Goods and Bads of Endocannabinoid System as a Therapeutic Target:
Lessons Learned after 30 Years

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Running title: Endocannabinoid System as a Therapeutic Target

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Number of text pages: 70
Number of tables: 15
Number of figures: 28
Number of references: 790
Number of words in the Abstract: 200

ABBREVIATIONS: AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; AEA, N-arachidonyl ethanolamine (anandamide); CADD, computer-aided drug discovery; CBD, cannabidiol; CB1R, cannabinoid receptor 1; CB2R, cannabinoid receptor 2; CNS, central nervous system; Covid-19, coronavirus disease of 2019; COX, cyclooxygenase; cPLA, cytosolic phospholipase A; CYP450, cytochrome P450; DAGL, diacylglycerol lipase; DSE, depolarization-induced suppression of excitation; DSI, depolarization-induced suppression of inhibition; eCB, endocannabinoid; eCBome, endocannabinoidome; ECS, endocannabinoid system; EMA, European medicines Agency; FAAH, fatty acid amide hydrolase; FABP, fatty acid binding protein; FDA, Food and Drug Administration; GPCR, G protein-coupled receptor; LAPS, Ligand assisted protein structure; LOX, lipoxygenase; MAGL, monoacylglycerol lipase; MS, multiple sclerosis; NAAA, N-acylethanolamine acid amide hydrolase; NAPE-PLD, N-acyl phosphatidylethanolamine-specific phospholipase D; NAT, N-acyltransferase; NOS, nitric oxide synthase; PAM, peptidyl-glycine alpha-amidating monooxygenase; PGE2-G, prostaglandin E2 glycercyl ester; PGH2-EA, prostaglandin H2 ethanolamide; PLC, phospholipase C; PPAR, peroxisome proliferator-activated receptor; SAR, structure-activity relationship; SERI, selective eCB reuptake inhibitor; THC, Δ9-tetrahydrocannabinol; TRP(V), transient receptor potential (vanilloid).
Abstract - The cannabis derivative marijuana is the most widely used recreational drug in the Western world, that is consumed by an estimated 83 million individuals (~3% of the world population). In recent years, there has been a marked transformation in society regarding the risk perception of cannabis, driven by its legalization and medical use in many states in the USA and worldwide. Compelling research evidence and the FDA cannabis-derived cannabidiol approval for severe childhood epilepsy have confirmed the large therapeutic potential of cannabidiol itself, Δ⁹-tetrahydrocannabinol (THC) and other plant-derived cannabinoids (phytocannabinoids). Of note, our body has a complex endocannabinoid system (ECS) - made of receptors, metabolic enzymes and transporters - that is also regulated by phytocannabinoids. The first endocannabinoid to be discovered 30 years ago was anandamide (N-arachidonyl-ethanolamine); since then, distinct elements of ECS have been the target of drug design programs aimed at curing (or at least slowing down) a number of human diseases, both in the central nervous system and at the periphery. Here, a critical review of our knowledge of the goods and bads of ECS as a therapeutic target are presented, in order to define the benefits of ECS-active phytocannabinoids and ECS-oriented synthetic drugs for human health.

Significance Statement – The endocannabinoid system plays important roles virtually everywhere in our body and is either involved in mediating key processes of central and peripheral diseases or represents a therapeutic target for treatment. Therefore, understanding the structure, function, and pharmacology of the components of this complex system, and in particular of key receptors (like CB₁R and CB₂R) and metabolic enzymes (like FAAH and MAGL), will advance our understanding of endocannabinoid signaling and activity at molecular, cellular, and system levels providing new opportunities to treat patients.
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I. Introduction

Paleobotanical records date the beginning of human cannabis cultivation in Eurasia to >8000 years ago, while archaeological evidence anchors its use as a psychotropic substance to approximately 2500 years ago (Russo et al., 2008; Long et al., 2017). Today, cannabis is one of the world’s most widely used recreational drugs, after alcohol and tobacco, and is consumed by an estimated 83 million individuals (~3% of the world population) (United Nations Office on Drugs and Crime, 2017). Cannabis’ increasingly expanding legal status heightens the need for research into its therapeutic potential for a wide range of pathological conditions (National Academies of Sciences, Engineering, and Medicine, 2017; Cohen et al., 2019; Friedman et al., 2019; Cristino et al., 2020), but also raises concerns about its possible hazards to health. Indeed, medical and non-medical cannabis use has been associated with short-term and long-term adverse effects, including schizophrenia, disturbs in cognition, and mood disorders (Cohen et al., 2019), as well as an impact on adult neurogenesis (Oddi et al., 2020), and female (Cecconi et al., 2020) and male reproductive health (Maccarrone et al., 2021).

A. Phytocannabinoids

The trichomes, specialized structures in the inflorescences of the female cannabis plant, produce a family of terpenophenolic substances, called phytocannabinoids (pCBs), which contain tricyclic, bicyclic, and monocyclic structures. In most cannabis varietals, the most abundant pCBs are the acidic (i.e., carboxylic) precursors of Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which are converted to THC and CBD by drying or heating, but many others have been identified whose pharmacological properties are still awaiting clarification (Gomez-Cañas et al., 2023). Indeed, cannabis contains more than 110 pCBs as well as hundreds of terpenoids, flavonoids, sterols and other non-pCB substances (El Sohly and Gul, 2014; El Sohly et al., 2017; Solymosi and Köfalvi, 2017). THC and its analogs (including Δ8-tetrahydrocannabinol and the propyl derivative Δ9-tetrahydrocannabivarin), CBD and its analogs (including cannabidivarin), cannabinol and its analogs (including the propyl derivative cannabivarin), cannabigerol and its analogs are highly abundant. In addition, trace amounts of cannabinodiol, cannabichromene, cannabicyclol, cannabinoids, cannabintriol are also detectable (Mechoulam, 2005; El Sohly and Gul, 2014; El Sohly et al., 2017; Morales et al., 2017; Li et al., 2022). The structures of the main pCBs identified so far are shown in Table 1.

Table 1. Placeholder

To date, the therapeutic potential of THC and CBD, alone or in combination, seems apparent and has been critically discussed in recent reviews (Maccarrone et al., 2017; Friedman et al., 2019; Pacher et al.,
Here, the main applications of THC and CBD for human health are summarized in Table 2.

Table 2. Placeholder

By contrast, our understanding of the pharmacological properties of less prevalent pCBs has only scratched the surface, and very little information is available on their effect in the human body (Russo, 2018; Franco et al., 2020; Maccarrone, 2020; Rock et al., 2021; Mechoulam, 2022; Li et al., 2022). For instance, cannabidiolic acid and cannabichromene are used in creams, foods, and beverages (Straiker et al., 2021) and the methyl ester of cannabidiolic acid has been shown to suppress nausea and anxiety (Pertwee et al., 2018), to reduce depression-like effects (Hen-Shoval et al., 2018), and to have a potent anti-hyperalgesic effects (Zhu et al., 2020). Further research has shown that cannabiol exhibits neuroprotective effects in an experimental model of glaucoma (Somvanshi et al., 2022), cannabigerol reduces inflammation, pain, and obesity (Kogan et al., 2021), and both pCBs hold anti-cancer potential (Li et al., 2022). Humans and other mammals do not produce pCBs but can effectively dispose them via the cytochrome P450 and glucuronidation pathways in the liver and other organs (Huestis et al., 2007; Watanabe et al., 2007; Schafroth and Carreira, 2017; Solymosi and Köfalvi, 2017).

Overall, it is apparent that the term "phytocannabinoid" serves to cluster different plant-derived lipophilic compounds (Pertwee, 2014; Ligresti et al., 2016). It is also worth noting that different cannabis varietals can have distinct chemical profiles (referred to as "chemovars") and can thus display both qualitative and quantitative differences in their constituents. Because differences in genetics, cultivation technique, harvest, and extraction can affect the ultimate product consumed by humans, it is reasonable to conclude that there is no "one cannabis" and that caution must be taken in generalizing its effects (Hanuš et al., 2016; Procaccia et al., 2022). This variability may also confound our understanding of cannabis' pharmacological properties and, indeed, remaining uncertainties represent a serious obstacle to its clinical applications (Friedman et al., 2019). Unsurprisingly, despite its use for millennia, cannabis remains surrounded by controversies, debates and misconceptions related to its medical potential, legalization, and long-term health consequences.

Taken together, the complexity of cannabis extracts seems apparent. However, such a complexity is mirrored, and possibly even exceeded, by that of the ensemble of receptors, enzymes, and transporters of endocannabinoid (eCB) substances which together form the "eCB system (ECS)", recently discussed in comprehensive reviews (Iannotti et al., 2016; Maccarrone, 2017; Baggelaar et al., 2018; Cristiano et al., 2020; Kilaru and Chapman, 2020; Simard et al., 2022; Piomelli and Mabou Tagne, 2022). Notably, the main components of the ECS support and control the manifold actions of the eCBs both in the central nervous
system (CNS) (Maccarrone et al., 2014; Iannotti et al., 2016; Cristino et al., 2020) and the periphery (Maccarrone et al., 2015). Here, it should be stressed that little is still known on the effects that pCBs have on the ECS. Emerging evidence indicates that, even at low concentrations, THC can alter eCB signaling especially when administered during critical periods such as adolescence (Lee et al., 2022). Additionally, 24-hour treatment with cannabigerol, cannabichromene, Δ<sup>9</sup>-tetrahydrocannabivarin and cannabigerolic acid has been shown to modulate the function of distinct ECS elements in human HaCaT keratinocytes, where they all increase binding of [³H]CP55940 to cannabinoid receptors 1 and 2 (CB₁R and CB₂R), stimulation of transient receptor potential vanilloid 1 (TRPV1) channels, as well as catalytic activity of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) catabolic enzymes (Di Meo et al., 2022). These data extend previous studies on the effects of cannabinoid-enriched cannabis extracts on transient receptor potential (TRP) channels (De Petrocellis et al., 2011), and of cannabidiol- and cannabigerol-type pCBs on CB₁R and CB₂R (Navarro et al., 2020), suggesting that these minor pCBs could have an impact when present in various cannabis formulations (Di Marzo and Piscitelli, 2015; Turner et al., 2017).

