Systems pharmacology of the endocannabinoid system
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Chapter 4

Investigating the players of 2-arachidonyl glycerol biosynthesis in cellular system

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Abstract

The endocannabinoid 2-AG is an important signaling lipid, which interacts with the cannabinoid CB1 and CB2 receptors. 2-AG is synthesized by diacylglycerol lipase-α (DAGL-α) and DAGL-β, but their respective contribution to 2-AG synthesis remains unclear. In this study, we investigate which isoform of DAGLs takes lead in the biosynthesis of 2-AG using Neuro2A cellular model, a DAGL expressing murine neuroblastoma cell line. Activity based protein profiling (ABPP) was employed to assess DAGL activity and lipidomics to quantify downstream effects. We confirmed in situ DAGL-β inhibition by DH376, a potent in vivo active DAGL inhibitor, while DAGL-α could not be detected. Lipidomics revealed a dose-dependent reduction of 2-AG levels upon DH376 treatment, thereby confirming a functional effect of the inhibitor. Next, DAGL knock-out populations (DAGL-α KO, DAGL-β KO, DAGL-α, -β double KO) were generated in Neuro2A cells using CRISPR/Cas9 technology. The KO of DAGL-β was confirmed in the gel-based ABPP assay, whereas DAGL-α could not be detected in either WT or KO populations. Lipidomics, however, revealed no significant reduction of 2-AG levels in any of these DAGL KOs, whereas DH376 substantially suppressed 2-AG levels in these cells. These data suggest the presence of additional enzymes responsible for 2-AG synthesis, which can be targeted by DH376. Identification, validation and characterization of these additional enzymes is currently ongoing.
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Introduction

2-Arachidonylglycerol (2-AG) is an important signaling lipid and an intermediate in lipid metabolism. 2-AG and anandamide (AEA) are well-studied ligands in the endocannabinoid class, that interact with the cannabinoid CB1 and CB2 receptors. Endocannabinoids and cannabinoid receptors are part of an endogenous signaling system, the endocannabinoid system (ECS). ECS is involved in plethora of physiological and pathological processes and is emerging as a therapeutic drug target. 2-AG is the most abundant endocannabinoid present in the brain and is the primary mediator in neurogenesis, synaptic transmission, synapse formation thereby mediating synaptic plasticity. Several lines of evidence show the involvement of 2-AG in various pathological conditions like obesity, neuro-inflammation, anxiety, addiction and pain.

2-AG is synthesized primarily by two main different pathways. A signaling pathway via a two-step process starting from sn-2 arachidonoyl phosphatidylinositol 4,5-bisphosphate (PIP2). In the first step, PIP2 is hydrolyzed into arachidonyl-containing diacylglycerol (DAG) species by phospholipase Cβ (PLCβ), which is activated by various G-protein-coupled receptors. The second step is catalyzed by diacylglycerol lipase (DAGL-α and -β), in which DAG is converted into 2-AG in a sn-1 specific manner. A second metabolic pathway starting from PIP2 towards phosphatidyl inositol (PI) by PIP2 phosphatases, followed by hydrolysis of sn-1-ester by phospholipase A1 to generate 2-arachidonyl-LPI. In the final step, phosphate is removed by lysophospholipase C (LysoPLC) to form 2-AG.

2-AG is synthesized “on demand” mainly by DAGL-α (120 kDa) and DAGL-β (70 kDa). Both enzymes belong to the serine hydrolase family and structurally differ by the presence of a long C-terminal tail (~300 amino acids) in DAGL-α. Both enzymes are expressed in developing and adult brain and the localization of both enzymes markedly shift during the brain development. Genetic studies using DAGL knockout (KO) mice have shown that DAGL-α and DAGL-β regulate 2-AG production in a tissue-type-dependent manner. For instance, 2-AG levels in the CNS of DAGL-α and -β knockout mice were reduced 80% and 50% respectively. However, in the liver of DAGL-β knockout mice 2-AG levels were reduced 90%, while they were reduced only 50% in DAGL-α knockout mice. Previous genetic KO studies suggest that DAGL-α was the predominant 2-AG synthesizing enzyme in neurons and participates in endocannabinoid-mediate plasticity, while DAGL-β is dominant in microglia and in peripheral tissues like liver, and has been implicated in inflammatory response in macrophages. Another study in hippocampal neurons suggest
that DAGL-\(\beta\) is capable of eliciting endocannabinoid-mediated synaptic plasticity under certain conditions and both isoforms of DAGL can cooperatively mediate synaptic transmission at both excitatory and inhibitory synapses \(^{23}\). To this end, the above studies indicate that two isoforms of DAGL are responsible for production of 2-AG, but which isoform majorly contributes to 2-AG synthesis still remains to be elucidated.

