.0} assay within a concentration range from

10 μM to 0.1 μM . The tested inhibitors were categorized into two classes based on selectivity: non-selective DAGL and DAGL β selective inhibitors. The concentration-response curves of non-selective DAGL inhibitors are shown in Figure 5.5 and a summary of their biochemical and cellular results is presented in Table 5.1. Similar to LEI-106, these inhibitors exhibited lower cellular activity compared to their biochemical activity. Compounds **1-5** demonstrated cellular activity with a pEC_{50} higher than 6. Among them, compound **1** displayed the highest inhibitory activity with a pEC_{50} of 6.48 ± 0.12 . Compounds **6-11** were less active than LEI-106 with a pEC_{50} lower than 6. From these results, a general trend was observed that the inhibitor with a higher affinity for DAGL α would show a higher cellular activity. Notably, cellular activity can also be influenced by other factors such as lipophilicity and permeability.

The concentration-response curves of DAGL β selective inhibitors are depicted in Figure 5.6 and their biochemical and cellular results are summarized in Table 5.2. In general, DAGL β selective inhibitors showed lower cellular activity compared with the non-selective ones. Compounds **12-15** demonstrated activity in the Neuro2A GRAB $_{\text{eCB}2.0}$ assay with cellular activity in the micromolar range. However, the standard deviation of pEC_{50} of compounds **13-15** could not be determined due to the steep slope of the concentration-response curves. Despite being highly active for DAGL β in the biochemical assay, compounds **16, 17, 19** and **20** were inactive ($\text{pEC}_{50} < 5$) in the Neuro2A GRAB $_{\text{eCB}2.0}$ assay. In contrast, compounds **18** and **21** showed some cellular activity.

