8 Druggable Targets in Endocannabinoid Signaling

Ann M. Gregus and Matthew W. Buczynski

Abstract

Cannabis and cannabinoid-based extracts have long been utilized for their perceived therapeutic value, and support for the legalization of cannabis for medicinal purposes continues to increase worldwide. Since the discovery of Δ^9-tetrahydrocannabinol (THC) as the primary psychoactive component of cannabis over 50 years ago, substantial effort has been directed toward detection of endogenous mediators of cannabinoid activity. The discovery of anandamide and 2-arachidonoylglycerol as two endogenous lipid mediators of cannabinoid-like effects (endocannabinoids) has inspired exponential growth in our understanding of this essential pathway, as well as the pathological conditions that result from dysregulated endocannabinoid signaling. This review examines current knowledge of the endocannabinoid system including metabolic enzymes involved in biosynthesis and degradation and their receptors, and evaluates potential druggable targets for therapeutic intervention.

Keywords

Endocannabinoid · GPCR · CB_1 · CB_2 · FAAH

8.1 Cannabinoids as Therapeutics

For centuries, cannabis and cannabinoid-based extracts were thought to possess therapeutic value. In recent years, the use of medical marijuana has increased for a wide variety of disorders in the United States, and changes in the legal landscape and public opinion support expanding its recreational availability nationwide. The 2010, a resolution adopted by the American Medical Association advocated reconsideration of marijuana as a Schedule I controlled substance given the potential therapeutic value of marijuana and cannabis-based products. As of January 1, 2020, 33 states have legalized the sale of medical marijuana, with additional states considering similar legislation with possible enaction in the near future.

The primary psychoactive component of cannabis was identified as Δ^9-tetrahydrocannabinol (THC) by Yechiel Gaoni and Raphael Mechoulam in the 1960s [1, 2]. While hundreds of bioactive molecules have been identified in cannabis thus far [3], THC recapitulates many of the pharmacological properties attributed to marijuana in both rodent models and in humans [4, 5]. Subsequent

A. M. Gregus · M. W. Buczynski (✉)
School of Neuroscience, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
e-mail: mwb@vt.edu

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worldwide efforts were aimed at the discovery of both synthetic and semi-synthetic cannabinoids capable of producing cannabinoid-like effects *in vivo* for the eventual development of patentable drugs with verifiable therapeutic value. While many hundreds of cannabinoid compounds were created in subsequent years, the pharmacological properties of these compounds often retained or exacerbated psychoactive effects when compared with THC [6], and thus many of these early cannabinoid-mimetics were not heavily pursued in clinical trials. Alternatively, synthetic cannabinoids began to reemerge as a recreational alternative to traditional cannabis in convenience stores and online marketplaces during the mid-2000s under pseudonyms such as "Spice" and "K2". Synthetic cannabinoids were generally consumed by inhalation via cigarettes containing herbal substances along with these synthetic molecules to obtain euphoric, anxiolytic, and antidepressant-like effects. Whereas traditional cannabis products generally have been considered safe across a wide dose range, numerous case reports illustrate that synthetic cannabinoids produce deleterious effects including paranoia, tachycardia, panic, convulsions, psychosis, visual/auditory hallucinations, vomiting, and seizures [6].

Thus far, two cannabinoid-based therapeutics have obtained FDA approval: Marinol® (dronabinol or THC) and Cesamet® (nabilone), a synthetic cannabinoid [7, 8], for the treatment of chemotherapy-induced nausea and emesis. Marinol also has been indicated as an appetite stimulant to treat cachexia in AIDS patients. A third medication, formulated with equivalent concentrations of THC and cannabidiol (CBD) known as Sativex®, has been approved in several countries outside the United States for the relief of spasticity in multiple sclerosis (MS) patients [9]. While the widespread use of medical marijuana suggests potential therapeutic value for a number of diseases, psychoactive effects and addictive potential of cannabinoids with chronic usage may limit widespread use in clinical practice. Additionally, their CNS effects complicate interpretation of efficacy in clinical trials as patients can easily determine whether or not they are receiving the drug or a placebo. Thus, substantial efforts are directed toward evaluating alternative targets in the cannabinoid signaling pathway for the development of safe and effective therapeutics.

8.2 Selective Modulation of Cannabinoid Receptors

While significant adverse effects and lack of efficacy have hampered the development of cannabinoid receptor antagonists for clinical use [10–14], these compounds served as important tools for the discovery of endogenous cannabinoid receptors 1 (CB_1) and 2 (CB_2) and their classification as G protein-coupled receptors [15]. Binding studies conducted using radiolabeled versions of potent synthetic cannabinoids such as CP-55,940 revealed high-affinity cannabinoid-specific binding sites via radioactive displacement by THC or other synthetic cannabinoids [16, 17]. Subsequent efforts harnessed these approaches to discover CB_1 [18, 19] and CB_2 receptors [20], respectively. Both CB_1 and CB_2 receptors couple to $G\alpha_{i/o}$ proteins to inhibit adenylate cyclase activity and reduce production of cyclic AMP [21, 22]. While CB_1 receptors are enriched in neuronal synapses (where they inhibit neurotransmitter release), CB_2 is strongly expressed in immune cells and glia [23–25]. Many of the psychoactive effects of THC and other cannabinoids can be attributed to actions on the CB_1 receptor [26], yet mounting evidence paints a more complex picture of the cell-type specific expression patterns of cannabinoid receptors *in vivo*.