B. Cannabinoid Receptors, Endocannabinoids and their Congeners

The discovery of THC in the ‘40s (Adams et al., 1948) and its complete structural elucidation twenty years later (Gaoni and Mechoulam, 1964) allowed researchers to synthesize radiolabeled synthetic analogs that were instrumental to the identification and localization of specific cannabinoid binding sites in the brain (Devane et al., 1988; Herkenham, et al., 1990). In particular, a radiolabeled THC congener, the nonclassical bicyclic cannabinoid CP55940, allowed researchers to perform initial binding assays and structure-activity relationship studies of the receptor (Devane et al., 1988; Howlett et al., 1988). This was followed by development of radiolabeled 5’-(1,1-dimethylheptyl)-7-hydroxyhexahydrocannabinol (Devane et al., 1992a). The pharmacological characterization eventually led to the molecular cloning of the CB₁R from rat (Matsuda et al., 1990) and human (Gerard et al., 1990, 1991) orphan G protein-coupled receptor (GPCR) clones. CB₁R activation in mice led to a standard set of cannabimimetic responses, the so-called "tetrad test", which sequentially assesses antinociception, catalepsy, hypomotility, and hypothermia (Smith et al., 1994). Shortly afterwards a second molecular target of THC was found and named CB₂R (Munro et al., 1993), predominantly localized to the immune system (Lynn and Herkenham, 1994), where it leads to immune suppressive responses (Howlett et al., 2002; Klein and Cabral, 2006; Cabral and Griffin-Thomas, 2008; Cabral et al., 2008). For a comprehensive review of both cannabinoid receptors see report of the International Union of Pharmacology Cannabinoid Receptor Nomenclature Committee (Howlett et al., 2002).
The identification of CB₁R, the most abundant GPCR in the mammalian brain, and of CB₂R prompted intense research into the endogenous ligands for these receptors (Di Marzo and Fontana, 1995). Such ligands were identified as anandamide (N-arachidonoylethanolamine, AEA) (Devane et al., 1992b) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). The first endogenous ligand of CB₁R and CB₂R was named anandamide after the Sanskrit word “ananda” which means bliss, and on its chemical nature as an amide. Indeed, AEA and 2-AG are an amide and an ester of the ω-6 arachidonic acid (AA), respectively (Table 3), and remain the best studied eCBs.

Table 3. Placeholder

Other potential members of the eCB family have been discovered, including: (i) ω-6 fatty acid-derived eCBs like AEA, 2-AG, 2-arachidonoylglycerol (noladin) ether and the “inverted anandamide” virodhamine, reported to have various biological activities (Maccarrone, 2017; Baggelaar et al., 2018; Cristino et al., 2020); and (ii) ω-3 fatty acid-derived eCBs like N-eicosapentaenoylethanolamine and N-docosahexaenoylethanolamine, endowed with promising anticancer activity (Brown et al., 2010, 2020). In addition, various eCB-like fatty acid ethanolamides, including N-palmitoylethanolamine and N-oleoylethanolamine, have been described, which serve important functions in the control of energy metabolism (Rodriguez de Fonseca et al., 2001; Schwartz et al., 2008; Misto et al., 2019), pain (Calignano et al., 1998; Fotio et al., 2021), and inflammation (Solorzano et al., 2009) by engaging the nuclear receptor peroxisome proliferator-activated receptor (PPAR) α (Fu et al., 2003; Lo Verme et al., 2005). N-stearoylethanolamine also has anti-inflammatory activity, but via activation of PPARγ (Kosiakova et al., 2022). Finally, eCB-like amino acids (also known as lipoamino acids) have been isolated, such as N-arachidonoylglycine, N-arachidonoyldopamine, N-arachidonoylserine, N-oleoylglycine and N-oleoylalanine (Ayoub et al., 2020), which may have a number of distinct biological activities and hold therapeutic potential against vasodilation and osteoporosis (Table 3).

Although THC and AEA have completely different structures, with THC being a terpene-resorcinol derivative (Table 1) and AEA being an AA amide linkage with ethanolamine (Table 3), their biological activities were found to be closely related (Fride et al., 1993; Vogel et al., 1993). Also of note is the observation based on phylogenetic analyses that eCBs appear to be much older than pCBs. Cannabis (aged ca. 76–107 million years) is much younger than organisms like black truffles (Tuber melanosporum, aged ca. 156 million years) (Pacioni et al., 2015), hydra (De Petrocellis et al., 1999) and tetratymena (Anagnostopoulos et al., 2010) where eCBs can be detected.

C. Diverse pCB and eCB Targets and Signaling Pathways
The number of receptors activated by pCBs and eCBs in the same cell, both on the plasma membrane and in the nucleus, appears striking and is schematically depicted in Fig. 1.

Indeed, pCB- and eCB-binding receptors include: i) seven-transmembrane GPCRs CB₁ and CB₂ (Howlett et al., 2002), as well the recently de-orphanized GPCRs GPR55, GPR119 and GPR18 that can also bind cannabinoid-like ligands (Godlewski et al., 2009; Pertwee et al., 2010; Zhao and Abood, 2013; Shore and Reggio, 2015; Morales and Reggio, 2017; Alhouayek et al., 2018; Morales et al., 2020; Im, 2021); ii) receptors that are located on the plasma membrane and have intracellular binding sites, such as ionotropic TRP vanilloid 1, 2, 3, 4 channels, TRP cation channel A1 and melastatin 8, that are all six-transmembrane spanning receptors; and iii) nuclear PPARs α, γ and δ, that are transcription factors able to regulate gene expression (Maccarrone, 2020; Gomez-Cañas et al., 2023). Of note, CB₁R has been shown to move in and out distinct microdomains of the plasma membrane known as lipid rafts, which might contribute to control their G protein-dependent signaling (Oddi et al., 2017; Saumell-Esnaola et al., 2021). In addition, CB₁R appears to localize also in the outer membrane of mitochondria, where it modulates energy metabolism of neuronal (Benard et al., 2012) and non-neuronal cells (Pagano Zottola et al., 2022). GPCRs, TRPs and PPARs trigger different transduction pathways, summarized in Fig. 2.

Therapeutic benefits have been documented by targeting the pCB/eCB-binding receptors and signal transduction thereof, both in CNS and peripheral pathologies as detailed in the following sections. It is now widely appreciated that GPCRs instigate intracellular signaling by two transducer families, heterotrimeric G proteins and GPCR kinases/arrestin. These transducers interact with agonist-bound GPCRs to trigger alternative signaling cascades, so that biased agonists that favor either heterotrimeric G protein or GPCR kinases/arrestin signaling are of profound pharmacological interest (Chen and Tesmer, 2022). In this context, recent advances in understanding biased signaling and off-target activity of CB₂R (Soethoudt et al., 2017), also in living cells (Sarott et al., 2020), and molecular mechanism of allosteric modulation of CB₁R (Yang et al., 2022) suggest that biased signaling driven by eCBs might be better appreciated in the next future and usher in a new generation of drugs with greatly reduced side effects.

D. Metabolic Routes

Metabolism of AEA and 2-AG has been intensely investigated since their discovery in the mid ‘90s, whereas little information is as yet available on the metabolic routes of the additional eCBs and congeners. AEA and 2-AG are metabolized by a complex array of distinct biosynthetic and catalytic enzymes, and are
transported through the plasma membrane, intracellularly and extracellularly by distinct and poorly understood mechanisms that engage putative protein carriers.

In general, it is of paramount importance that all biological activities of eCBs, either receptor-dependent or independent, are subjected to a stringent “metabolic control”, which means that they depend on the cellular concentration of eCBs that in turn depends on a balance between synthesis and degradation by multiple regulated enzymes (Friedman et al., 2019; Cristino et al., 2020; Maccarrone, 2020).

1. Metabolism of AEA. AEA can be produced by membrane phospholipid precursors via multiple pathways, as schematically depicted in Fig. 3. Among these, N-acyltransferase (NAT), either Ca^{2+}-dependent or independent (iNAT), and N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) catalyze the most classical route for the release of AEA from phosphatidylethanolamine and phosphatidylcholine precursors. In addition, soluble phospholipase A_2, α/β hydrolase domain protein 4, phospholipase C, lyso-phospholipase D, protein tyrosine phosphatase non-receptor type 22, SH2 domain-containing polyniositol-5-phosphatase 1, and various glycerophosphodiesterase family members catalyze parallel routes for the biosynthesis of AEA.

Fig. 3. Placeholder

Multiple pathways also exist for the degradation of AEA, which can be cleaved into ethanolamine and AA, thus terminating its biological activity. This hydrolysis is primarily catalyzed by fatty acid amide hydrolase-1 (FAAH-1), but also by the less widespread FAAH-2 or by the lysosomal enzyme N-acylethanolamine acid amidase (NAAA) (Piomelli et al., 2020), as shown in Fig. 4.

Fig. 4. Placeholder

As an alternative to degradation, AEA can be biotransformed by oxygenation (i.e., addition of molecular O_2) of the AA moiety catalyzed by lipoxygenase 5, 12, 15 isozymes (5-, 12-, 15-LOX), cyclooxygenase-2 (COX-2) or cytochrome P450 (CYP450), as summarized in Fig. 4 and recently reviewed (Rouzer and Marnett, 2011; Fezza et al., 2014; Simard et al., 2022). Remarkably, COX-2-generated prostamides and the other oxidative derivatives of AEA are endowed with biological activities on their own (Van der Stelt et al., 2002; Simard et al., 2022). To date, their pathophysiological roles remain rather elusive, but apparently they include neuroprotection of the brain (Veldhuis et al. 2003).

2. Metabolism of 2-AG. Much like AEA, membrane phospholipid precursors like phosphatidylinositol and phosphatidic acid are cleaved via phospholipase A_1 or phosphohydrolase, respectively, to release 2-arachidonoylglycerol-3-phosphate or diacylglycerol, respectively (Fig. 5). Then, a Ca^{2+}-dependent phospholipase C (PLC) or Ca^{2+}- and glutathione-dependent DAG lipases (DAGL) α and β release 2-AG. The
latter DAGLα/β-dependent pathway is the classical biosynthetic route for 2-AG (Bisogno et al., 2003), and glutathione seems to be a key regulator in the brain (Maccarrone et al., 2008).

Fig. 5. Placeholder

Alternative pathways have been discovered for the degradation of 2-AG, which is primarily cleaved to glycerol and AA by monoacylglycerol lipase (MAGL), as shown in Fig. 6. In addition, α/β hydrolase domain proteins 2, 6 and 12, carboxylesterases 1 and 2 and palmitoyl-protein thioesterase 1 can degrade 2-AG to AA and glycerol (Baggelaar et al., 2018; Maccarrone, 2020), as shown in Fig. 6. Much like AEA, 2-AG can be oxygenated by COX-2, 12- and 15-LOX (Rouzer and Marnett, 2011; Fezza et al., 2014; Simard et al., 2022), leading to oxidative derivatives like prostaglandin- or thromboxane-glyceryl esters with their own biological activities (Baggelaar et al. 2018; Simard et al., 2022).