To study the physiological role of DAGL, highly selective potent inhibitors are required. Selective inhibitors for the DAGL subtypes have not been identified yet. Dual DAGL-\(\alpha\) and -\(\beta\) inhibitors were used to study the physiological role of 2-AG in health and disease models \(^{24-26}\). Recently, Ogasawara et al. reported the first CNS in vivo active dual DAGL-\(\alpha/\beta\) inhibitor DH376. Lipid analysis in brain revealed extensive reorganization of lipid signaling networks (including anandamide signaling) upon DAGL inhibition by DH376, which largely mirrors the myriad lipid changes observed in the brains of DAGL\(\alpha--\) mice \(^{27}\). This indicates the key role of DAGLs in orchestrating the lipid cross-talks in the brain. In addition, the effect of DH376 was assessed in Neuro2A cellular model \(^{28}\) but which isoform of DAGL is taking lead in 2-AG synthesis is not studied yet.

To investigate the activity and selectivity of the enzyme inhibitors, Activity-based protein profiling (ABPP) can serve as a powerful profiling technique for discovering selective inhibitors acting in their physiological context. ABPP is unique in its ability to rapidly identify inhibitor activity and selectivity within large enzyme families in complex protein samples \(^{29}\). To monitor the activity of DAGL, it is also important to quantify the downstream effects, endocannabinoid production in this case. Therefore, an LC-MS/MS platform was developed and applied to quantitatively profile the endocannabinoids, 2-AG and AEA and other related congeners in neuronal cells which allows better understanding of DAGL physiological role at molecular level. Generating specific DAGL subtype knockouts allows to better understand which subtype of DAGL is contributing more for 2-AG synthesis. To this end, CRISPR-Cas9 technology was employed that allows efficient targeted genome editing in all organisms without leaving any foreign DNA. This RNA-guided DNA endonuclease, Cas9 method exhibits higher fidelity and low cell toxicity when compared with traditional gene editing techniques. CRISPR-Cas9 can be used for knocking out specific gene expression, so that the function of that particular gene can be elucidated \(^{30,31}\).
The objective of this study is to investigate which DAGL isoform takes lead in 2-AG biosynthesis in a cellular system. The functional effect of DAGLs in Neuro2A cell model was assessed using DH376, a dual inhibitor for DAGL-α and-β, by ABPP and lipidomics. CRISPR/Cas9 technology was used to generate DAGL-α and DAGL-β single and double knockout Neuro2A lines to study the role of the two DAGL isoforms in this cellular system.
**Materials and Methods**

**General cell culture**

Neuro2A wildtype and knockout cell lines were used as a cell model. Cells were cultured at 37 °C under 7% CO₂ in DMEM containing phenol red, glutamax, 10% (v/v) Newborn Calf Serum (Thermo Fisher), and penicillin and streptomycin (200 µg/mL each; Duchefa). Medium was refreshed every 2-3 days and cells were passaged twice a week at 80-90% confluence by resuspension in fresh medium. In the treatments methods below, the term *in situ* is used to designate experiments in which live cells are treated with inhibitors.

**Neuro2A treatment for Lipidomics**

2.5 million cells in 4 mL complete DMEM per 6 cm dish were seeded 40-48 h prior to treatment initiation. Cell viability was checked by Trypan Blue staining in a TC20 automated Cell Counter (Bio-Rad) and was 95% or higher at time of cell seeding.

Prior to treatment, culture medium was aspirated and cells were washed carefully (to prevent detachment of the cells) with 2 mL PBS. Subsequently 2 mL treatment medium (serum-free DMEM, 0.1% DMSO (vehicle), 0-10 µM DH376) was added to each dish and cells were incubated (1h or 2 h, 37 °C, 7% CO₂). After incubation, treatment medium was aspirated and cells were carefully washed with 2 mL PBS. Cells were harvested in 1250 µL PBS using a P1000 micropipette. Cell viability was checked for part of the samples by Trypan Blue staining in a TC20 automated Cell Counter (Bio-Rad) and was 90-99%. 1000 µL of the cell suspension was transferred to a low binding Eppendorf tube and cells were pelleted (1000 g, 3 min, RT). The supernatant was aspirated and cell pellets were snap frozen in liquid nitrogen and stored at -80 °C until lipidomics analysis.