Following the discovery of endogenously expressed cannabinoid receptors, investigators raced to develop the first potent and selective CB_1 and CB_2 receptor modulators. From the many compounds identified, SR141716A (rimonabant) represents the most well-characterized drug in this class [27]. Subsequently, it was demonstrated that CB_1-selective rimonabant blocks acute cannabinoid-induced tetrad behaviors in mice [27], alters dopamine release in rats [28, 29], precipitates withdrawal in THC-dependent rats [30–32], and inhibits long-term potentiation in rodent brain slices [33, 34]. It has been shown in multi-

ple preclinical models of nociception that rimonabant exacerbates hyperalgesia [35–37] and attenuates cannabinoid-induced analgesic effects [35, 37] suggesting an opportunity for newer generation therapeutics that modify endocannabinoid signaling in the treatment of chronic pain. Rimonabant approved in 2006 as an anti-obesity medication in Europe (Acomplia, Zimulti), but was later associated with increased incidence of severe adverse psychiatric consequences during Phase III clinical trials to examine its efficacy as an obesity treatment and smoking cessation therapy [38, 39]. As a result, rimonabant did not garner FDA approval in the United States, and was subsequently pulled from the market worldwide in 2008.

The localization of CB_2 primarily in immune cells with limited expression in neurons may underlie its implication in several diseases with an inflammatory component including neurodegenerative and autoimmune diseases [40–42]. While a suite of CB_2 agonists have been synthesized to date, a collaborative effort between multiple academic and industry laboratories identified substantial differences in their mechanisms of action, pharmacokinetic properties, and off-target effects *in vivo* [43]. Based on their collective findings on a wide range of compounds, this research team recommends using HU910, HU308, or JWH133 as potent and *in vivo* active agonists of the CB_2 receptor for subsequent drug discovery efforts of clinically useful CB_2-based therapeutics. This endeavor has proven to be more challenging than initially expected, as only a few synthetic CB_2 agonists have reached clinical trials (GW842166X, CP-55,940, S-777469, and JTE-907), with none completing phase II for chronic pain indications [42]. Currently, the CB_2 agonist JBT-101 is undergoing Phase II testing for efficacy in autoimmune diseases including systemic lupus erythematosus (NCT03093402) and diffuse scleroderma, where it has shown some beneficial effects (NCT02465437).

8.3 Two Primary Endogenous Cannabinoids: Anandamide and 2-Arachidonoylglycerol

Nearly 30 years ago, two derivatives of arachidonic acid were identified as the endogenous cannabinoid receptor ligands. Anandamide (AEA) was the first endocannabinoid (eCB) to be discovered [44], closely followed by identification of a second endogenous molecule, 2-arachidonoylglycerol (2-AG), signaling via CB_1 and CB_2 receptors [45, 46]. AEA and 2-AG retain an arachidonoyl moiety that imparts a significant amount of their bioactivity. While endocannabinoid-related lipids generated from other fatty acids substrates, including palmitoylethanolamide [47] and oleoylethanolamide [48] have described as eCBs, these molecules do not interact with cannabinoid receptors [49, 50]. Thus, AEA and 2-AG are still viewed as the primary endogenous mediators of cannabinoid signaling.

Historically, evidence for an eCB mechanism *in vivo* was determined indirectly using cannabinoid receptor antagonists, without certainty of the identity of the signaling molecule(s). Most studies quantified eCB content primarily by lipid extraction and purification from bulk tissue, followed by subsequent analysis with liquid chromatography coupled with mass spectrometry. Several excellent articles outline this process [51–54]. However, there is significant debate regarding the physiological range of eCB concentrations in various regions, as there is considerable variability in estimates of brain AEA and 2-AG content. Notably, a significant pool of 2-AG serves as an intracellular substrate for triacylglycerol formation in energy metabolism and may not participate in cannabinoid signaling [55]. An alternate approach utilizing *in vivo* microdialysis samples of interstitial, signaling-competent eCBs from awake, behaving animals with exquisite sensitivity [55, 56]. Using this method, basal interstitial AEA and 2-AG in the brain are estimated at low to mid-nanomolar levels, physiologically relevant concentrations for activating cannabinoid receptors *in vivo* [55, 57].

8.4 Selective Inhibitor Development Using Activity-Based Protein Profiling (ABPP)

Drug selectivity presented a major challenge in early efforts in the discovery of drug candidates that modulate endocannabinoid metabolism. Initial pharmacology studies suggested that these enzymes were not rate-limiting, and thus near-complete inhibition is required to produce therapeutic effects [58–61]. Moreover, many of these enzymes utilized the same mechanism of action (serine hydrolase), so compounds used at doses needed for complete inhibition were more likely to exhibit off-target effects. Thus, it was particularly challenging to develop inhibitors for the serine hydrolases, a class of over 200 enzymes with a wide range of biological functions [62]. For example, partial inhibition of acetylcholinesterase by donepezil can improve cognitive function in patients with Alzheimer's disease [63]. However, acetylcholinesterase knockout mice typically do not survive to adulthood [64], and complete inhibition by non-selective nerve agents such as Sarin produce lethal neurotoxicity [65]. Given the large number of unannotated serine hydrolases in the human body [62], it is plausible that inhibition of additional off-target serine hydrolases may have similar safety and toxicity issues. Thus, the development of a safe and effective therapeutic targeting endocannabinoid metabolism requires substantial preclinical pharmacokinetic and pharmacodynamic validation prior to entering clinical trials.