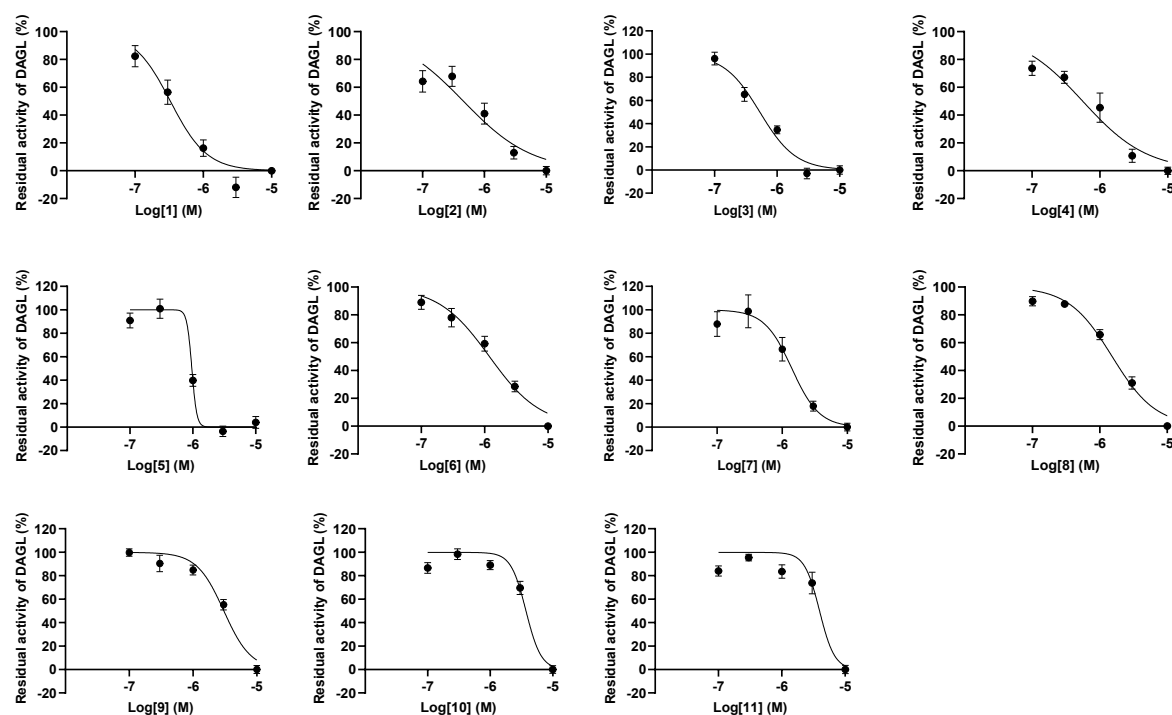


Figure 5.5 Concentration-response curves of non-selective DAGL inhibitors **1-11**. Data shown are mean $\pm$ SEM ($n = 5$, $N = 2$).

Table 5.1 Biochemical and cellular results of non-selective DAGL inhibitors **1-11**.^a

Compound	pIC ₅₀ DAGL β	pIC ₅₀ DAGL α	Apparent selectivity	cLogD	tPSA (Å ²)	pEC ₅₀
DH376	8.79 ± 0.08	8.90 ± 0.04	0.8	4.9	80	7.51 ± 0.20
LEI-106	6.69 ± 0.14	7.35 ± 0.06	0.2	1.9	104	5.82 ± 0.12
1	7.22 ± 0.17	7.60 ± 0.08	0.4	4.5	95	6.48 ± 0.12
2	7.01 ± 0.09	7.41 ± 0.09	0.4	3.8	95	6.34 ± 0.18
3	7.22 ± 0.12	8.17 ± 0.21	0.1	3.8	95	6.28 ± 0.10
4	7.34 ± 0.18	7.89 ± 0.11	0.3	2.3	95	6.25 ± 0.15
5	7.36 ± 0.10	7.75 ± 0.11	0.4	3.2	104	6.02 ^b
6	7.73 ± 0.09	7.83 ± 0.09	0.8	4.5	95	5.93 ± 0.11
7	6.96 ± 0.10	7.36 ± 0.14	0.4	4.2	95	5.85 ± 0.13
8	7.17 ± 0.10	7.20 ± 0.03	0.9	2.3	95	5.82 ± 0.07
9	7.07 ± 0.11	7.19 ± 0.04	0.8	3.0	95	5.52 ± 0.08
10	6.66 ± 0.07	6.91 ± 0.05	0.6	2.3	95	5.43 ± 0.08
11	6.66 ± 0.18	6.82 ± 0.11	0.7	4.5	95	5.40 ± 0.10

^aThe negative logarithms of half maximal inhibitory concentration (pIC₅₀) and half maximal effective concentration (pEC₅₀) were determined in DAGL EnzChek lipase substrate assay and Neuro2A GRAB_{eCB2.0} assay, respectively. The calculated logarithm of the *n*-octanol-water partition coefficient at pH 7.4 (cLogD) and the topological polar surface area (tPSA) were calculated using DataWarrior 5.0.0. ^bThe pEC₅₀ could not be reliably determined due to the steep slope of the concentration-response curves.