Fig. 6. Placeholder

E. Trafficking of Endocannabinoids

The stringent metabolic control of eCB tone is further modulated by distinct transporters that facilitate the movement of eCBs across the plasma membrane (possibly via a purported and as yet elusive eCB membrane transporter), as well as intracellularly and extracellularly. Moreover, not only can eCBs be released from membrane precursors when the cell receives a stimulus “on demand”, but they can be stored in cytosolic organelles like adiposomes (Maccarrone, 2020b). The mechanisms underlying the membrane transport of eCBs have been extensively investigated, leading to two prevailing models whereby eCBs are transported either by passive diffusion (Fasia et al., 2003) or by facilitated diffusion through a membrane carrier (Di Marzo et al., 1994; Beltramo et al., 1997). The mechanism(s) of transmembrane transport of eCBs remain(s) a highly debated issue in the field, and has/have been the subject of comprehensive critical reviews (Fowler, 2013; Nicolussi and Gertsch, 2015; Kaczocha and Haj-Dahmane, 2022). In addition to passive or facilitated diffusion, eCBs can leave a cell as part of microvesicles that undergo exocytosis, and indeed such a mode of extracellular transport has been demonstrated in the synaptic cleft for both AEA (Gabrielli et al., 2015) and 2-AG (Nakamura et al., 2019). The different modalities of transmembrane transport of eCBs are schematically depicted in Fig. 7A.

Fig. 7. Placeholder

The eCBs are lipids and as such they cannot travel the aqueous cytosol without a suitable carrier (Maccarrone et al., 2010). Unsurprisingly, cytosolic AEA-binding proteins have been demonstrated over the last few years and include structurally unrelated proteins like heat shock protein 70 and albumin (Oddi et al., 2009), fatty acid binding proteins (FABPs) 1, 5 and 7 (Kaczocha et al., 2009; Elmes et al., 2019), FAAH-like
anandamide transporter (Fu et al., 2011), sterol carrier protein 2 (Hillard et al., 2017) and retinol-binding protein 2 (Plau et al., 2022). These eCB transporters are schematically depicted in Fig. 7B.

While the pathophysiological relevance of intracellular and extracellular trafficking of eCBs remains elusive (Jacobson et al., 2019; Fauzan et al., 2022), it appears that carriers of these lipids should be actively investigated, because they might be major players in driving eCB signaling. Indeed, these carriers can ferry the right eCB to the right target, at the right time and in the right concentration, thus holding potential as primary action points for the development of effective eCB-oriented therapeutics. Of note, these novel therapeutics should be devoid of unwanted side-effects often associated to drugs that target receptors or metabolic enzymes of eCBs (Ciaramellano et al., 2023).

On a final note, to date 3D structures of only 23 major components of the ECS have been resolved, whereas many other elements still await clarification of their structural features (Maccarrone, 2020b). Among the latter, key receptors (e.g., GPR55, GPR119 and TRPV4), enzymes (e.g., NAT, DAGLα/β, GDE1,4,7, ABHD2,4,6,12) and the putative eCB membrane transporter can be listed. It is apparent that such an information gap is particularly troubling for drug discovery programs, and must be urgently filled.

In the following sections the main properties and therapeutic potential of some of the main ECS components are detailed, whereas the other elements suffer from a lack of information.

II. Cannabinoid Receptor Physiology and Pharmacology

The eCBs and THC are dual effectors at both CB₁R and CB₂R, which share a ligand binding domain sequence identity of 44% (Matsuda et al., 1990; Munro et al., 1993; Howlett et al., 2002; Mackie, 2005). The absolute stereochemistry of THC was deciphered in 1967 (Fig. 1) (Mechoulam and Gaoni, 1967), and this was followed by the development of many analogs by academic chemists (Razdan, 1986). THC is a dual CB₁R and CB₂R partial agonist exhibiting multiple therapeutically interesting physiological properties involving both receptor types, that include anti-inflammatory, immunosuppressive and analgesic effects. THC was the first cannabinoid agonist approved as a medication by the US Food and Drug Administration (FDA) under the generic name dronabinol (Marinol), although its use was restricted due to CNS-mediated psychotropic side effects.

Many additional non-selective cannabinoid agonists have provided insights into pharmacotherapeutic potential (reviewed by Robson, 2001; Pertwee 2008b, 2012). With a goal to develop cannabinoid, non-opioid analgesics, Pfizer produced a series of A-C-bicyclic and A-C-D-tricyclic analogs of THC’s CNS-active metabolite 11-OH-THC, and these are referred to as “non-classical cannabinoids” because of their origin and
similarity to the A-B-C-tricyclic structure of THC (Johnson et al., 1981; Howlett et al., 1990; Melvin et al., 1993; Melvin et al., 1995). Of these, levonantradol, was taken to clinical trials for postoperative pain, but the project was discontinued due to prominent sedative and euphoric/dysphoric properties (Jain et al., 1981). The primary outcome of the Pfizer effort was the development of the CB1R/CB2R non-selective full agonist CP55940, outperforming THC with regard to CB1R/CB2R binding affinity and analgesic activity (Devane et al., 1988; Howlett et al., 1988; Showalter et al., 1996) (Fig. 8). CP55940 is a research tool that has been invaluable in identifying cannabinoid receptor cellular and systems physiology (Devane et al., 1988). Tritiated CP55940 was critically involved in the deorphanization of both CB1R (Matsuda et al., 1990) and CB2R (Munro et al., 1993), and has been broadly applied to quantitate the structure-activity relationships of most novel ligands developed for the investigation of cannabinoid receptors.

Sterling-Winthrop discovered that structural modifications of the non-steroidal anti-inflammatory agent, pravadoline, resulted in greater antinociceptive activity with diminished potential to block prostaglandin production (Bell et al., 1991). Although the Sterling-Winthrop project was terminated in the preclinical stages, the introduction of the CB1R/CB2R non-selective full agonist WIN55212-2 has contributed greatly to investigations of cannabinoid receptor physiology and pharmacology (Fig. 9). WIN55212-2 in its tritiated form is a standard CB1R/CB2R radioligand (D’Ambra et al., 1992; Eissenstat et al., 1995), and with its derivatives is referred to as “aminoalkylindoles” because their structure is built on indole or indene platforms.

Selective activation of either CB1R or CB2R by THC or the other non-selective agonists seems to be controlled by differential expression (induction or desensitization/down-regulation) of the receptors on a wide variety of cells that control differentiated functions (reviewed by Howlett and Abood, 2017).

Research work using Ligand Assisted Protein Structure (LAPS) methodology had characterized the sites of action at CB1R and CB2R (Janero et al., 2017). However, a more detailed CB1R structure was reported in 2016 in its inactive conformation by using the long-acting CB1R antagonist AM6538 (Hua et al., 2016), shown in Fig. 10, and the antagonist/inverse agonist taranabant (Shao et al., 2016). This allowed the docking of several CB1R antagonist analogs and the study of their interactions with the receptor. This work was followed by structures of the agonist-bound CB1R (Hua et al., 2017; Krishna Kumar et al., 2019; Hua et al., 2020) which demonstrated that activation of CB1R induces dramatic conformational changes of both extracellular and intracellular domains of the receptor, accompanied by a serious contraction of the binding
pocket. This more expansive conformation of CB1R in its inactive state explains how several antagonists can be accommodated in the receptor structure.

Figure 10. Placeholder

The high-resolution crystal structure of antagonist-bound CB2R was determined in 2019, which firstly discloses the binding mode of antagonist AM10257 (Li et al., 2019). The latter locates at the orthosteric ligand-binding pocket and mainly forms hydrophobic and aromatic interactions with residues from extracellular loop 2, as well as the cytoplasmic parts of transmembrane helices 2, 3, 5 and 6 of CB2R (Fig. 11A). However, the antagonist AM10257 adopts a constrained binding pose in CB2R, which is quite different from the extended binding conformation of antagonists in CB1R (Hua et al., 2016). Of note, the adamantyl moiety of AM10257, adapting a vertical conformation, would clash with the residue Phe102N-term of CB1R when two structures are superimposed (Fig. 11B-D). That is the reason why the N-terminus of CB2R forms a short helix over the orthosteric pocket, instead of the V-shaped loop that directly interacts with antagonist in CB1R (Hua et al., 2016; Shao et al., 2016). In addition, the extracellular part of transmembrane helices 1 and 2 in CB2R is more compact compared with the conformations of the same helices in CB1R, resulting in a much smaller antagonist-binding pocket than that of CB1R (Hua et al., 2016; Shao et al., 2016). The structural analysis provides the basis for the high-degree of antagonist selectivity between CB1R and CB2R.

Fig. 11. Placeholder

In spite of the high selectivity of antagonists or inverse agonists, most agonists can bind both CB receptors with comparable affinity (Pertwee et al., 2010). The recently determined structures of agonist-bound CB2R provide valuable information at the molecular level for subtype-selective agonist design (Hua et al., 2020; Xing et al., 2020) and subtype-selective receptor activation (Li et al., 2023). Both the synthetic THC-like agonist AM12033 and aminoalkylindole agonist WIN55212-2 form mainly hydrophobic and aromatic interactions with CB2R, including residues from transmembrane helices 2-3, 5-7 and the extracellular loop 2 with similar binding mode in the orthosteric ligand-binding pocket (Fig. 11E-F). Although the core of WIN55212-2 forms π-π interactions with F1173.36 and W2586.48 of CB2R, the rotamers of F1173.36 and W2586.48 are very similar in these two structures (Fig. 11G). The superposition of agonist-bound CB1R and CB2R structures shows that the agonist binding pocket volume, as well as the key residues that form interactions with ligands, are almost identical (Fig. 11H-M). This accurate molecular information of the CB receptors’ orthosteric binding pockets obtained so far should aid the design of selective agonists for safer therapeutics.
The CB$_2$R activation mechanism was revealed by the comparison of active and inactive structures. Though the antagonists and agonists of CB$_2$R share similar binding pockets, including the key interaction residues with the receptors, the interaction of CB$_2$R ligands with the "toggle residue" W2586.48 is related to their efficacies. Compared with antagonist AM10257, agonist AM12033 lacks the moiety that extends deeper into the binding cavity to constrain W2586.48 rotation, which can trigger receptor activation (Fig. 12A). Subsequently, the classical rearrangements of N7.49 P7.50 x x Y 7.53 and D3.49 R3.50 Y3.51 motifs were observed that contribute to the conformational change of the intracellular part of CB$_2$R, eventually forming the G-protein binding cavity (Fig. 12B-C). However, in contrast to agonist-bound CB$_1$R, only the intracellular part of CB$_2$R exhibits obvious conformational changes while the extracellular part including the N-terminus of CB$_2$R undergoes minor changes during its activation (Fig. 12D-F). The balloon-like plasticity of CB$_1$R during its activation indicates its higher ability to respond to a diverse array of ligands than CB$_2$R, which may explain the low selectivity compared with CB$_1$R for most classical THC-like agonists of CB$_2$R.