The use of activity-based protein profiling (ABPP) approaches has greatly facilitated the development of many of the selective inhibitors currently used in academic research and clinical trials [66]. While non-selective serine hydrolase inhibitors such as organophosphates would raise safety concerns as chronically administered therapeutics, their broad-spectrum capacity to covalently capture a wide range of endogenous serine hydrolases render them an excellent tool for evaluating potency and selectivity of potential drug candidates *in vivo*. The first broad-spectrum fluorophosphonate probes contained a rhodamine or biotin tag [67], which allowed any serine hydrolases captured by these probes to be visualized by in-gel fluorescence or identified using mass spectrometry. In competition experiments, any serine hydrolase inhibited by a drug would fail to be captured by the fluorophosphonate probe under those treatment conditions and the corresponding fluorescence or mass spectra would be diminished. In addition to broad-spectrum probes that capture high abundance serine hydrolases, a number of more selective ABPP probes have been synthesized for discovery of inhibitors for difficult targets, such as diacylglycerol lipases [59, 68–71], with procedural details outlined in several excellent reviews [71, 72]. It follows that potential drug-like molecules can be modified to contain alkyne moieties which have minimal effect on their selectivity but allow their direct targets to then be bound by an azide-functionalized rhodamine or biotin using click chemistry techniques. Collectively, this approach can provide an *in vivo* readout of both potency and selectivity while significantly facilitating the identification of off-targets for novel inhibitors.

These techniques have been employed to great effect in the development of a selective fatty acid amide hydrolase (FAAH) inhibitor by Pfizer. Systemic delivery of the initial lead compound (PF-3845) exhibits minimal serine hydrolase off-target effects in both brain and liver tissue at doses that abolish FAAH activity [58]. Moreover, an alkyne-functionalized PF-3845 provides direct evidence for the selectivity of this compound, as minimal off-target binding was identified. While minor modifications of the lead compound were made to improve efficacy and reduce interactions with liver cytochrome P450s (CYPs) that cause unwanted drug-drug interactions [73], the final candidate to enter clinical trials (PF-04457845) is based on the chemotype PF-3845, retaining the selectivity profile of the original lead compound [74].

8.5 Targeting Endocannabinoid Degradation

Increasing endogenous cannabinoid signaling represents an alternative therapeutic approach to using cannabinoid receptor agonists such as THC. By inhibiting the natural breakdown of

endogenous AEA and/or 2-AG, this approach would ideally recapitulate some of the beneficial therapeutic effects of cannabinoids while reducing undesirable side effects. In support of this approach, Long and colleagues used a combination of chemical inhibitors and genetic approaches to show that inhibiting all of the primary endocannabinoid degradative enzymes (FAAH, MGLL, ABHD6) in mice produces similar cannabinoid-appropriate responding in a drug discrimination test to THC [75]. Importantly, selective inhibition of either AEA or 2-AG degradation failed to recapitulate THC-like responsivity. This study provides clear support for the therapeutic viability of selective inhibition of specific endocannabinoid pathways with potential for reduced side-effect profile. Accordingly, the following sections will evaluate current clinical and preclinical studies evaluating inhibitors fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MGLL), and α/β hydrolase domain 6 (ABHD6).

Fatty Acid Amide Hydrolase (FAAH) Following the discovery of AEA as an endogenous ligand for the CB_1 receptor [44], the search began for the enzyme(s) regulating its metabolism. In 1996, the fatty acid amide hydrolase (FAAH) was identified as the primary enzyme responsible for AEA degradation [76, 77]. Perturbation of FAAH activity via genetic deletion or pharmacological inhibition markedly reduces AEA hydrolysis, thereby elevating AEA levels in multiple organ systems in rodents [78]. In addition to AEA, FAAH metabolizes a number of other fatty acid amide substrates such as oleoylethanolamine and palmitoylethanoamine [58, 73], resulting in a battery of CB_1-dependent and CB_1-independent behavioral changes in rodents. These effects include, but are not limited to, decreased anxiety-like [79–81] and depression-like behaviors [82, 83], gastrointestinal function [84–88], altered expression of drug and alcohol withdrawal [89–93], as well as diminished inflammatory and neuropathic pain states [74, 94–97]. This substantial body of preclinical evidence inspired clinical development of FAAH inhibitors by several pharmaceutical companies including PF-04457845 (Pfizer), JNJ-42165279 (Janssen), ASP3652 (Astellas), V158866 (Vernalis) and BIA 10–2474 (Bial). Likewise, FAAH inhibitors have been evaluated for treatment of several disease indications, including cannabis use disorder [98], fear memory extinction [99], Tourette Syndrome (NCT02134080 – terminated for lack of funding), chronic pain due to spinal cord injury (NCT01748695), osteoarthritis [100], and prostatitis [101].

The most notable failure in FAAH drug development to date is BIA 10-2474, a potent and long-acting CNS-active inhibitor of FAAH which produced acute neurotoxicity in 5 patients, one of which resulted in death in the multiple ascending dose part of the study in Phase I [102]. The expression of severe adverse events had not yet been observed at such a late stage of a first-in-human study [103]. It was later revealed that BIA 10-2474 inhibits several lipases that are not targeted by PF04457845, and produces substantial changes in lipid networks in human cortical neurons that may lead to metabolic dysregulation [104]. One of these off-targets is Aldehyde Dehydrogenase 2, which has been implicated in neuroprotection from oxidative stress-related damage [105]. While BIAL has since conducted a series of toxicity studies in animals [106], in-depth analysis of the trial PK/PD parameters and study periods may help determine conclusively the drug and metabolite concentrations underlying these adverse events.