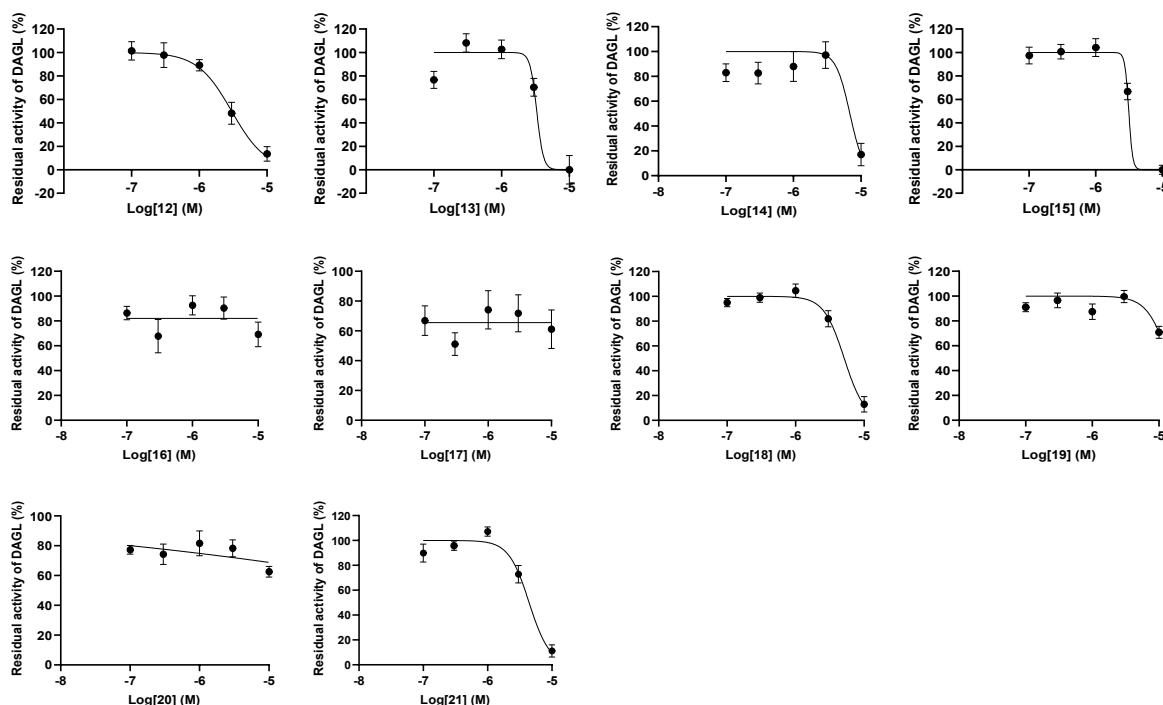


Figure 5.6 Concentration-response curves of DAGL β selective inhibitors **12-21**. Data shown are mean ± SEM (n = 5, N = 2).

Table 5.2 Biochemical and cellular results of DAGL β selective inhibitors **12-21**.^a

Compound	pIC ₅₀ DAGL β	pIC ₅₀ DAGL α	Apparent selectivity	cLogD	tPSA (Å ²)	pEC ₅₀
12	7.89 ± 0.12	7.22 ± 0.04	4.7	2.3	86	5.52 ± 0.14
13	7.85 ± 0.14	7.03 ± 0.03	6.6	2.9	99	5.48 ^b
14	7.61 ± 0.12	6.50 ± 0.12	13	2.9	104	5.17 ^b
15	8.29 ± 0.08	7.15 ± 0.08	14	3.2	104	5.50 ^b
16	7.59 ± 0.06	6.29 ± 0.05	20	1.1	104	< 5 ^c
17	7.48 ± 0.09	5.93 ± 0.16	36	1.4	104	< 5 ^c
18	7.96 ± 0.06	6.37 ± 0.05	39	2.5	104	5.29 ± 0.09
19	7.94 ± 0.08	6.34 ± 0.09	40	2.2	104	< 5 ^c
20	7.88 ± 0.09	6.27 ± 0.07	41	1.9	104	< 5 ^c
21	8.07 ± 0.09	6.36 ± 0.05	51	2.3	104	5.35 ± 0.10

^aThe negative logarithms of half maximal inhibitory concentration (pIC₅₀) and half maximal effective concentration (pEC₅₀) were determined in DAGL EnzChek lipase substrate assay and Neuro2A GRAB_{eCB2.0} assay, respectively. The calculated logarithm of the *n*-octanol-water partition coefficient at pH 7.4 (cLogD) and the topological polar surface area (tPSA) were calculated using DataWarrior 5.0.0. ^bThe pEC₅₀ could not be reliably determined due to the steep slope of the concentration-response curves. ^cThe compound was inactive in Neuro2A GRAB_{eCB2.0} assay.