Fig. 12. Placeholder

A. Therapeutic Potential of CB$_1$R

The epigenetic regulation of CB$_1$R expression and signal transduction pathways following Gi/o or ß-arrestin activation is related to differentiated cell functions, as reviewed recently (Kendall and Yudowski, 2016; Ligresti et al., 2016; Howlett and Abood, 2017; Lutz, 2020; Schurman et al., 2020). The CB$_1$R is highly abundant in the CNS and many peripheral tissues and organs (Howlett et al., 2002; Pacher et al., 2006). For instance, it is critically involved in the regulation of mood and appetite, pain perception, learning and memory, as well as motor control (Marsicano and Lutz, 2006; Kano et al., 2009; De Laurentiis et al., 2014). The CB$_1$R has been recognized as a target for pharmacotherapeutic development based upon a wealth of preclinical data (for reviews see Mackie, 2008; Pertwee, 2008b, 2012; Tsang and Giudice, 2016; Lu and Anderson, 2017; Amin and Ali, 2019; Schurman et al., 2020; Wilkerson et al., 2021). However, bringing CB$_1$R agonists and antagonists to market has been fraught with the challenges of selectivity resulting from the abundance of CB$_1$R throughout all areas of the brain, including expression by neuronal and non-neuronal cells. This broad distribution increases the probability of unwanted side effects accompanying the therapeutic benefits.

1. CB$_1$R Agonists and Positive Allosteric Modulators. The only FDA-approved CB$_1$R agonists are THC itself (synthesized as dronabinol) and its dimethylheptyl analog nabilone (LY-109514), specifically to treat cancer chemotherapy-induced nausea and vomiting, and these medicines remain within the US Pharmacopeia (Clarivate CortellisTM data base dronabinol, 2022; Adis Insight Web Page nabilone, 2022).
The European Medicines Agency (EMA) approved the mixture of THC and CBD extracted and purified from cannabis (nabiximols) for the treatment of spasticity and pain in multiple sclerosis (MS). Dronabinol, nabilone and nabiximols exhibit agonist activity at both CB₁R and CB₂R, though therapeutic responses and untoward side effects can be attributed to one or both CB receptors, as determined by pharmacological characterization in \textit{in vivo} or \textit{in vitro} models. Nevertheless, targeting CB₁R for unmet therapeutic needs has evolved based upon preclinical investigations, and these opportunities will be considered in this section.

Dronabinol was developed to counteract the nausea and vomiting in cancer chemotherapy and was later approved to promote appetite stimulation and metabolic maintenance to counteract cachexia in AIDS patients (Plasse et al., 1991). Dronabinol is synthetically-produced THC formulated in a sesame oil capsule and marketed as Marinol (Adis Insight Web Page nabilone, 2022). Dronabinol is also available in a liquid formulation solubilized in ethanol and propylene glycol and marketed as SYNDROS. The pharmacokinetics, dosage recommendations, and drug interactions are available at Prescribers Digital Reference-Marinol-dronabinol-2726 (PDR, 2022a). The warnings reported include bradycardia and seizures in vulnerable populations. Mild to moderate adverse reactions include: emotional lability in 8% to 24% of users; impaired cognition, dysphoria or euphoria, depression, hypotension, drowsiness, paranoia, dizziness or nausea in 3% to 10% of users; and conjunctivitis, hallucinations, confusion, amnesia, ataxia, tinnitus, nightmares or diarrhea in 0.3% to 1% of users (PDR, 2022a).

Nabilone is synthesized as a 9-ketocannabinoid with a dimethylheptyl side chain (Fig. 13), and is enzymatically reduced in the liver to the hydroxylated S(axial) isomer believed to be the active form (Archer et al., 1977; Rubin et al., 1977; Billings et al., 1980). Nabilone was approved as an antiemetic for cancer chemotherapy, but also exhibits anxiolytic properties (Lemberger and Rowe, 1975; Ward and Holmes, 1985). Nabilone is used off-label for treatment of the symptoms of Huntington’s chorea (PDR, 2022b). The warnings and adverse reactions are similar to those reported for dronabinol: seizures in vulnerable populations, early euphoria or dysphoria, delayed depression, ataxia, hypotension, drowsiness, vertigo, dizziness, asthenia, or headache.

\textit{Fig. 13. Placeholder}

Nabiximols is a mixture of THC and CBD (1:1) in ethanol and propylene glycol solvent as an oromucosal spray formulation marketed as Sativex (see the EMA electronic medicines compendium (emc) for information, GW_Pharma_Ltd, 2022). The spray is intended to be applied at the onset of muscle contractions in order to reduce spasticity and pain in MS patients. Each application provides some fraction of dosage to be absorbed via the mucosal membranes and the remainder is swallowed and absorbed from the
gastrointestinal tract. Sativex was granted orphan designation by the EMA for treatment of glioma patients while clinical trials were being conducted; however, this status was later withdrawn - see EMA notices (EMA, 2022). The EMA emc reports pharmacokinetic data and recommended dosing schedules for use in MS patients (GW_Pharma_Ltd, 2022). The report includes warnings/precautions for use in patients with histories of seizures or cardiovascular disease. Adverse reactions found in clinical trials include: appetite changes, dizziness, disorientation, mood swings, depression, amnesia/memory impairment, somnolence or blurred vision in 1% to 10% of users; and pharyngitis, syncope, anxiety, illusions, paranoia, hallucinations, or delusional beliefs in 0.1% to 1% of users. Adverse effects at the site of application include oral discomfort/pain, altered taste, mouth ulceration and accompanying pain.

Prior to the recognition of CB receptors, clinical trials provided positive indications for CBD for seizure control and movement disorders (Cunha et al., 1980; Carlini and Cunha, 1981; Consroe et al., 1986; Consroe et al., 1991). CBD entered the market for the treatment of the Dravet syndrome, infantile severe myoclonic epilepsy, Lennox-Gastaut syndrome and tuberous sclerosis (Clarivate CortellisTM data base cannabidiol, 2022). In addition, CBD and THC combinations have been approved for MS-associated spasticity and pain management (Clarivate CortellisTM data base nabiximols, 2022; PharmaprojectsTM data base tetrahydrocannabinol plus cannabidiol, 2022). CBD in the nabiximols formulation may or may not exert its cellular actions via processes involving CB1R. CBD exerts both negative and positive interactions with THC over a range of biological and behavioral responses in animal models and humans (Pertwee, 2008a; McPartland et al., 2015). In a cloned neuronal cell model, CBD competed with the CB1R against \({}^{3}H\)CP55940 in binding to the receptor at concentrations nearly three orders of magnitude greater than did THC (Devane et al., 1988); however, CBD failed to inhibit cAMP production via the CB1R-coupled G, protein as does THC (Howlett, 1984; Mukhopadhyay et al., 2002). Similar findings of low potency binding to CB1R and inability to stimulate CB1R cellular signaling were reported in multiple studies using other models as compiled in a meta-analysis from a pool of >200 research publications (McPartland et al., 2015). Two influences of CBD on CB1R pharmacology are most compelling: 1) CBD could exert a non-competitive antagonism at CB1 receptor (Petitet et al., 1998; Thomas et al., 2007; Laprairie et al., 2015); and 2) CBD could indirectly modulate CB1R activity by FABP competition (Elmes et al., 2015) and FAAH inhibition (Bisogno et al., 2001; De Petrocellis et al., 2011) or activation (Massi et al., 2008), thereby changing eCB tone. Non-CB1R mechanisms proposed for CBD’s neurological actions minimally include: facilitation of serotonin signaling, activation of TRPV1 or PPARy receptors, neuroprotection via antioxidant activity, and attenuation of pro-inflammatory processes (Campos et al., 2012; Ibeas Bih et al., 2015; Campos et al., 2012; Ibeas Bih et al., 2015).
2017). Other molecular targets for CBD include additional GPCRs (e.g., GPR55, GPR18, μ and δ opioid receptors) and TRP channels A1, V2, M8 (McPartland et al., 2015; Ligresti et al., 2016).

Because dronabinol, nabilone and nabiximols are currently approved medicines by regulatory agencies, it is acceptable to repurpose these preparations for treatment or amelioration of other disease symptoms based upon preclinical evidence that justifies their use. Table 4 lists the double-blind clinical trials that have been registered with ClinicalTrials.gov, and are completed or ongoing at the date of publication of this review.

**Table 4. Placeholder**

Appropriate preclinical data justify these putative uses, and warrant evaluation of both efficacy of these cannabinoid agonists for these purposes, and relative safety given the risk:benefits assessment and the circumstances of patient treatment. Review articles are cited which summarize research evidence in animal models, address implications and challenges, and provide original references.

Nausea and vomiting that accompany surgical procedures, retroviral therapy, and neoplasms are unmet needs that build upon the original usage approved by regulatory agencies for patients undergoing cancer chemotherapy (Abrams and Guzman, 2015). Preclinical studies using animal models of nausea and vomiting (“retching” or “gaping”) have demonstrated effective attenuation with CB1R agonists, although the exact neurological mechanism has not been established (Parker et al., 2011; Sticht et al., 2015). In contrast, in the current population of recreational cannabis users, a novel cannabis-induced hyperemesis syndrome has been attributed to ingestion of very high doses of THC. The mechanism is poorly understood, but it has been suggested that prolonged exposure to high doses of THC might down-regulate CB1R or otherwise perturb the endogenous regulation of vomiting centers in the brain stem and/or elicit stress mechanisms at the hypothalamic-pituitary axis (Galli et al., 2011; DeVuono et al., 2020). Thus, there may be a “bell-shaped” dose-response curve suggesting multiple mechanisms for the anti-versus pro-nausea/vomiting endpoints.

The appetite stimulation response was the impetus for regulatory approval of CB1R agonists as “orphan” drugs for treatment of cachexia in cancer (Plasse et al., 1991). However, it is the appetite suppression by CB1R antagonism that prompted clinical trials for weight loss in morbidly obese individuals and resulted in an explosion of basic science research linking the CB1R to metabolic processes associated with energy storage (Piazza et al., 2017; DiPatrizio, 2021; Miralpeix et al., 2021). Studies of CB1R-mediated inhibition of gut mobility (Pertwee et al., 1992; Pertwee, 2001) led to the consideration of agonist treatments for irritable bowel syndrome and other gastrointestinal pathologies (Lee et al., 2016; Sharkey and Wiley, 2016). Conversely, detrimental influences of CB1R stimulation on pancreatic β-cell function, diabetic insuli-
Control of chronic and episodic pain continues to be an unmet therapeutic need. The development of CB1R agonists as antinociceptive agents by Pfizer Central Research (Johnson et al., 1981; Howlett et al., 1990; Melvin et al., 1993; Melvin et al., 1995) was meant to fulfill this need, but the effort was discontinued due to untoward side effects in patients during clinical trials (Jain et al., 1981). A resurgence of interest in cannabinoid analgesics as adjunctive or second/third-line treatments has reassessed the benefits versus risks ratio for pain conditions associated with cancer, neuropathy, fibromyalgia and spasticity (Tsang and Giudice, 2016; Woodhams et al., 2017). Recent clinical trials suggest that cannabinoid-mediated analgesia in humans could be attributed to a moderate reduction in affective response but not a reduced perception of the experimental pain (Lötsch et al., 2018).