In contrast to BIA 10-2474, most clinical candidates were generally safe and well-tolerated [101, 107–109], and some report efficacy of FAAH inhibition in primary outcome measurements for reducing stress reactivity [99] and cannabis withdrawal symptoms [98]. However, PF-04457845 and ASP3652 failed to attenuate osteoarthritis or prostatitis pain, respectively [101, 110] despite considerable data demonstrating antihyperalgesic effects of FAAH inhibition in several preclinical models of chronic pain states. It has been hypothesized that premature termination of the osteoarthritis study due to lack of efficacy, while necessary, may have prevented

the adequate assessment of contribution of non-responders and placebo effects [110]. Furthermore, the human trial focused on assessment of affective measures of pain that generally were not evaluated in rodent models, with exception of one study reporting no effect of FAAH inhibition on osteoarthritis-induced burrowing behavior [111]. Thus, it may be advantageous to include spontaneous and functional output measures of pain-like behaviors (e.g., locomotor activity, grip force, nesting, sucrose preference) in preclinical studies in order to help inform selection of disease indication for clinical trials.

Monoacylglycerol Lipase (MGLL) The discovery of 2-AG as a bonafide endocannabinoid led to the search for enzymes regulating its metabolism *in vivo*. While FAAH was briefly considered a potential candidate, both chemical and genetic inhibition of FAAH failed to substantially elevate 2-AG compared with AEA. Monoacylglycerol lipase was identified as the primary metabolic driver of cannabinergic 2-AG breakdown [112, 113], and genetic deletion of MGLL in mice confirmed these findings [114]. Genetic and pharmacological analyses demonstrate ubiquitous expression of MGLL across most tissues including brain, liver, kidney, lung, heart, muscle, intestines, and adipose tissue [60, 115, 116]. These mice exhibited decreased body weight as adults, hastened increased latency to inflammatory thermal hyperalgesia, and alterations in basal pain sensitivity [117]. Genetic inactivation of MGLL enhanced extinction and reversal learning [118] and facilitates anxiety-like behavior [119], neurophysiological analyses reveals cannabinoid-dependent changes in excitatory and inhibitor synaptic plasticity in multiple brain regions in mice [119–121]. Accordingly, selective chemical inhibitors would be necessary to distinguish between role of 2-AG signaling in adults with the critical role of this pathway in neuronal development.

The early chemical inhibitors developed in academic labs suggested a prominent role for MGLL in 2-AG metabolism and neuronal signaling [122–127]. The first ABPP-validated selective murine MGLL inhibitor JZL-184 confirmed many of these findings, including enhancement of depolarization-induced 2-AG release and reduction of cannabinoid-sensitive pain states [128]. The development of JZL-184 greatly accelerated the evaluation of the molecular and behavioral role of MGLL in mice, with over 200 publications using this compound to date. A number of potential physiological roles have been discovered using JZL-184 including, but not limited to, drug withdrawal [91, 129, 130], pain states [131–133], stress [134, 135], immune function [136–138], cancer [139–141], gastrointestinal function [137, 142, 143], and neurodegeneration [144–147]. Importantly, chronic administration of JZL-184 produces CB_1 receptor desensitization and functional antagonism [148], suggesting that pharmacological tolerance of an MGLL inhibitor may produce a therapeutic profile more similar to a CB_1 antagonist than with cannabinoid-based therapeutics such as THC. However, limitations in JZL-184 pharmacology including partial inhibition of ABHD6 and FAAH during chronic dosing procedures [149] and limited efficacy in rats [60] have helped open the door for next-generation compounds that address these concerns [149–152]. Thus, future studies should validate the pharmacological selectivity of these compounds and dosing procedures for proper interpretation of results.

Recently, Abide Therapeutics discovered ABX-1431 (acquired by Lundbeck, and rebranded as Lu AG06466) as the potent first-in-class, orally bioavailable and selective inhibitor of MGLL, now under development for as a therapeutic for movement disorders, neurodegenerative diseases and pain [153]. A Phase I Experimental Hyperalgesia study of ABX-1431 may yield insight into its viability in this therapeutic space (NCT02929264), as this model is generally highly predictive of clinical success for Neuropathic Pain [154]. At present, this molecule is undergoing a Phase IIa trial for the treatment of Tourette syndrome (NCT03625453) and a Phase I trial for neuropathic pain (NCT03138421). Likewise, Pfizer has developed a selective cova-

lent MGLL inhibitor [155] and a corresponding ^{11}C-PET tracer [156] for evaluating the pharmacokinetics of this compound as part of a Phase 1 clinical trial (NCT03100136). Additionally, both Takeda Pharmaceutical Co. [157] and Janssen Research [158] recently have developed non-covalent MGLL inhibitors that show preclinical viability for target engagement and increased 2-AG levels in CNS and peripheral tissue, but these candidates have not yet entered clinical trials.