5.2.3 DAGL α is responsible for ATP-stimulated 2-AG production in Neuro2A

The cellular activity of DAGL inhibitors in the Neuro2A GRAB_{eCB2.0} assay enabled a correlation analysis between biochemical and cellular activities to elucidate the isoform of DAGL involved in ATP-stimulated 2-AG production. For this purpose, the pEC₅₀ values of the inhibitors obtained in the Neuro2A GRAB_{eCB2.0} assay were plotted against the biochemical pIC₅₀ values of the inhibitors for DAGL α or DAGL β . A significant correlation between the pEC₅₀ values and the pIC₅₀ values for DAGL α was observed with a Pearson correlation coefficient (Pearson *r*) of 0.91 (*p* < 0.0001) (Figure 5.7A). Generally, inhibitors with higher affinity for DAGL α exhibited better inhibitory activity in the Neuro2A GRAB_{eCB2.0} assay. In contrast, there was a low correlation (Pearson *r*) of -0.04 (*p* = 0.87) between the pEC₅₀ values and the pIC₅₀ values for DAGL β , as indicated by the scattered data points in Figure 5.7B. The non-selective and DAGL β selective inhibitors were clustered separately, with DAGL β selective inhibitors tending to have lower pEC₅₀ than non-selective ones. Additionally, the correlation between pEC₅₀ values and physiochemical properties of inhibitors, such as cLogD (representing lipophilicity) and tPSA (representing topological polar surface area), was analyzed (Figure 5.7C, D). The pEC₅₀ exhibited a significant positive correlation with cLogD while displaying a negative correlation with tPSA. In this context, it cannot be ruled out that the reduced cellular activity of DAGL β selective inhibitors in the Neuro2A GRAB_{eCB2.0} assay could be attributed to generally lower lipophilicity and higher tPSA resulting in reduced cell permeability of these compounds compared to the non-selective inhibitors. Despite LEI-106 and compound **20** sharing the same tPSA and cLogD values, LEI-106 demonstrated activity in the Neuro2A GRAB_{eCB2.0} assay

while compound **20** was inactive. Furthermore, compounds **14**, **15**, **18**, **19**, and **21**, possessing the same tPSA and higher cLogD compared to LEI-106, all exhibited significantly lower cellular activity than LEI-106. The reduced cellular activity in these cases was dominantly attributed to the lower potency of these compounds for DAGL α compared to LEI-106. Consequently, it can be concluded that ATP-stimulated 2-AG production in Neuro2A cells is most likely mediated by DAGL α rather than DAGL β . Previous immunostaining studies provided valuable insights into the cellular localization of DAGL α and DAGL β in Neuro2A cells.²⁰ These studies revealed that DAGL α was located on plasma membranes, whereas DAGL β was predominantly found in perinuclear lipid droplets. The distinction in localization between DAGL α and DAGL β may offer a plausible explanation for DAGL α 's role in ATP-stimulated 2-AG production.