The remaining cell suspension (250 µL) was transferred to an Eppendorf tube and snap frozen in liquid N₂ immediately. This suspension was thawed on ice and homogenized using a probe sonicator (5 sec/sample, 30% amplitude). Protein concentrations of these lysates were determined by Bradford Protein Assay and used for normalization purposes.

**Neuro2A treatment for ABPP**

Cells were seeded at 1:6 from an 80% confluent 10cm dish to a 6-well plate 48 hours prior to treatment initiation. Prior to treatment, culture medium was aspirated and cells were carefully washed with 1 mL PBS. Subsequently 1 mL treatment medium (serum-free DMEM, 0.1% DMSO (vehicle), 0/100 nM DH376) was added to each dish and cells were incubated (2 h, 37 °C, 7% CO₂). After incubation, treatment medium was aspirated and cells were carefully
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washed with 1 mL PBS. Cells were harvested in 1 mL PBS using a P1000 micropipette. The cell suspension was transferred to a low binding Eppendorf tube and cells were pelleted (1000 g, 3 min, RT). The supernatant was aspirated and cell pellets were snap frozen in liquid nitrogen and stored at -80 °C until whole lysate preparation and ABPP analysis.

ABPP

Whole cell lysate preparation

Cell pellets were thawed on ice, resuspended in cold lysis buffer (20 mM HEPES pH 7.2, 250 mM sucrose, 1 mM MgCl₂, 2.5 U/mL benzonase) and incubated on ice (~15 min). Protein concentrations were determined by a Bradford Protein Assay (Bio-Rad), samples were diluted to 2.0 mg/mL in buffer (20 mM Hepes, pH 7.2) and are analyzed immediately.

ABPP: Direct activity based probes

Gel-based activity based protein profiling (ABPP) was performed and analyzed with minor adaptations on previously reported procedures. In situ treated cells (15 µL whole lysate, 2.0 mg/mL) were directly incubated with the activity based probe (1 µM or 2 µM MB064, 24 or 500 nM FP-TAMRA, 20 min, RT). Reactions were quenched with 4 × Laemmli buffer (5 µL, 240 mM Tris (pH 6.8), 8% (w/v) SDS, 40% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.04% (v/v) bromophenol blue). Samples were resolved on a 10% acrylamide SDS-PAGE gel (180 V, 80 min), scanned using Cy3 and Cy5 multichannel settings (605/50 and 695/55, filters respectively) (ChemiDoc MP, Bio-Rad) and subsequently stained with Coomassie as a protein loading control. Fluorescence was normalized to Coomassie staining and quantified with Image Lab (Bio-Rad). IC₅₀ curves were fitted with Graphpad Prism®.

LC-MS/MS based lipidomics

The endocannabinoid extraction and LC-MS/M analysis was adapted from our previously developed method. The concentration using 2-2.5 million cells of Neuro2A was determined after correction with isotopically labeled internal standards and individual calibration curves and normalized to protein concentrations. A targeted list of 24 endocannabinoids and NAEs were monitored out of which 16 were detected and quantified. The method was validated in terms of precision, recovery and matrix effects (Fig. S1-S3). The absolute values of endocannabinoids and NAEs in Neuro2A cells are listed in Table. S1.
Results and discussion

Function of DAGLs in cellular system

To investigate whether DAGLs are responsible for 2-AG synthesis in a cellular system, a dual inhibitor for DAGLs, DH376, was used in Neuro2A. Neuro2A is a murine neuroblastoma cell line known to express DAGL-α and -β. Initially we performed a competitive gel-based ABPP to investigate the activity of endogenous DAGLs in Neuro2A cells. These studies were performed using a tailored activity-based probe MB064 which can label active DAGL. In situ treatment with DH376 dose-dependently reduced DAGL-β (70 kDa) labelling with a pIC$_{50}$ of 8.4 ± 0.1 (Fig. 1A, B), which is similar to that measured in mouse brain membrane proteome. Of note, dose-dependent inhibition of ABHD6, a hydrolyzing enzyme of 2-AG, upon DH376 treatment was also observed. It was reported that the mRNA levels of DAGL-α protein were 128 fold lower compared to DAGL-β in Neuro 2A cells. In line with this, DAGL-α activity cannot be monitored as its activity was below the detection limits of our ABPP assay.