α/β Hydrolase Domain 6 (ABHD6) While MGLL drives the majority of 2-AG breakdown *in vivo*, emerging evidence suggests that other enzymes play a supportive role in this process. Initial studies using immortalized BV-2 microglial cells, which do not express MGLL, provide clear evidence of alternative 2-AG metabolism pathways [159]. While compensation by FAAH accounted for about half of the 2-AG metabolism, the use of selective inhibitors revealed that the remaining activity could be attributed to unknown enzyme(s). Using a functional proteomic approach, two additional serine hydrolases (ABHD6 and ABHD12) were identified as potential 2-AG metabolic enzymes [160]. Ultimately, the unknown 2-AG activity in BV-2 microglia was attributed to ABHD6, which regulates endocannabinergic signals to alter excitatory [161] and inhibitory [162] synapses in the brain.

Our understanding of the significance of ABHD6 in regulating endocannabinoid signaling and other lipid pathways has emerged from studies of targeted inactivation using genetic and chemical tools [163]. Constitutive deletion of ABHD6 reveals its critical role in energy metabolism [164, 165] but not in lysosomal storage disorders [166]. ABHD6 knockout mice are protected from high-fat diet-induced obesity [164] and displayed increased decreased body weight, increased energy expenditure, improved glucose tolerance and insulin sensitivity, and changes in white and brown adipose tissue composition [165]. Many of these metabolic effects can be recapitulated by antisense oligonucleotides or treatment with WWL70, the first chemical inhibitor designed to target ABHD6. Early studies using WWL70 implicate ABHD6 function in traumatic brain injury [167], obesity and type II diabetes [165, 168], seizure activity [169, 170], inflammation and pain [171, 172]. Selective blockade of ABHD6 with the newer generation peripherally-restricted inhibitor KT-203 decreases pancreatic cancer cell metastasis [173], while KT-182 modestly attenuates autoimmune demyelination [174, 175], in contrast with a previous study reporting significant reduction of clinical signs in the experimental autoimmune encephalitis model of Multiple Sclerosis with WWL70 [176]. At present, it is not clear if ABHD6 drives metabolic changes by enzymatic regulation of 2-AG signaling [177] or other lipid pathways [164]. Since ABHD6 exhibits promiscuity in its acceptance of lipid substrates [164], the process of determining the specific lipid mediators responsible for its effects presents a substantial challenge. Thus, studies utilizing inhibitors with off-target effects should be interpreted with caution for future drug development efforts.

α/β Hydrolase Domain 12 (ABHD12) Since its discovery as a serine hydrolase with in vitro 2-AG metabolic activity [160], the physiological role of ABHD12 has remained poorly understood. Loss-of-function mutations in ABHD12 cause the rare neurodegenerative disorder PHARC (polyneuropathy, hearing loss, retinosis pigmentosa, and cataract) in humans [178–182], which is phenocopied in ABHD12 knockout mice [178]. While ABHD12 knockout mice have increased levels of 2-AG in multiple brain regions [183], the primary function of this enzyme *in vivo* is likely a lysophosphatidylserine lipase. Indeed, both genetic deletion [178, 184] and selective inhibition [184, 185] of ABHD12 generate substantially elevated levels of very long chain lysophosphatidylserine levels *in vivo* [186]. ABHD12 and downstream target lysophosphatidylserine receptors are coexpressed in glia and in immune cells, and treatment of macrophages with the selective ABHD12 inhibitor DO264 exacerbated immune responsivity [178, 184,

187]. Although acute chemical inactivation of ABHD12 did not produce the severe behavioral deficits found in knockout mice [184, 187], it is presently unclear if the PHARC phenotype would emerge following chronic, long-term drug treatment. Given its restricted contribution to endocannabinoid signaling and potential role in neuroprotection, ABHD12 may offer limited opportunity for therapeutic development when compared with other targets in the endocannabinoid metabolic pathway.

8.6 Targeting Endocannabinoid Biosynthesis

Due to concerns regarding safety and tolerability with cannabinoid receptor antagonism, efforts have emerged to selectively decrease cannabinoid signaling as an alternative therapeutic approach. While CB_1 receptor antagonists or inverse agonists such as rimonabant and taranabant demonstrated efficacy in the treatment of obesity, type II diabetes and nicotine dependence [188–192], serious adverse psychiatric consequences significantly limit clinical utility [10–14], ultimately precluding their approval by the FDA. Instead, by inhibiting the natural production of endogenous AEA and/or 2-AG, targeting endocannabinoid biosynthesis might recapitulate some of the beneficial therapeutic effects of receptor antagonists while mitigating undesirable side effects.

The FDA-approved drug Orlistat (tetrahydrolipstatin, sold over-the-counter as Alli®) indicated for the treatment of obesity was designed as a pancreatic and gastric lipase inhibitor [193, 194], but nonetheless has a number of potential off-target effects including inhibition of the two diacylglycerol lipases α (DAGLα) and β (DAGLβ) that generate 2-AG *in vivo*. Unlike rimonabant, Orlistat does not produce serious psychiatric events, suggesting that inhibition of endocannabinoid biosynthesis may represent a viable alternative to CB_1 receptor antagonists. While the study of endocannabinoid biosynthetic pathways and the subsequent discovery of corresponding selective chemical inhibitors is in its nascent stage, a number of recent advances have bolstered this field and may yield unique candidates for future drug development. Several selective ABPP probes have been developed to facilitate inhibitor development against DAGLs and other potential endocannabinoid biosynthases, including some based on the tetrahydrolipstatin structure [195]. Accordingly, the following sections will evaluate current clinical and preclinical studies evaluating inhibitors of DAGLα and β (DAGLβ, n-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), and other enzymes involved in endocannabinoid biosynthesis.