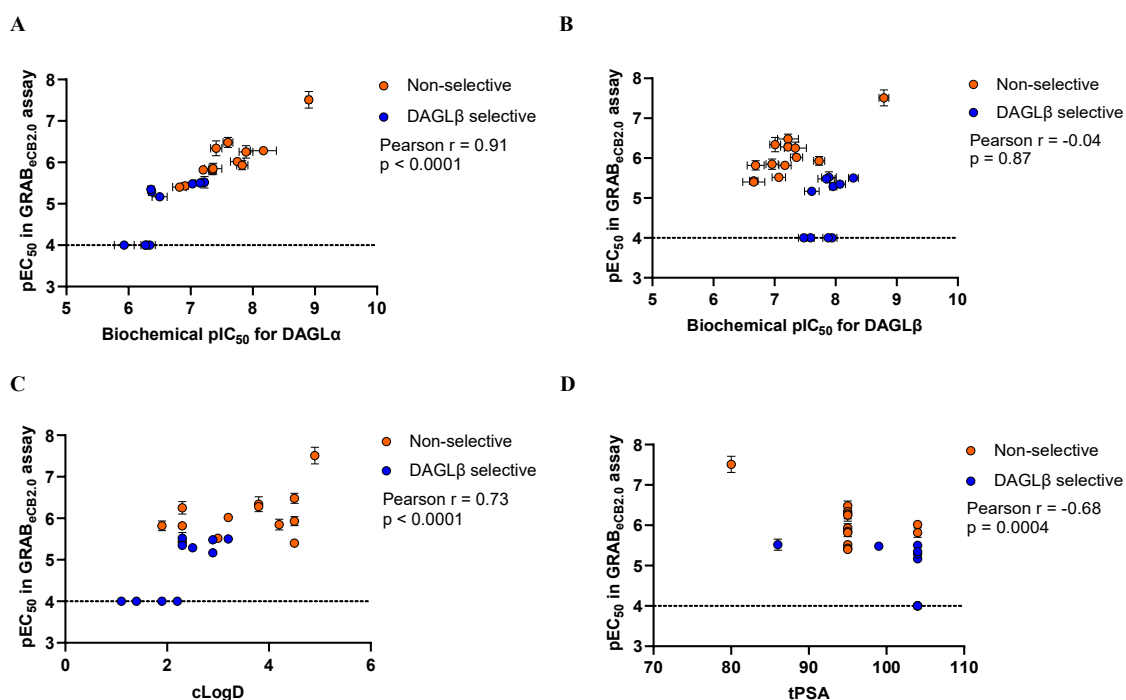


Figure 5.7 Correlation plots. (A) pEC₅₀ vs biochemical pIC₅₀ for DAGL α . (B) pEC₅₀ vs biochemical pIC₅₀ for DAGL β . (C) pEC₅₀ vs cLogD. (D) pEC₅₀ vs tPSA. The pEC₅₀ value for compounds that were inactive (pEC₅₀ < 5) in the Neuro2A GRAB_{eCB2.0} assay is set at 4.

5.3 Conclusion

In this Chapter, the GRAB_{eCB2.0} sensor, whether transiently or stably expressed in Neuro2A cells, demonstrated exquisite sensitivity to the concentrations of 2-AG. This facilitated the kinetic measurement of ATP-induced 2-AG production in a 96-well plate format and the assessment of DAGL inhibitors in inhibiting 2-AG production. A total of 23 DAGL inhibitors with diverse activity and isoform selectivity were evaluated in the Neuro2A GRAB_{eCB2.0} assay. These inhibitors concentration-dependently blocked the production of 2-AG by inhibiting DAGL with varying potencies. Correlation analysis revealed that the cellular activity of these inhibitors in the Neuro2A GRAB_{eCB2.0} assay was influenced by their lipophilicity, permeability

and potency for DAGL α . Parallel comparison of the cellular activity of DAGL β selective inhibitors and non-selective DAGL inhibitor LEI-106 with similar tPSA and cLogD values enables the conclusion that DAGL α is the dominant isoform in Neuro2A responsible for 2-AG production upon ATP stimulation. These findings further underscore the importance of isoform-specific DAGL inhibitors in unraveling the intricate mechanisms of DAGL and their roles in biological processes.

5.4 Acknowledgements

Prof. dr. Nephi Stella is gratefully acknowledged for providing plasmids of eCB2.0 and eCBmut. Tom van der Wel and Verena Straub are kindly acknowledged for generating the Neuro2A cell line stably expressing GRAB_{eCB2.0}. Matthijs R. van Wijngaarden is kindly acknowledged for his contribution to profiling the DAGL inhibitors in the Neuro2A GRAB_{eCB2.0} assay.