CB1R agonist efficacy in symptomatic relief in MS and amyotrophic lateral sclerosis is related to the reduced spasticity and tremors, as investigated in an animal model of chronic relapsing experimental allergic encephalomyelitis, as well as reports from patients (Pryce and Baker, 2015; Pertwee, 2002). In addition to relieving the spastic pain, cannabinoid agonists at CB1R and CB2R slow the progression of the disease as a result of neuroprotective mechanisms and oligodendrocyte development to promote myelination (Pryce and Baker, 2015; Ilyasov et al., 2018; Khan et al., 2022). Similarly, agonist stimulation of both CB receptors reduces symptomology and disease progression in other neurodegenerative diseases such as Parkinson’s disease, Huntington’s chorea, Alzheimer’s disease and stroke (Fernández-Ruiz et al., 2015a; Fernández-Ruiz et al., 2015b).

Numerous “cannabinoid products” that are not approved by regulatory agencies are being tested for their potential therapeutic value. It is difficult to discern the composition and concentration of active agents in these herbal preparations, which are variously described as: cannabis, cannabis oil, smoked cannabis (cigarettes), inhaled cannabis, vaporized cannabis, cannabis extract, or “CBD-rich”/”THC-rich” marijuana or extracts. These preparations are not further discussed here, because of the lack of quantitative analyses of the materials being used by the patients. Of note, these herbal studies are registered in ClinicalTrials.gov as assessments (blinded or unblinded) for symptomatic improvements in neuropsychiatric and neurological disorders, including attention deficit and hyperactivity disorder, dementia, anxiety, depression, post-traumatic stress disorder, autism spectrum disorder, obsessive-compulsive disorder, refractory epilepsy, MS, amyotrophic lateral sclerosis, Tourette’s syndrome, pain (migraine, neuropathic, fibromyalgia, pre- and post-
surgical, back, and cancer), agitation associated with aging dementia, irritable bowel disease, chronic obstructive pulmonary disease, and retinitis pigmentosa with degeneration. The rationale for using plant products is that the effects of multiple chemical entities (including “cannabinoids”, terpenes and flavonoids) may synergize, a concept referred to as an “entourage effect”. The idea of combining medicines - referred to as polypharmacology - that provide different but complementary pharmacological responses, such as anti-inflammatory plus analgesic agents, is not new and is often a preferred treatment strategy (Brodie et al., 2015; Ligresti et al., 2016). However, the challenges of determining the active synergistic agents, appropriate dosing schedule, specificity of therapeutic use, and safety profile remain to be overcome when herbals are used as medicinal products.

In an effort to address selectivity for the CB1R, modifications have been made to pCB, aminoalkyindole, and eCB ligands. For example, AEA analogs arachidonylcyclopropylamide (ACPA) and arachidonyl-2-chloroethylamide (ACEA) exhibit 1-2 nM affinity for the CB1R but 1-3 μM affinity for the CB2R, and both inhibit cAMP CB1R selectivity of the ACPA (Hillard et al., 1999). This selectivity led to the CB1R selective (CB1R/CB2R Ki ratio = 0.1) dual CB1R/CB2R agonist CMX-020, which is currently being explored in phase 2 clinical trials for the treatment of osteoarthritis (ANZCTR Web Page, 2022), pain including sciatica, and diabetic neuropathy (Clarivate CortellisTM data base CMX-020, 2022).

Another mechanism for achieving selectivity is found in the recent development of allosteric modulators to modify the CB1R response. Exploiting allosteric modulation is a broadly used approach for targeting GPCRs (Wold et al., 2019). It allows addressing target selectivity issues and associated off-target side effects of orthosteric ligands by binding to a topographically distinct site. Allosteric ligands modify the conformation of the receptor protein, which allows for modulating the affinity of orthosteric ligands. Allosteric ligands can either augment (positive allosteric modulation) or diminish (negative allosteric modulation) the effect of endogenous ligands. Importantly, this provides the opportunity for tissue-specific modulation of ECS signaling, e.g. via a local eCB increase as consequence of an inflammatory stimulus. In contrast to the high evolutionary conservation of orthosteric binding domains, allosteric sites exhibit a greater sequence difference allowing for the generation of ligands with high subtype selectivity (Kenakin and Miller, 2010). In addition, an interaction of cholesterol was also observed with CB1R, suggesting its endogenous allosteric modulating role (Hua et al., 2020). This observation extended previous in vitro (Bari et al., 2005) and ex vivo (Maccarrone et al., 2009) functional data showing that membrane cholesterol controls CB1R dimerization and binding activity.
Positive allosteric modulation of CB, R is likely to play an increasingly important role for drug discovery (Saleh et al., 2018; Garai et al., 2021). For instance, ZCZ011 increased the potency and reduced tolerance development in the anti-nociceptive activity of CB, agonists (Ignatowska-Jankowska et al., 2015); GAT211 synergized with FAAH- or MAGL-inhibitor-mediated eCB accumulation to attenuate inflammatory and neuropathic pain (Slivicki et al., 2020; Slivicki et al., 2018). Preclinical studies of CB, R allosteric modulators have been reviewed recently (Khurana et al., 2017; Hryhorowicz et al., 2019; Manning et al., 2021).

Another promising approach to selectivity is the development of “biased agonists”. The binding mechanism for a biased agonist would be expected to alter the conformation of the CB, R to prefer either an interaction with the Gi/o family, or alternatively allow phosphorylation of the receptor via G-protein receptor kinases to facilitate interaction with β-arrestins 1 or 2 (Priestley et al., 2017; Al-Zoubi et al., 2019). The selectivity would be for the signal transduction pathway involved in the beneficial effects while diminishing the signal for unwanted side effects. Preclinical studies that explore possible CB, R biased agonists have been reviewed recently (Laprairie et al., 2016; Ibsen et al., 2017; Leo and Abood, 2021; Manning et al., 2021).

2. CB, R Antagonists. Sanofi discovered the first CB, R selective antagonist in the early 2000s (SR141716) and the compound was initially earmarked for use as a medication for loss of weight (rimonabant, marketed as Acomplia or Zimulti). It was reasoned that since the activation of CB, R increased food intake with weight gain, the use of its antagonist as a drug would result in weight loss. The potential success of such a medication prompted other drug companies to produce their own compounds that were structurally different but pharmacologically identical. The first CB, R antagonist to enter clinical trials for several of the above indications was rimonabant (Sanofi), followed by tarianabant (Merck), otenabant (Pfizer), ibipinabant (Solvay), and surinabant (Sanofi) as shown in Fig. 13. Additional indications that have been explored clinically include hepatic fibrosis and non-alcoholic fatty liver disease, renal diseases, as well as alcohol dependence and smoking cessation (Cinar et al., 2020). Recently, a CB, R antagonist, ANEB-001 (Anebulo) has been under clinical development as an antidote for acute cannabis intoxication.

Based on results showing weight loss and improved cardiometabolic markers in overweight and obese patients (Despres et al., 2005), rimonabant was accepted by the EMA in 2006 as an adjunct to diet and exercise for the treatment of obesity and related metabolic risks. However, approval by the FDA failed because of its unexpected neuropsychiatric side effects, namely depression and suicidal ideation (Christensen et al., 2007). Some additional side effects of CB, R antagonists are related to the gastrointestinal tract and include nausea, vomiting, and frequent bowel movements (Addy et al., 2008;
Limebeer et al., 2010). When the use of rimonabant was withdrawn by Sanofi in 2008, the development of CB₁R antagonists was discontinued by other pharmaceutical companies. Notwithstanding the failure of rimonabant, its availability allowed research toward understanding the mechanism of action of CB₁R antagonists and the potential use of such compounds for other indications. Ligands like SR141716 and AM251 (Rinaldi-Carmona et al., 1995; Lan et al., 1999) were used to establish the role of CB₁R in physiology (Varga et al., 1995; Petitet et al., 1996; Gatley et al., 1997; Liu et al., 2000; Di Marzo et al., 2001; Wang et al., 2003).

The apparent therapeutic value of CB₁R blockade led to much of the research in developing selective CB₁R antagonists and their preclinical and clinical testing for a variety of disorders related to metabolism, the cardiovascular system and addiction (Pacher et al., 2008; Cinar et al., 2020). Given the clinical efficacy shown by CB₁R blockade for several conditions with unmet medical needs, additional approaches have been explored in order to retain efficacy and circumvent the unwanted neuropsychiatric side effects. Among these, CB₁R antagonist/inverse agonists that cannot enter the CNS and CB₁R neutral antagonists have shown promising results in preclinical models.

The discovery of functional CB₁Rs in the periphery and the realization that they mediate many processes of cardiovascular system, metabolism, and fibrotic conditions (Liu et al., 2000; Di Marzo et al., 2001; Jourdan et al., 2014; Bowles et al., 2015), have led to the hypothesis that peripherally selective CB₁R antagonist/inverse agonists may retain the therapeutic effects of CB₁R blockade without the unwanted CNS effects. Small-molecule CB₁R antagonist/inverse agonists with minimal brain exposure have shown efficacy in animal models of obesity and metabolic syndrome, alcoholic and non-alcoholic liver steatosis, liver fibrosis, and renal diseases, as recently reviewed by Kunos’ group (Cinar et al., 2020). The primary methods used to determine brain permeability are pharmacokinetic studies (Zhang et al., 2018; Iyer et al., 2022), while for the specific engagement of brain CB₁R positron emission tomography tracers are used (Tam et al., 2012; Chang et al., 2019), as well as antagonism of the tetrad effects induced by CB₁R agonists (Fulp et al., 2013; Amato et al., 2018). Although many peripherally restricted ligands have minimal brain permeability after acute administration, it remains to be ascertained whether chronic administration would lead to an increase in brain permeability that can affect the profile of unwanted CNS side effects. Furthermore, only a few peripheral CB₁R antagonists/inverse agonists have been evaluated in detail for their unwanted effects, with the most extensively-studied being JD5037 (Kale et al., 2019). This compound exhibited only minor side effects such as repetitive grooming at doses much higher than the therapeutic doses, which is translated into
a safer therapeutic window compared to the brain permeant CB₁R antagonist/inverse agonists (Kale et al., 2019).

In a different approach to achieve peripheral restriction, monoclonal antibodies that act as CB₁R antagonists/inverse agonists have been developed and entered clinical evaluation. The two candidates that have been in clinical development for renal diseases and diabetic complications are Nimacimab (Bird Rock Bio) and GFB-024 (Goldfinch Bio), both listed in Table 4. However, there are no publicly available data regarding the efficacy and safety of this innovative approach.