Diacylglycerol Lipase α (DAGLα) The endogenous biosynthesis of signaling competent 2-AG is driven by the enzymes diacylglycerol lipase α (DAGLα) and diacylglycerol lipase β (DAGLβ). While both of these serine hydrolases convert diacylglycerol into 2-AG, they exhibit unique cellular and tissue specific expression. Specifically, DAGLα is predominantly expressed in neuronal tissue [196] and DAGLα knockout mice exhibit approximately 80% lower levels of 2-AG in the brain and spinal cord, compared with a 50% reduction of 2-AG in liver and adipose tissue [197, 198]. Within the central nervous system, neurons contain the predominant amount of DAGLα as compared with glial cells [196, 199–201]. In addition, many of the metabolic and behavioral phenotypes found in CB_1 receptor knockout mice are recapitulated by genetic deletion of DAGLα. Mice with genetic inactivation of either CB_1 or DAGLα both exhibit signs of enhanced metabolic function including lower body weight and decreased body fat, as well as reduced fasting insulin release and blood lipid levels [197, 202]. However, both genotypes also showed signs of psychiatric dysfunction including less marble-burying and shorter latencies in the forced swim test [202–204]. DAGLα mice also exhibited increased mortality beginning around 8–10 weeks of age [202]. Mounting evidence indicates that DAGLα plays a critical role in neuronal developmental processes [205–210], suggesting that psychiatric behavioral phenotypes may results from neurodevelopmental defi-

ciencies as opposed to the direct signaling actions of DAGLα. Collectively, these findings highlight the necessity for rigorous behavioral evaluation of a selective DAGLα inhibitor prior to initiation of clinical development.

Early DAGLα inhibitors such as tetrahydrolipstatin were utilized mainly in electrophysiological studies, yet the recent development of *in vivo*-active diacylglycerol lipase inhibitors has offered important insight into the consequences of global chemical inactivation of these enzymes [211]. Ogasawara and colleagues created a suite of CNS-active covalent inhibitors of diacylglycerol lipases, most notably DO34 and DH376 [61]. Compared with DH376, DO34 exhibits enhanced selectivity for DAGLα, however both compounds block DAGLα and DAGLβ following systemic administration and are blood-brain barrier permeable. Despite their off-target effects, each molecule targets a unique group of serine hydrolases. Importantly, DO53 blocks all spurious targets of DO34 while sparing DAGLα and DAGLβ, thus serving as a critical negative control compound for *in vivo* pharmacology studies. Furthermore, Baggelaar et al. report LEI105 as a reversible inhibitor of diacylglycerol lipases, with potent inactivation of DAGLα and DAGLβ and minimal cross-reactivity with other endocannabinoid metabolic enzymes in mice [69]. Surprisingly, intracerebroventricular administration in rats of the DAGLβ inhibitor KT172 (originally validated in mice) actually reduces activity of DAGLα (~80%) and DAGLβ (~50%) in the brain [162], suggesting this approach as a potential alternative to rimonabant antagonism of CB_1 for smoking cessation. While the selectivity of these compounds generally has been evaluated against the human and mouse orthologs, there is precedent for species-specific selectivity when targeting enzymes in the eCB pathway [60]. Thus, optimization is needed in order to fully validate effects of these compounds in rats for behavioral studies and in multiple species for future clinical development.

Diacylglycerol Lipase β (DAGLβ) With the initial cloning in 2003 of DAGLα and DAGLβ [196], it was demonstrated that DAGL expression pattern shifts from axonal tracts to dendritic fields, consistent with later reports that DAGL activity is required for synaptic plasticity [125, 126, 212, 213], axonal growth and guidance [214], adult neurogenesis [215, 216] and oligodendrocyte differentiation [217]. Several studies suggest that while DAGLα is the predominant 2-AG synthesizing enzyme for endocannabinoid-mediated modulation of neurotransmission in adults [197, 198, 218], DAGLβ is more abundantly expressed in the developing CNS [196, 219]. DAGLβ contributes to depolarization-induced suppression of excitation in early postnatal hippocampal autaptic neurons [209] and neurite outgrowth in culture models [208]. Outside of the CNS, DAGLβ is widely expressed in multiple sites including white blood cells [220], liver [197] and adipose tissue [221], where it may be correlated with serum high-density lipoprotein cholesterol levels. Most notably, while LPS-induced eCB-eicosanoid crosstalk is dependent on DAGLβ in microglia [201], macrophages [59] and dendritic cells [222]. These observations indicate a crucial role for DAGLβ in immune function and inflammation, consistent with the implication of this enzyme in pathologies associated with alcoholic fatty liver disease [223], Alzheimer's disease [224] as well as inflammatory, neuropathic and post-surgical pain [225–227].

The development of *in vivo*-active DAGLβ inhibitors has facilitated significantly our understanding of the role of this enzyme. Systemic administration of currently available blood-brain barrier-permeable DAGLβ inhibitors exhibit cross-reactivity with both DAGLα and DAGLβ [211], however Hsu and colleagues have developed peripherally-restricted inhibitors KT109 and KT172, which exhibit ~60-fold selectivity for DAGLβ versus DAGLα in mice [59]. To account for its limited off-target effects on ABHD6 and to determine DAGLβ-specific biology, the negative control compound KT195 was utilized as a selective ABHD6 inhibitor. Acute treatment with KT109 or KT172 reveals a role for DAGLβ in 2-AG metabolism, as well as in

downstream eicosanoid production and inflammatory signaling in peripheral macrophages [59]. Delivery of liposome-encapsulated KT109 produces macrophage-specific targeted inhibition of DAGLβ, with no apparent activity of other tissues *in vivo* [228]. This mode of administration substantially enhances anti-nociceptive potency of KT109 compared with traditional systemic treatment, thereby demonstrating potential for DAGLβ as a novel druggable target with potential indications in inflammatory diseases.