We next assessed whether DAGL inhibition reduces 2-AG production in Neuro2A cells. Initially a time dependency curve was generated to select the appropriate time point of DH376 treatment required to achieve full inhibition. LC-MS/MS based lipidomics results showed significant reduction of 2-AG levels after 30 min of treatment (Fig. 1C). However, we chose 1hr time point for easy sample handling. Furthermore, we investigated whether longer incubation of DH376 has an effect on 2-AG levels. No further reduction in 2-AG levels were observed up to 24h of inhibitor treatment (Fig. 1C). Using 1h incubation, dose-dependence data was generated. Lipidomics results showed a concentration-dependent reduction of 2-AG levels in Neuro2A cells with a pIC$_{50}$ of 8.0 ± 01 (Fig. 1D). Taken together these data showed that 2-AG levels in Neuro2A cells can be dose- and time-dependently reduced by DH376.
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Figure 1: DH376 dose-dependently inhibits DAGL-β and reduces 2-AG levels. (A) Representative gel of concentration dependent inhibition of endogenous DAGL-β inhibition in \textit{in situ} treated Neuro2A cells as determined with ABPP using MB064 (B) Dose-response curve of DAGL-β inhibition as determined with ABPP using MB064. (C) Time course decrease in the levels of 2-AG in Neuro2A cells treated with vehicle (DMSO) or DH376 (1 μM, 1h, 37°C). The response in (C) are expressed as ratio between DH376 treated to vehicle treated (DMSO) to compensate for sample changes over time. (D) Dose-dependent decrease in the levels of 2-AG upon DH376 treatment (1h, 37°C). The responses in (B) and (D) are normalized and expressed in percentages (%). N = 3 independent experiments per group.
Activity of DAGLs in Neuro2A knockout cells

Validation of DAGL KOs in Neuro2A cells

To investigate which isoform of DAGLs is predominantly responsible for 2-AG synthesis, Neuro2A populations lacking either DAGL-α or DAGL-β or both DAGL-α and β, generated via the CRISPR/Cas9 technology, were used. Genetic modification was confirmed by a T7E-assay for DAGL-α and DAGL-β. ABPP and western blot (WB) were used to check activity and abundance of DAGL-β in the knockout line, showing a decrease of ~80-100% in both assays (Fig. 2A for ABPP and data not shown for WB). DAGL-α was not detected by ABPP or western blot in both WT and KO. Other serine hydrolases were not affected by knockout of DAGL and their treatment with DH376, as was shown by ABPP using TAMRA-FP probe which can label a broader range of serine hydrolases (Fig. 2B).

DH376 substantially reduced 2-AG levels in DAGL KO’s

Next, we evaluated the endogenous levels of 2-AG in Neuro2A DAGL KOs cells using LC-MS/MS based lipidomics. As a control, wildtype cells were treated with DH376. Surprisingly, in DAGLα -/-, DAGLβ -/- and dual DAGLα, β-/- cells, no significant reduction of 2-AG and AEA levels were observed compared to wild-type cells (Fig. 2C, 2D). Importantly, we observed that DH376 was still able to substantially suppress 2-AG levels, but not AEA-levels, in all three DAGL knock-out cell lines (Fig. 2C, 2D). These results suggest the presence of additional enzymes targeted by DH376 responsible for the synthesis of 2-AG in Neuro2A cells.
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Figure 2: ABPP and lipidomics on DAGL Neuro2A knockouts and the effect of DH376 on KO’s. Representative gel-based ABPP on the selectivity and activity of DAGL in Neuro2A KO’s and in situ treatment of WT and KO’s with DMSO (0.1%) or DH376 (100 nM, 2 hours, 37°C) as measured using competitive-ABPP with MB064 (A) and FP-TAMRA (B) probes. Scatter plot of 2-AG (C) and AEA (D) levels in WT and Neuro2A KO’s and their treatment with DMSO (-) (0.1%) or DH376 (+) (100 nM, 2 hours, 37°C; n=6 for WT; n=4 for others). The values in scatter plots are normalized to wild type and expressed in percentages (%).
Conclusions and future prospects

In summary, the in vitro functional activity of DAGLs was evaluated in wildtype and DAGL knockouts in Neuro2A cells using ABPP and lipidomics analysis with DH376 inhibitor. The results of DH376 treatment to DAGL knockouts revealed substantial suppression of endogenous 2-AG levels. This study paves the way to the identification and characterization of new enzyme targets responsible for 2-AG biosynthesis.