5.5 Experimental methods

Cell culture

Wild type Neuro2A and Neuro2A GRAB_{eCB2.0} cells were cultured at 37 °C under 7% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich) containing phenol red, GlutaMax (2 mM), penicillin/streptomycin (200 μ g/mL each, Duchefa) and 10% newborn calf serum (Thermo Fischer). Cells were passaged every 3 to 4 days and for no longer than 25 generations and the medium was refreshed every 2 to 3 days. Blasticidin S was added to the cultured Neuro2A GRAB_{eCB2.0} cells once a week at a concentration of 5 μ g/mL.

EVOS fluorescent microscopic imaging of Neuro2A

One day before transfection, 3×10^4 Neuro2A cells were seeded to each inner well of a clear-bottom black 96-well plate (Greiner-Bio-One, REF 655090). The outer wells were filled with 100 μ L PBS (Sigma Aldrich). The cells were incubated at 37 °C under 7% CO₂ for 24 h. After that, 40 μ L of medium was removed and cells were transfected with 0.3 μ g PEI and 0.1 μ g DNA (eCB2.0, eCBmut, GFP or empty vector) in 10 μ L of serum-free DMEM. 24 h post-transfection, the medium was replaced with 100 μ L PBS containing 1 mM CaCl₂ and 0.55 mM MgCl₂ and cells were incubated at rt for 20 min. Background fluorescence images were taken on Invitrogen EVOS FL Auto 2 in GFP channel. 1 μ L of CB₁R agonist CP55,940 in DMSO (final concentration 1 μ M) was added and fluorescence images were taken again.

2-AG, AEA and ATP Activation of GRAB_{eCB2.0} in Neuro2A

Transient transfection was done as previously described. 24 h after transfection, the medium was replaced with 95 μ L PBS containing 1 mM CaCl₂ and 0.55 mM MgCl₂ and cells were incubated at rt for 20 min. After incubation, baseline fluorescence was measured (30 sec/cycle, 5 cycles) in CLARIOstar[®] (excitation 470-15 nm, emission 515-20 nm, bottom optical, focal height $\sim$ 4.1 mm). 5 μ L of 2-AG or AEA at different concentrations (dissolved in PBS with 2 mg/mL BSA) was then added and kinetic fluorescence measurement (30 sec/cycle, 61 cycles) was started immediately. PBS with 2 mg/mL BSA (final concentration of 0.1 mg/mL) was used as a vehicle control. The assay was performed in $n = 3$. For ATP activation, 1 μ L of DMSO or rimonabant at 100 μ M (final concentration of 1 μ M) was added followed by the baseline fluorescence measurement. 5 μ L of 20 mM ATP (final concentration 1 mM) or MilliQ (vehicle) was added and kinetic fluorescence measurement started immediately. The assay was performed in $n = 6$. The $\Delta F/F_0$ value was calculated by the formula: $\Delta F/F_0 = (F - F_0)/F_0$, where F and F_0 represent the measured fluorescence at any time point and the baseline fluorescence, respectively. Area under the curve (AUC) of $\Delta F/F_0$ was calculated in GraphPad Prism 9.0.0, which was corrected by the AUC of vehicle to generate the concentration-response curves using GraphPad Prism 9.0.0 (nonlinear regression, log(agonist) vs. response with variable slope).