N-Acyl Phosphatidylethanolamine Phospholipase D (NAPE-PLD) While multiple enzymatic pathways have been implicated in the endogenous biosynthesis of AEA, none has been definitively nominated as the "AEA synthase" to date. The initial evaluation of n-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) as a zinc hydrolase capable of producing n-acylethanolamines (including AEA) offered the first example of a potential anandamide synthase [229]. Consistent with this purported role of NAPE-PLD *in vivo*, overexpression of this enzyme in mammalian culture systems increases AEA and other n-acylethanolamines [230]. However, while NAPE-PLD knockout mice express lower levels of saturated and monounsaturated n-acylethanolamines, reduction of AEA is inconsistent between reports [231–233].

Recent studies utilizing global and spatiotemporal genetic inactivation of NAPE-PLD have attempted to elucidate the physiological role of this enzyme. Mice expressing a constitutive knockout of NAPE-PLD exhibit no changes in body mass composition or glucose tolerance on a normal chow diet, yet inactivation of NAPE-PLD selectively in either adipose [234] or intestinal tissue [235] leads to an exaggerated obese phenotype on a high-fat diet. Spatio-temporal deletion of NAPE-PLD produces significant decreases in AEA and other n-acylethanolamines in the targeted tissues and alters the composition of gut microbiota in these animals [234, 235]. NAPE-PLD may limit development of obesity through non-cannabinergic pathways, as other n-acylethanolamines such as oleoylethanolamine produce robust anorexic effects [48] through multiple receptors including GPR119 [236] and PPARα [237]. However, it should be noted that given the potential for compensatory lipid metabolic pathways in constitutive knockout models, selective chemical inhibitors are critical in order to clarify the function of NAPE-PLD in activity-dependent AEA signaling. Unfortunately, potent CNS-active inhibitors are currently not available as existing compounds lack the necessary pharmacological properties to inhibit NAPE-PLD *in vivo* [230, 238, 239]. Future work will evaluate if these metabolic changes can be recapitulated and treated using pharmacological tools and subsequently translated into a potential therapeutic for obesity.

Other Potential Anandamide Biosynthetic Enzymes In addition to NAPE-PLD, multiple enzymatic pathways leading to the biosynthesis of n-acylethanolamines, a family of lipid species that includes AEA, have been discovered. However, current evidence highlights several obstacles to therapeutic drug discovery in this arena. For example, the serine hydrolase ABHD4 can act in concert with glycerophosphodiesterase GDE1 to produce anandamide and other n-acylethanolamines *in vitro* [240], however levels of AEA in brain tissue are unaltered in GDE1 knockout mice [241]. Likewise, ABHD4 regulates multiple lipid classes, and genetic inactivation of this enzyme elicits comparatively greater changes in lysophosphatidylserine levels [242]. An additional pathway for AEA production utilizing sequential activity of phospholipase C and tyrosine phosphatases such as lymphoid-specific tyrosine phosphatase (PTPN22) was discovered in macrophages [243, 244]. However, targeting either phospholipases C or PTPN22 alone is expected to exert substantial non-cannabinergic effects, as these enzymes are broadly involved in lipid metabolism and in responsiveness of B and T cells, respectively. Collectively, research on AEA biosynthesis suggests that multiple redundant pathways likely exist *in vivo* [241, 244], as illustrated by biological compensation when one

of these enzymes is inactivated. It follows that the physiological source(s) of AEA and thus the therapeutic utility of inhibitors for these enzymes remain to be fully elucidated.

8.7 The Interaction Between Endocannabinoids and Eicosanoid Production

Precursors for Eicosanoid Production The primary focus of research on endocannabinoid metabolism centers on changes in 2-AG signaling via cannabinoid receptors. However, recent work has uncovered an important role for prostaglandins and other arachidonic acid metabolites that are derived from endocannabinoid precursors. Although it was previously thought that prostaglandins arise mainly from actions of cytosolic phospholipase A_2, genetic inactivation of this enzyme exerts only minimal effect on arachidonic acid levels in the brain [245]. Instead, metabolic breakdown of 2-AG by MGLL supplies arachidonic acid for production of proinflammatory prostaglandins by cyclooxygenases during neuroinflammation [138]. Both genetic and chemical inactivation of MGLL attenuate lipopolysaccharide-induced cytokine release and protect against neurodegeneration of dopamine neurons through a CB_1-independent mechanism of reduced prostaglandin synthesis. Accordingly, MGLL activity liberates prostaglandins in the brain to facilitate neurodegeneration in mouse models of Alzheimer's disease [246–248], while deletion or pharmacological inhibition of MGLL facilitates cannabinoid receptor-independent blunting of disease progression [246]. MGLL-dependent prostaglandins also mediate the fever response in mice, as genetic or pharmacological inactivation of MGLL blunts LPS-induced elevations in body temperature without altering core body temperature in control mice [249, 250].