Table 4. Placeholder

CB₁R is a constitutively active receptor that even in the absence of ligands exists in equilibrium between active and inactive states; this condition is translated into increased basal activity (Pertwee, 2005; Fong, 2014) and may be important for cellular homeostasis. While inverse agonists reduce the basal activity of receptors, neutral antagonists do not significantly affect it (Bond and Ijzerman, 2006; Sink et al., 2008). Additionally, the ECS as a whole exhibits an endogenously active tone controlled by the cellular production of eCBs (Howlett et al., 2011). Therefore, CB₁R neutral antagonists can compete with the endogenous cannabinoid ligands without affecting the basal activity of the receptor. For this reason, it was hypothesized that CB₁R neutral antagonists could produce the therapeutic phenotypes of CB₁R antagonism without the unwanted CNS and gastrointestinal side effects. To this regard, the most extensively studied CB₁R neutral antagonist, AM4113, exhibited therapeutic efficacy with a better tolerability profile. In animal models of obesity, AM4113 was shown to reduce food intake and weight gain, as well as to suppress food-reinforced operant responding and feeding (Chambers et al., 2007; Sink et al., 2008; Gueye et al., 2016). In addiction-related models, AM4113 was effective in suppressing alcohol consumption, reducing drug-seeking behavior of nicotine and THC, as well as in inhibiting the self-administration of heroin (Gueye et al., 2016; Schindler et al., 2016; Balla et al., 2018; He et al., 2019). Moreover, AM4113 did not induce anxiety-like behaviors in elevated plus maze and electrical brain-stimulation reward paradigm, unlike the CB₁R antagonist/inverse agonist AM251 (Sink et al., 2010; Gueye et al., 2016; He et al., 2019). Additionally, in contrast to CB₁R inverse agonists AM4113 did not produce gastrointestinal side effects such as nausea, potentiation of vomiting, and increase in whole gut transit (Chambers et al., 2007; Sink et al., 2008; Storr et al., 2010). Other CB₁R neutral antagonists, such as the peripherally restricted AM6545 and NESS06SM, have been shown to suppress food intake and improve cardiometabolic risk factors (Cluny et al., 2010; Randall et al., 2010; Tam et al., 2010; Mastinu et al., 2013). AM6545 also exhibited efficacy in animal models of experimental diabetic nephropathy, alone and in combination with the CB₂R agonist AM1241 (Barutta et al., 2017; 2018).
On a final note, a novel and attractive dual-targeting approach is represented by the combination of CB₁R antagonists and CB₂R agonists, as evidenced by the synergy shown by co-administration of AM6545 and AM1241 for treating diabetic nephropathy (Barutta et al., 2017). Indeed, there is early evidence that CB₁R and CB₂R promote opposing functions in fibrotic and inflammatory conditions of peripheral organs (Gruden et al., 2016), as well as in some preclinical models of addiction (Delis et al., 2017; Gobira et al., 2019) that could be leveraged for a therapeutic benefit by dual-acting CB₁R antagonists/CB₂R agonists.

B. Therapeutic Potential of CB₂R

The CB₂R is a class A (rhodopsin-like) GPCR (Fig. 14). It is an essential element of the ECS and indeed CB₂R-mediated signaling plays an important role in many human health and disease conditions (Pacher and Mechoulam, 2011; Gasperi et al., 2023). Therefore, CB₂R holds tremendous therapeutic potential for treating major pathologies affecting humans.

A plethora of preclinical evidence demonstrating the anti-inflammatory and tissue protective effects of CB₂R activation has been generated triggering the design, synthesis and evaluation of multiple CB₂R ligands. Based on their chemical structure, they can be characterized as pCBs, eCBs and congeners, or synthetic ligands (Han et al., 2013; Guba et al., 2020; Brennecke et al., 2021). While the majority of these molecules are CB₂R activators, multiple antagonists/inverse agonists and a few allosteric ligands have also been discovered. Of these, more than twenty CB₂R-selective agonists have been advanced to clinical trials. Recently, several 3D structures of CB₂R in complex with ligands have been reported (Li et al., 2019; Hua et al., 2020; Xing et al., 2020). Furthermore, a broad variety of labelled chemical probes was generated and applied in mechanistic studies (Basagni et al., 2020; Haider et al., 2020; Sarott et al., 2020; Gazzi et al., 2022; Guberman et al., 2022), and have contributed to understand the structural basis of selective CB₂R activation (Li et al., 2023). Together this knowledge will facilitate the design of novel further improved ligands. Here, efforts were made to recognize the full range of studies that have contributed to progress CB₂R research since the discovery of the receptor. Due to space limitations the content of this section highlights only foundational studies and key aspects.

The CB₂R is primarily expressed in immune cells, including macrophages, T and B cells, monocytes and polymorphonuclear neutrophils, as well as tissues like spleen (Bouaboula et al., 1993; Galiègue et al., 1995; Atwood and Mackie, 2010; The ImmGen Web Page, 2022), bone (Ofek et al., 2006) and the gastrointestinal tract (Atwood et al., 2012). CB₂R is expressed both on the cell surface and intracellularly (Kleyer et al., 2012; Brailoiu et al., 2014; Castaneda et al., 2017), and is highly inducible, for instance, in microglia upon
neuroinflammation (Cabral et al., 2008). The CB2R is a G_{i/o} coupled GPCR and its activation leads to an inhibition of cAMP production. In addition, the CB2R recruits β-arrestins, controls the activation and phosphorylation of different mitogen-activated protein kinase family members (ERK1/2, p38 MAPK, JNK), and interacts with PLC as well as G-protein-coupled inwardly rectifying K⁺-channels (Bouaboula et al., 1993; Felder et al., 1995; Howlett et al., 2002; Cabral et al., 2008; Atwood and Mackie, 2010). Surface and intracellular CB2R might be able to activate distinct signaling responses (Brailoiu et al., 2014). In addition, agonists binding to the orthosteric site exhibit different transduction profiles that might translate into distinct pharmacodynamics read-outs (Oyagawa et al., 2018; Yuan et al., 2021). Downstream effects of CB2R activation encompass the differentiation of B and T lymphocytes (Ziring et al., 2006), the suppression of T cell receptor signaling (Börner et al., 2009), the induction of natural killer cell migration (Kishimoto et al., 2005) and the modulation of cytokine release (Cencioni et al., 2010; Correa et al., 2011). CB2R interactions at the molecular level and its resulting downstream effects translate toward modulation of disease pathogenesis. CB2R ligands have demonstrated a huge therapeutic potential in a large variety of disease models, e.g. in liver (Mallat and Lotersztajn, 2008; Pacher and Gao, 2008), kidney (Mukhopadhyay et al., 2010a; Mukhopadhyay et al., 2010b; Zoja et al., 2016; Mukhopadhyay et al., 2016), lung (Pacher et al., 2006), and heart disorders (Pacher et al., 2008), skin pathologies (Bíró et al., 2009; Maccarrone et al., 2015), neurodegenerative diseases (Centonze et al., 2007; Fernández-Ruiz et al., 2007) and pain (Guindon and Hohmann, 2008; Anand et al., 2009). Generally, the reported effects are a consequence of CB2R-mediated immunosuppressive and anti-inflammatory effects leading to a dampening of tissue injury. In hypo-activated immune states, CB2R activation might however enhance tissue damage (Pacher and Mechoulam, 2011). Under these pathological conditions, CB2R inverse agonists/antagonists might provide therapeutic options.

1. CB2R Agonists. Due to the huge therapeutic potential of CB2R, multiple ligands have been developed. In 1996, a first patent for a CB2R-selective antagonist was filed (Rinaldi et al., 1996). Since then, more than 1150 CB2R patent applications have been registered. CB2R targeting molecules covered by these papers and patents encompass agonists, modulators, neutral antagonists, inverse agonists, and allosteric ligands.

While the majority of these ligands are classical small molecules, including many labelled chemical probes, some are of a peptidic nature. Multiple comprehensive and excellent reviews on this subject have been published (Thakur et al., 2009; Riether, 2012, Han et al., 2013; Han et al., 2014; Morales et al., 2016; Aghazadeh Tabrizi et al., 2016; Manera et al., 2016; Cooper et al., 2017; Guba et al., 2020; Brennecke et al., 2021). Focus within this section has been placed on representative molecules that describe the development of a “CB2R ligand space” with a strong emphasis on those that made it into clinical development, all of them
being activators of CB2R. CB2R agonists that are launched or under active development and registered with ClinicalTrials.Gov are listed in Table 5.

Table 5. Placeholder

1.1 Endogenous Cannabinoids and Related Fatty Acid Derivatives. Polyunsaturated C20 fatty acids such as AA are the basic building blocks of eCBs and related fatty acid derivatives, which include amides such as AEA, esters like 2-AG, and ethers like noladin ether (Hanus et al., 2001) (Figs 1 and 2). 2-AG was first isolated from canine gut and rat brain (Mechoulam et al., 1995; Sugiura et al., 1995) and is considered as the most relevant signaling component of the ECS. Like AEA, it can be generated by several pathways and enzymes (Fezza et al., 2014; Baggelaar et al., 2018; Tsuboi et al., 2018). These key eCBs are synthesized and released on demand following CB1/2R activation (De Petrocellis et al., 2004; Lambert and Fowler, 2005; Di Marzo, 2018; Cristino et al., 2020). Besides CB1/2R (Fig. 15), they interact also with further molecular targets, e.g. the vanilloid TRPV1 ligand-gated ion channel (De Petrocellis et al., 2000).

In the meantime, further eCBs and a multitude of eCB-like mediators have been isolated. Generally, the eCBs are relatively short acting ligands, especially due to their hydrolysis through FAAH and MAGL. Therefore, synthetic efforts were undertaken to improve the hydrolytic stability of eCBs, e.g. by modifying the amide residue of AEA, which provided ligands such as ACPA (Fig. 16) (Hillard et al., 1999).

1.2. Plant-derived Cannabinoids. THC and its thermodynamically more stable and similarly potent regioisomer Δ9-THC served as a starting point for generating further classical cannabinoids (Razdan, 1986; Mechoulam et al., 1998). Dual CB1R/CB2R agonist Lenabasum, also known as Anabasum, Resunab, ajulemic acid, JBT-101, or CT-3 (Tepper et al., 2014), demonstrated efficacy in reducing chronic neuropathic pain in a phase 2 clinical trial (Karst et al., 2003) (Fig. 17). Currently the ligand is being evaluated in phase 3 for the treatment of dermatomyositis and scleroderma (Adis Insight Web Page Lenabasum, 2022). Although Lenabasum activates CB1R in addition to CB2R, it is not psychoactive (Zurier et al., 1998). Presumably, this is the consequence of its low brain penetrance.

Preferential CB2R activation can be achieved by omitting the phenolic C-1 hydroxyl of THC (Reggio et al., 1990; Gareau et al., 1996; Huffman et al., 1996), a strategy that was successfully applied for the generation of JWH133. The ligand is one of the first CB2R-selective agonists (10^8(pKi CB2R-pKi CB1R) >153), and thus it has been exploited to interrogate CB2R pharmacology (Pertwee, 1999; Soethoudt et al., 2017).
reference compound CP55940 is a potent dual CB1R/CB2R agonist outperforming THC with regard to CB1/2R binding affinity and analgesic activity (Showalter et al., 1996). Its tritiated congener has been broadly applied for the discovery and profiling of many CB1/2R ligands (Devane et al., 1988).