96-well plate Neuro2A GRAB_{eCB2.0} assay

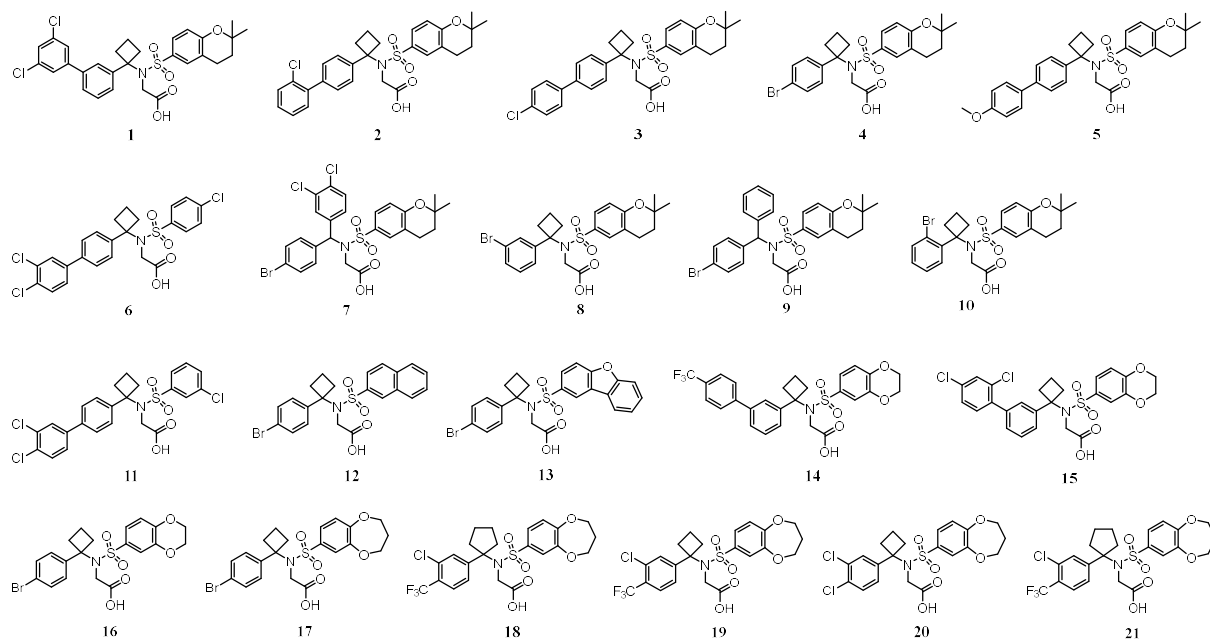
Neuro2A GRAB_{eCB2.0} cells were harvested in fresh DMEM medium without phenol red (Sigma Aldrich) and 6×10^4 cells in 100 μ L DMEM medium were seeded to each inner well of a clear-bottom black 96-well plate (Greiner-Bio-One, REF 655090). The outer wells were filled with 100 μ L PBS (Sigma Aldrich). The cells were incubated at 37 °C under 7% CO₂ for 24 h. The medium was removed by aspiration and 100 μ L of Hank's balanced salt solution (HBSS) with Ca²⁺ and Mg²⁺ with DMSO or inhibitor (0.1% DMSO) was added. The plate was incubated at 37 °C without CO₂ for 1 h. After incubation, baseline fluorescence was measured (60 sec/cycle, 5 cycles) in CLARIOstar® (excitation 470-15 nm, emission 515-20 nm, bottom optical, focal height ~4.1 mm, full plate gain adjustment at 25%, spiral scan, 3 mm, 25 flashes). 5.2 μ L of 20 mM ATP (final concentration of 1 mM) or MilliQ (vehicle) was added and kinetic fluorescence measurement (60 sec/cycle, 31 cycles) started immediately. The $\Delta F/F_0$ value was calculated by the formula: $\Delta F/F_0 = (F - F_0)/F_0$, where F and F₀ represent the measured fluorescence at any time point and the baseline fluorescence, respectively. Area under the curve (AUC) of $\Delta F/F_0$ was calculated in GraphPad Prism 9.0.0, which was converted to the residual activity of DAGL with the formula: residual activity (%) = $(AUC - AUC_{\text{background}})/(AUC_{\text{DMSO}} - AUC_{\text{background}}) \times 100\%$, where AUC_{background} represents the AUC of the vehicle control or the highest inhibitor concentration. Residual activities were used to generate the concentration-response curves using GraphPad Prism 9.0.0 (nonlinear regression, log(inhibitor) vs. normalized response with variable slope). The assay was performed in n = 5, N = 2.