One patent has been filed claiming that purified ABHD11 protein extracted from pig brain exhibits similar activity to DAGLs, however the endogenous activity of this enzyme remains to be elucidated. In addition, DDHD2 protein has been reported to possess DAGLs activity, however the endogenous activity is not well studied. It was previously reported that two additional enzymes, 1-LPA phosphatases and 2-AG-LPI are also responsible for 2-AG synthesis, but these enzymes are not targeted by DH376.

Click chemistry ABPP is a chemoproteomics method which can be used to identify target engagement and potential off-targets of newly developed small molecule inhibitors. Click chemistry ABPP can be performed to discover and identify new targets labelled by DH376 treatment in vivo with an Cy5-azide ABPP probe that can detect labeled proteins in whole proteomes by copper(I)-catalyzed ligation (CuAAC). Since DH376 already contains an alkyne moiety which can be clicked to CY5-azide, it is an ideal inhibitor for performing click chemistry.

After performing click-chemistry ABPP, MS-based chemoproteomics can be used to identify the proteins inhibited by DH376 treatment on wild type and knockouts. This methodology allows for a more accurate quantification avoiding band overlap (as observed in gel-based ABPP) and enables screening over a broader range of particular serine hydrolases. Once the enzyme targets are identified, specific Neuro2A knockouts of identified enzymes can be generated to understand their role and contribution to 2-AG synthesis. Identification of all the players and their contribution to 2-AG synthesis is important to understand the regulatory mechanism of 2-AG signalling and their contribution in different cell types. Targeting specific enzymes in particular cell type to tune endocannabinoid signalling is highly essential to reduce off-target effects and to provide therapeutic solutions for diseases such as metabolic disorders, neurological disorders, where 2-AG levels are upregulated. Overall, ABPP and lipidomics served as versatile techniques that can be used to identify enzyme targets.
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References

27. Deng, H. Chemical tools to modulate endocannabinoid biosynthesis. (Leiden University, 2017).
Supplementary Information

**Figure S1**: The precision values of endocannabinoids and associated N-acylethanolamines (NAEs). The values are expressed in RSD (relative standard deviation). Intra-day is analysed using 5 biological samples and inter-day is analysed by analysing 5 biological samples in three consecutive days.

**Figure S2**: Recovery and matrix effect values are expressed in percentages. Recovery: Higher values indicate better recoveries. Matrix effect: Values above 100% implies ion enhancement and below 100% implies ion suppression.
Table S1: Concentrations of endocannabinoids and related NAEs quantified in Neuro2A cells (n=4)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Compound</th>
<th>Mean (fM/1M cells)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2&amp;1AG (20:4)</td>
<td>2.3E+04</td>
<td>1.0E+03</td>
</tr>
<tr>
<td>2</td>
<td>AEA (20:4)</td>
<td>8.7</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>DHEA (22:6)</td>
<td>4.8</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>LEA (18:2)</td>
<td>52.3</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>OEA (18:1)</td>
<td>39.2</td>
<td>2.1</td>
</tr>
<tr>
<td>6</td>
<td>PEA (16:0)</td>
<td>104.3</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>SEA (18:0)</td>
<td>119.0</td>
<td>2.6</td>
</tr>
<tr>
<td>8</td>
<td>DEA (22:4)</td>
<td>22.3</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>DGLEA (18:3)</td>
<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>2LG (18:2)</td>
<td>5.1E+04</td>
<td>1.2E+03</td>
</tr>
<tr>
<td>11</td>
<td>1LG (18:2)</td>
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<td>3.4E+02</td>
</tr>
<tr>
<td>12</td>
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<tr>
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<tr>
<td>15</td>
<td>PDEA (15:0)</td>
<td>1.6</td>
<td>0.3</td>
</tr>
<tr>
<td>16</td>
<td>AA (20:4)</td>
<td>1.1E+05</td>
<td>2.5E+03</td>
</tr>
</tbody>
</table>

The concentrations are determined using individual calibration curves. Labelled internal standards were used to correct for losses. SEM- Standard error of mean.