In contrast, non-psychotropic (-)CBD exhibits moderate affinity for CB2R (Showalter et al., 1996). CBD has been suggested to function as an inverse agonist of CB2R (Thomas et al., 2007), but interacts with multiple other targets as well (Ibeas Bih et al., 2015). Second-generation CBD derivative EHP-101 (VCI-004.8) is a dual CB2R and PPARγ agonist and activator of protein phosphatase 2A, which is currently investigated in phase 2a clinical trials (Del Río et al., 2016; Emerald Health Pharmaceuticals, 2022). Indications in focus are systemic and multiple sclerosis, for which preclinical proof of concept e.g. in fibrosis models (García-Martín et al., 2018) and in neuroinflammation (Navarrete et al., 2018) has been demonstrated.

Cannabinoid fumaric acid ester PRS-211375 (Cannabinor) is a selective CB2R agonist (CB2R EC\(_{50}\) cAMP = 17.4 nM - 98% efficacy) (Gratzke, 2010). It showed efficacy in various rodent \textit{in vivo} disease models including pain readouts in a chronic constriction injury model (Clarivate CortellisTM data base PRS-211375, 2022). Analgesic effects were translated into the clinic. In patients undergoing third molar dental extraction, nociceptive pain was reduced at 12 mg (i.v.) in a phase 2a study (Clarivate CortellisTM data base PRS-211375). Interestingly, no effect was observed at higher doses. This bell-shaped curve behavior is characteristic of the pharmacodynamics studies with other cannabinoid-derived CB1/2R ligands (Martellotta et al., 1998; Linares et al., 2019). Converting the phenolic C-1 hydroxyl group of CBD-dimethylheptyl into a methoxy moiety can enhance selectivity for CB2R as exemplified for HU-308. This potent, selective, and bioavailable CB2R agonist (Soethoudt et al., 2017) has demonstrated anti-inflammatory and tissue protective effects in multiple rodent disease models such as formalin-induced inflammation (Hanus et al., 1999) and hepatic ischemia/reperfusion injury studies (Rajesh et al., 2007). Attenuated leukostasis, chemotaxis and oxidative stress associated with reperfusion damage suppressed the acute inflammatory response (Pacher and Haskó, 2008). Structurally close analog HU-910 exhibited high binding and functional selectivity for CB2R over CB1R (Soethoudt et al., 2017). In addition, it is highly selective against a representative set of further off-targets and displays favorable pharmacokinetic properties. Therefore, HU-910 was recommended as a preferred CB2R agonist for studying the role of the receptor in biological and disease processes (Soethoudt et al., 2017). HU-910 \textit{in vivo} efficacy studies opened the door for exploring the potential of CB2R activation for the treatment of type 2 diabetic nephropathy (Zoja et al., 2016) and eye diseases such as uveitis (Porter et al., 2019). Importantly, HU-910 exhibits a different signaling preference in the five CB2R
signal transduction pathways in human and mouse. In contrast to being an unbiased agonist for the human CB2R, HU-910 exhibited a preference towards G-protein activation as compared to cAMP signaling and β-arrestin recruitment in mice (Soethoudt et al., 2017). Such interspecies differences in signaling preference might influence the translation of preclinical models to the clinic.

The vast majority of synthetic cannabinoids exhibit high lipophilicity, low aqueous solubility and tight plasma protein binding which translates into poor pharmacokinetic properties, such as high in vivo clearance and low oral bioavailability (McGilveray, 2005; Huestis, 2007). To overcome these issues, molecules were developed to exhibit favorable physicochemical properties and improved oral bioavailability. In the following paragraphs, key representatives from the most important scaffolds were selected to illustrate the progress made on synthetic CB2R ligands.

Aminoalkylindoles were among the earliest discovered CB2R scaffolds. In particular, dual CB1R and CB2R agonist WIN55212-2 (Eissenstat et al., 1990; Bell et al., 1991) was very important for identifying and deciphering the role of cannabinoid receptors (Fig. 9). It displays antihyperalgesic activity in multiple rodent pain models (D'Ambra et al., 1992; Fox et al., 2001; Johanek and Simone, 2004).

Initial aminoalkylindoles were structurally simplified. Furthermore, CB2R selectivity was improved to lead to CB2R agonists such as A-796260 (Fig. 9) (Frost et al., 2008), which achieved efficacy in various rodent pain models upon i.p. administration (Yao et al., 2008). Importantly, these antihyperalgesic effects could be blocked by pre-treatment with a CB2R antagonist.

Bicyclic (het)aryl scaffolds were investigated for CB2R selectivity or minimal CB1R efficacy. Several high throughput screening campaigns were conducted on the search for potent, selective and orally bioavailable CB2R agonists, e.g. providing benzimidazole (Pagé et al., 2008) and triazolopyrimidine (Nettekoven et al., 2016) derived starting points. Subsequent lead optimization efforts provided development candidates such as dual CB1R/CB2R agonist ART-27.13 (AZD-1940) (Pagé et al., 2010) (Fig. 18). This molecule is currently assessed as oral treatment of cachexia in phase 2 and cancer-related anorexia in phase 1 trials (Clarivate CortellisTM data base ART-27.13, 2022; Artelo Biosciences, 2022). However, due to CNS-related side effects, phase 2 studies for the treatment of nociceptive and neuropathic pain were terminated (Kalliomäki et al., 2013; AstraZeneca, 2022).

Phase 2 clinical trials for the oral treatment of osteoarthritic knee pain were conducted with CB2R agonist LY-2828360 (Fig. 17), but they were terminated despite an acceptable side effect profile (Hollinshead et al., 2013; ClinicalTrials LY-2828360, 2022; Clarivate CortellisTM data base LY-2828360, 2022).
imidazopyrimidine is brain penetrant and exhibits an excellent selectivity over CB₁R (ratio CB₁R/CB₂R EC₅₀ for GTPγS binding was >5'000). Recently reported triazolopyrimidine-derived CB₂R agonist (at 1 nM) RG7774 (Fig. 17) is under active development in phase 2 as an innovative oral treatment for diabetic retinopathy with limited potency for CB₁R (ratio CB₁R/CB₂R EC₅₀ for cAMP >6'940) (Grether, 2022). Further bicyclic (het)aryl derived ligands reached advanced preclinical stages and were successfully explored in various disease models with an inflammatory pathology. PF-03550096 (Kikuchi et al., 2008) and RQ-00202730 (Iwata et al., 2015) were tested in 2,4,6-trinitrobenzene sulfonic acid-induced colonic pain rat models, and RO6871304 was tested in rodent models of kidney ischemia–reperfusion, renal fibrosis and endotoxin-induced uveitis (Nettekoven et al., 2016; Porter et al., 2019).

Multiple organizations developed bicyclic aliphatic (het)aryl arrays with at least one aliphatic ring. Five-five, five-six, and five-seven systems were elaborated and four of these CB₂R agonists made it into clinical trials. Tedalinab was investigated for the oral treatment of neuropathic pain and osteoarthritis (Clarivate CortellisTM data base tedalinab, 2022) (Fig. 19). The CB₂R-selective pyrazole carboxamide exhibits similar binding affinities for human and rat CB₂R (human CB₂R Ki = rat CB₂R Ki ≈ 12 nM) and bioavailabilities >50% across species. Despite favorable safety and tolerability data in single ascending dose (doses up to 1200 mg) and multiple ascending dose studies (doses up to 300 mg once daily for 14 days), development was halted for unknown reasons.

Fig. 19. Placeholder

Lead optimization toward olorinab was guided by a β-arrestin efficacy assay (Han et al., 2017). This highly potent CB₂R full agonist is a peripherally acting molecule that is devoid of psychotropic effects (Adis Insight Web Page olorinab, 2022; Captivate, 2022). Olorinab (Fig. 19) was clinically assessed as an oral treatment for pain related to irritable bowel syndrome in phase 2. While the drug was well-tolerated, it did not meet the primary efficacy endpoint of statistically significant improvement in the overall average abdominal pain score (Citeline Informa Pharma Intelligence olorinab, 2022). The ligand exhibits a short human half-life and was therefore administered three times a day (Clarivate CortellisTM data base olorinab, 2022). Dual CB₁R/CB₂R agonist TAK-937 (Fig. 19) was developed as an injectable for the treatment of stroke after observing cerebroprotective effects in rat and non-human primate in vivo efficacy studies (Suzuki et al., 2012; Clarivate CortellisTM data base TAK-937, 2022). Yet, due to a narrow safety margin, development was halted. CB₂R-selective agonist dihydro-benzofuran NTRX-07 (Fig. 19) is being explored in phase 1 as an oral drug for the treatment of memory loss in Alzheimer's disease, cognitive disorder and neuropathic pain (NeuroTherapia, 2022; Clarivate CortellisTM data base NTRX-07, 2022). Follow-up studies targeting
MS and amyotrophic lateral sclerosis are foreseen. NTRX-07 preserves CB₂R potency across species and showed efficacy in multiple rodent efficacy studies (Naguib et al., 2008). The (S)-enantiomer is the active stereoisomer (Diaz et al., 2009).

CB₂R modulators containing aromatic and aliphatic five, six and seven-membered central cores have been described by multiple organizations. Pyrimidine-based agonist GW-842166X displays high CB₂R selectivity over CB₁R, and favorable pharmacokinetic properties, translating into potent analgesic effects (ED₅₀ = 0.1 mg/kg) in the Complete Freund's adjuvant rat model of inflammatory pain without initiating tetrad-like effects such as catalepsy or hypothermia (Giblin et al., 2007) (Fig. 20). The ligand reached phase 2 clinical trials for pain associated with osteoarthritis of the knee (ClinicalTrials GW-842166X osteoarthritis, 2022) and dental pain (ClinicalTrials GW-842166X dental pain, 2022).

Fig. 20. Placeholder

Disubstituted phenyl derivative KN 387271 (Fig. 20) is a dual CB₁R/CB₂R agonist. Neuroprotective effects in rat models of cerebral ischemia and traumatic brain injury (Mauler et al., 2002; Mauler et al., 2003) enabled phase 1 stroke and phase 2 traumatic brain injury studies in humans (Clarivate Cortellis™ data base KN 387271, 2022). The selective orally bioavailable CB₂R agonist S-777469 exhibited efficacy in rodent models of scratching and skin inflammation (Odan et al., 2012; Haruna et al., 2015; Haruna et al., 2017). However, these effects did not translate into therapeutic benefits in phase 2a trials with patients suffering from atopic dermatitis and pruritus (Clarivate Cortellis™ data base S-777469, 2022; Shionogi, 2022). Many additional CB₂R modulators with different central cores such as pyrazoles (Ohta et al., 2007), thiazoles (Yao et al., 2009), diazepanes (Zindell et al., 2011), piperidines (Bartolozzi et al., 2015), pyrrolidones (Riether et al., 2015), imidazolide-2,4-diones (Mukhopadhyay et al., 2016), pyridines (Porter et al., 2019) and 4-oxo-1,4-dihydropyridines (El Bakali et al., 2015) have been evaluated in detail. In some cases, minor structural changes triggering a switch from agonism to inverse agonism have been reported (Sellitto et al., 2010; Porter et al., 2019). Insights on how to design CB₂R agonists with favorable kinetic profiles were disclosed in a structure kinetics relationship study on a biaryl imidazolide-2,4-dione-based scaffold (Soethoudt et al., 2018). An adamantyl-derived series was investigated for functional activity on the Q63R variant of CB₂R (Nettekoven et al., 2013), which is associated with the risk of schizophrenia (Ishiguro et al., 2010), and an increased risk of celiac disease and liver damage in obese children (Rossi et al., 2011).