References

1. Piomelli, D. The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci.* **4**, 873–884 (2003).
2. Alger, B. E. & Kim, J. Supply and demand for endocannabinoids. *Trends Neurosci.* **34**, 304–315 (2011).
3. Südhof, T. C. Neurotransmitter release: The last millisecond in the life of a synaptic vesicle. *Neuron* **80**, 675–690 (2013).
4. Kavalali, E. T. The mechanisms and functions of spontaneous neurotransmitter release. *Nat. Rev. Neurosci.* **16**, 5–16 (2015).
5. Stella, N. Cannabinoid signaling in glial cells. *Glia* **48**, 267–277 (2004).
6. Hebert-Chatelain, E. *et al.* A cannabinoid link between mitochondria and memory. *Nature* **539**, 555–559 (2016).
7. Jimenez-Blasco, D. *et al.* Glucose metabolism links astroglial mitochondria to cannabinoid effects. *Nature* **583**, 603–608 (2020).
8. Baggelaar, M. P., Maccarrone, M. & van der Stelt, M. 2-Arachidonoylglycerol: A signaling lipid with manifold actions in the brain. *Prog. Lipid Res.* **71**, 1–17 (2018).
9. Buczynski, M. W. & Parsons, L. H. Quantification of brain endocannabinoid levels: Methods, interpretations and pitfalls. *Br. J. Pharmacol.* **160**, 423–442 (2010).
10. Marchioni, C. *et al.* Recent advances in LC-MS/MS methods to determine endocannabinoids in biological samples: Application in neurodegenerative diseases. *Anal. Chim. Acta* **1044**, 12–28 (2018).
11. Sun, F. *et al.* A Genetically Encoded Fluorescent Sensor Enables Rapid and Specific Detection of Dopamine in Flies, Fish, and Mice. *Cell* **174**, 481–496 (2018).
12. Jing, M. *et al.* A genetically encoded fluorescent acetylcholine indicator for *in vitro* and *in vivo* studies. *Nat. Biotechnol.* **36**, 726–737 (2018).
13. Wan, J. *et al.* A genetically encoded sensor for measuring serotonin dynamics. *Nat. Neurosci.* **24**, 746–752 (2021).
14. Labouesse, M. A. & Patriarchi, T. A versatile GPCR toolkit to track *in vivo* neuromodulation: not a one-size-fits-all sensor. *Neuropsychopharmacology* **46**, 2043–2047 (2021).
15. Vardy, E. & Roth, B. L. Conformational ensembles in GPCR activation. *Cell* **152**, 385–386 (2013).
16. Dong, A. *et al.* A fluorescent sensor for spatiotemporally resolved imaging of endocannabinoid dynamics *in vivo*. *Nat. Biotechnol.* **40**, 787–798 (2021).
17. Yang, N. *et al.* Deficiency in endocannabinoid synthase DAGLB contributes to Parkinson’s disease and dopaminergic neuron dysfunction. *Nat. Commun.* **13**, 3490 (2021).

18. Liput, D. J. *et al.* 2-Arachidonoylglycerol mobilization following brief synaptic stimulation in the dorsal lateral striatum requires glutamatergic and cholinergic neurotransmission. *Neuropharmacology* **205**, 108916 (2022).
19. Singh, S. *et al.* Pharmacological characterization of the endocannabinoid sensor GRAB_{eCB2.0}. *Cannabis Cannabinoid Res.* (2023).
20. Jung, K. M., Astarita, G., Thongkham, D. & Piomelli, D. Diacylglycerol lipase- α and - β control neurite outgrowth in neuro-2a cells through distinct molecular mechanisms. *Mol. Pharmacol.* **80**, 60–67 (2011).

Supplementary Figures



Supplementary Figure S5.1 Chemical structures of glycine sulfonamide DAGL inhibitors **1-21**.