Upstream of MGLL, DAGLα and DAGLβ both contribute to release of arachidonic acid [197, 201] and subsequent production of prostaglandins [201], with a prominent role of DAGLα in the brain [59, 197, 201] and DAGLβ in microglia and other immune cells [59, 201], respectively. For example, inhibition of DAGLβ following injury decreases local PGE_2 production and attenuates chronic pain-like behaviors in mouse models of neuropathic and inflammatory pain [225]. Furthermore, targeted delivery of the DAGLβ inhibitor KT-109 using liposomes produces 80% inactivation in macrophages without altering activity in other tissues such as brain and heart, and reduces LPS-induced allodynia [228], thus providing more direct evidence for peripheral immune DAGLβ in the inflammatory response. Alternatively, pharmacological or genetic inactivation of DAGLα abrogates production of prostaglandins in brain tissue, blocks central LPS-induced prostaglandin release and blunts the fever response in these mice [225]. While the role of eCBs in prostaglandin signaling has become clear, our understanding of their contribution(s) to other eicosanoid pathways remains limited, and future studies will establish potential links with lipoxygenases and cytochrome P450s.

Eicosanoid-Like 2-AG Metabolites Endocannabinoids also may interact directly with cyclooxygenases as substrates to produce prostaglandin-like compounds with unique biological effects [251]. While the (S)-enantiomers of nonsteroidal anti-inflammatory drugs ($NSAID_S$) such as ibuprofen and naproxen inhibit cyclooxygenase to prevent the formation of proinflammatory prostaglandins [252], the (R)-enantiomers of these compounds accomplish only minimal inhibition of enzymatic activity against arachidonic acid substrate. However, (R)-NSAIDs attenuate cyclooxygenase-dependent activity with AEA and 2-AG, thereby acting as potent substrate-selective inhibitors of this class of lipid signals [253]. Importantly, (R)-NSAIDS exhibit antihyperalgesic activity in models of neuropathic pain that is superior to that of traditionally prescribed (S)-$NSAID_S$ [254, 255]. Furthermore, levels of endogenous prostaglandin E_2 glycerol ester are elevated in the carrageenan model of inflammatory pain, contributing to thermal hyperalgesia that is not fully reversed by prostaglandin receptor antagonists [256]. Accordingly, prostaglandin

E_2 glycerol ester functions as an agonist for the g-protein coupled receptor P_2Y_6, with almost four orders of magnitude more potency than the prototypical agonist uridine diphosphate [257]. In a model of colon inflammation, prostaglandin D_2 glycerol ester derived from 2-AG, but not related metabolites arising from arachidonic acid or AEA, reduced dextran sulfate sodium-induced colitis in mice and are blocked by antagonists of traditional prostaglandin receptors, DP_1 and PPARy [258].

Eicosanoid-Like Anandamide Metabolites While serving only a limited role in colon inflammation, prostaglandin D_2 ethanolamine induces skin cancer apoptosis independent of the putative DP receptors, but the precise mechanism remains unclear [259]. Perhaps the most well-established endogenous prostamide, prostaglandin $F_2\alpha$ ethanolamine exerts minimal activity through the prostaglandin $F_{2\alpha}$ receptor and instead likely acts through an FP receptor variant [260]. A structural analogue of prostaglandin $F_{2\alpha}$ ethanolamine, Bimatoprost is an FDA-approved drug marketed under the name Lumigan® (Allergan) for reducing intraocular pressure as a treatment for glaucoma [261], and a sustained-release formulation of Bimatoprost is currently undergoing testing in Phase I/II clinical trials [262]. Patients reported longer and fuller eyelashes during administration of Bimatoprost, so the drug was repurposed as Latisse® and received FDA approval for the treatment of eyelash hypotrichosis [263] In contrast, an antagonist of the prostamide $F_{2\alpha}$ receptor AGN211336 reduces inflammatory pain in mice [264]. Substrate-selective inhibitors affect a number of different pathologies in mice including stress and anxiety-like behaviors (23912944), and future research likely will uncover additional biological roles for these lipids as signaling molecules.

While it has become clear that cyclooxygenases utilize 2-AG and AEA as substrates, research investigating the biological activity of corresponding lipoxygenase and cytochrome P450 enzymes on these lipid species is limited [251] and future studies will help clarify their role(s) in endocannabinoid biology. Collectively, these results suggest a number of novel potential therapeutic avenues for endocannabinoid metabolism inhibitors.

8.8 Conclusions and Future Directions

Considerable efforts have been concentrated on targeting endocannabinoid biosynthetic and degradative enzymes as alternatives to CB_1-receptor-based therapeutics that can produce serious adverse effects associated with a number of failures in clinical trials. In general, potent and selective inhibitors of FAAH or MGLL that are devoid of off-target effects have demonstrated safety and tolerability in human volunteers. These drug candidates could be met with success in the treatment of neurological diseases and pain if caution is exercised in interpreting preclinical data as well as selection of clinical indication and output measures. Small molecule inhibitors of other eCB enzymes remain in the preclinical discovery stage, but current research suggests some potential druggable targets. DAGLα serves an important physiological role in metabolism and brain function, yet its temporal inhibition may improve smoking cessation. Chemical inactivation of DAGLβ by liposome-mediated delivery of a peripherally-restricted inhibitor reduces inflammation and pain-like behaviors in mice. DAGL inhibitors undoubtedly will benefit from further chemical optimization in order to improve both selectivity and brain-barrier permeability for CNS indications. In addition, future studies elucidating pathways of anandamide synthesis and exploring substrate-specific inhibitors of cyclooxygenases, lipoxygenases and cytochrome P450s may yield additional promising targets for drug discovery in the cannabinoid therapeutic space.

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