2. CB₂R Antagonists and Allosteric Ligands. Selective CB₂R antagonist/inverse agonist SR144528 (human CB₂R selectivity ratio = 129; mouse CB₂R selectivity ratio 10^4(pKi CB₂R-pKi CB₁R) = 6'026) (Rinaldi-Carmona et al., 1998; Portier et al., 1999; Soethoudt et al., 2017) is an important pharmacological tool for
antagonizing effects triggered by CB₂R agonists \textit{in vitro} and \textit{in vivo} (Nackley et al., 2003). Interestingly, the ligand shows a bias in suppressing different signal transduction pathways. It effectively blocks the modulation of cAMP signaling but is less potent with regard to antagonizing CB₂R-mediated signal transduction pathways (Soethoudt et al., 2017).

Few ligands targeting postulated CB₂R allosteric sites (Feng et al., 2014; Pandey et al., 2020) are known. An allosteric CB₂R interaction has been suggested for CBD (Martinez-Pinilla et al., 2017). Conversely, it was also experimentally shown that CBD acts as an orthosteric partial agonist (Tham et al., 2019), although it does not follow a simple one-site competition model. An overlap of allosteric and orthosteric binding pockets might provide a suitable explanation for these findings. In contrast, 1,1'-dimethyl heptyl CBD (CBD-DMH) was shown to act as a pathway-specific CB₂R allosteric modulator (Fig. 21). While positively modulating the cAMP response, it negatively modulated β-arrestin₁ recruitment by CP55940 and SR144528. Interaction with a high affinity allosteric binding site has been postulated by 5XRA- and 5TGZ-based \textit{in silico} docking studies.

Endogenously occurring RVD-hemopressin peptide pepcan-12 (Fig. 21) exhibits positive allosteric modulation of CB₂R (Petrucci et al., 2017). It was shown to increase binding of orthosteric ligands and to potentiate 2-AG- and CP55940-induced CB₂R signaling. Synthetic ligand C2 shows positive allosteric modulation of CB₂R \textit{in vitro} (Gado et al., 2019). Importantly, these effects translated into dose-dependent efficacy in a mouse model of neuropathic pain upon oral administration. Neither an X-ray crystal nor a cryo-electron microscopy structure of a CB₂R allosteric modulator in complex with the receptor has been reported. Therefore, the design of novel ligands is mostly aided by \textit{in silico} predictions including molecular dynamics simulations that can lead to the identification and ranking of multiple putative allosteric binding sites (Yuan et al., 2022). Furthermore, molecular dynamics simulations suggest that cholesterol exerts an allosteric effect on the intracellular CB₂R regions that interact with the G-protein complex thus altering the recruitment of G-protein (Yeliseev et al., 2021). Therefore, cholesterol levels might influence the screening for novel allo- and orthosteric CB₂R ligands, which should be taken into account in designing selective drugs directed towards CB₂R.

3. CB₂R Chemical Probes for Research and Diagnostics. A labelled chemical probe is a small molecule that is a ligand for a respective target and carries a reporter unit, e.g. a radio-, fluorescent- or biotin-label that allows characterization of ligand-target interactions. Optionally a linker connects target recognition element and reporter unit (Prevet and Collins, 2019). Labelled probes are of utmost importance for all research and
Due to a major debate regarding the specificity of CB2 receptor (CB2R) antibodies (Marchalant et al., 2014; Cécyre et al., 2014; Zhang et al., 2019), labelled chemical CB2R probes are highly important tools for determining CB2R protein expression. While radioligands are generally used for studying binding affinity (Cascio et al., 2016) or drug-target binding kinetics (Martella et al., 2017) of unlabelled ligands, positron emission tomography tracers focus on determining receptor expression in tissues and non-invasively measuring the distribution and receptor occupancy of drug candidates in patients (Homer et al., 2014). Non-selective [3H]CPC55940 and [3H] WIN55212-2 are the most relevant probes for measuring equilibrium binding affinities of novel CB2R ligands applying radioligand competition-binding assays. Selective CB2R inverse agonist [35S] SCH225336 (Lavey et al., 2005; Gonsiorek et al., 2006) was successfully applied for quantifying CB2R expression in various cell lines and hemopoietic cells making use of the superior specific activity of its 35S reporter unit, as compared to tritiated cannabinoids (>1400 versus ~20 Ci/mmol) (Fig. 22).

Fig. 22. Placeholder

Tritiated pyridine [3H]RO6957022 exhibits high binding selectivity targeting CB2R (Martella et al., 2017). The CB2R inverse agonist was utilized for studying drug-target binding kinetics. Its 11C-labelled analog [11C] RSR-056 carrying the carbon-11 reporter unit at the methoxy group is a CB2R-specific brain-penetrant positron emission tomography tracer that displayed a higher brain radioactivity in mice with lipopolysaccharide-induced neuroinflammation than in the control group (Slavik et al., 2014). 2-Oxoquinoline-derived [11C]NE40 is the first tracer that has been used for CB2R in vivo positron emission tomography in humans (Ahmad et al., 2013). In agreement with the known expression of CB2R, major uptake was observed in lymphoid tissue. Despite a rapid brain uptake and washout, no CB2R upregulation was detected in the brains of Alzheimer's disease patients (Ahmad et al., 2016). [18F]RoSMA-18-d6 exhibits subnanomolar affinity for CB2R across species and a remarkable selectivity factor of >12'000 over CB1R (Haider et al., 2020). It showed specific and reversible target binding in vitro and in vivo and was successfully utilized for detecting CB2R upregulation on post mortem human amyotrophic lateral sclerosis spinal cord tissues.

Fluorescently labelled CB2R ligands are highly versatile tools for studying receptor-ligand interactions and cellular trafficking, e.g. applying techniques such as flow cytometry, confocal fluorescence microscopy and time-resolved fluorescence resonance energy transfer. N-Alkyl isatin acylhydrazone NMP6 was among the first fluorescently labelled ligands that showed selectivity for CB2R over CB1R (Petrov et al., 2011). In flow cytometry and confocal microscopy studies specific binding to endogenously-expressed CB2R in CD4+ T cells and B-lymphocytes was demonstrated. Cy5-labelled (Cy5-) probe is a CB2R inverse agonist with an
extended linker moiety showing low levels of non-specific fluorescence in live-cell experiments (Singh et al., 2019). Combination of favorable structural elements of the two cannabinoid ligands HU-308 and AM841 provided a privileged chimera motif that was functionalized with a range of fluorophores while retaining excellent affinity and selectivity for CB2R (Sarott et al., 2020; Westphal et al., 2020). Coumarin fluorophore-labelled DY480-XL probe allowed for setting up a novel assay based on fluorescence resonance energy transfer, able to characterize equilibrium and kinetic binding constants and to visualize in real-time CB2R in endogenously expressing murine splenocytes and human macrophages. The reverse-design approach, in which small molecules previously optimized in medicinal chemistry programs form the basis for the generation of high-quality probes (Guberman et al., 2022), was applied for the generation of cell-permeable agonist-based SiR probe that was used for real-time in vivo tracing of CB2R in zebrafish larvae (Gazzi et al., 2022). Near-infrared fluorophores are best suited for in vivo imaging in higher species due to their deeper light penetration of biological tissues (Hong et al., 2017). Pyrazolopyrimidine derivative NIR760-XLP6 displays high selectivity over CB1R and improved specific binding as compared to predecessors such as NIR760-mbc94, and therefore holds promise for visualizing CB2R in in vivo imaging studies (Ling et al., 2015). Alternatively to fluorescent probes, biotinylated CB2R ligands have been applied for visualization of the receptor after conjugation with streptavidin-AlexaFluor488 (Martin-Couce et al., 2012).

While reversible non-covalent interaction with CB2R can easily be disrupted under experimental conditions resulting in the washout of the probe from the binding site, a covalent attachment can surmount these issues (Weichert and Gmeiner, 2015; Yang et al., 2019). The water-stable isothiocyanate group, which reacts preferentially with the nucleophilic amino acid side chains of cysteines was exploited to covalently attach cannabinoids to CB2R (Szymanski et al., 2011; Mallppeddi et al., 2017). Furthermore, CB2R-selective photoaffinity probes carrying benzophenone (Dixon et al., 2012) or azide (Szymanski et al., 2018) groups as photoreactive moiety have been reported. Two-step photoaffinity-based protein profiling probe LEI121 elegantly combines the covalently modifying photoaffinity technique with a click chemistry approach allowing for target engagement studies in live human cells by covalent SDS-PAGE visualization, flow cytometry and mass spectrometry-based proteomics (Soethoudt et al., 2018).

C. Summary of Clinical Status of CB1R and CB2R Agonists

In summary, three phytocannabinoid preparations (dronabinol, nabiximols and CBD) are currently available for treatment of diseases via stimulation of CB1R, CB2R, both or neither (Table 6). Although need for selective full agonist stimulation of CB1R is limited due to side effects, selective CB2R agonists are in phase 2 clinical trials. We are at the stage of defining which human diseases can best be treated with these
Mixed CB<sub>1</sub>R/CB<sub>2</sub>R-directed agonist preparations and numerous selective CB<sub>2</sub>R ligands are either on the market or under clinical development (reported in Table 6). Overall, more than 20 new molecular entities that activate CB<sub>2</sub>R have been investigated in humans for a wide range of indications. Structurally, they cover a huge chemical space including fatty acid derivatives, classical and non-classical cannabinoids as well as multiple diverse synthetic ligands, thus resulting also in the coverage of a broad range of physicochemical properties.

**Table 6. Placeholder**

Dronabinol, nabilone and CBD, exerting their action through both CB<sub>1</sub>R and CB<sub>2</sub>R activation, have been introduced to the market. Oral THC is used for the treatment of anorexia, cachexia and chemotherapy-induced emesis (Clarivate Cortellis data base dronabinol, 2022). Buccal THC has been launched for cancer pain (Adis Insight Web Page dronabinol buccal, 2022). Other routes of administration, e.g. inhalable and sublingual formulations, are under exploration. Nabilone was launched for treating patients that suffer from chemotherapy-induced nausea and vomiting (Adis Insight Web Page nabilone, 2022). Clinical trials for the treatment of Parkinson’s disease and pain are in advanced stages. CBD, a non-classical cannabinoid for which the main mode of action is still a matter of debate, is marketed for the treatment of infantile severe myoclonic epilepsy, Dravet and Lennox-Gastaut syndrome, and tuberous sclerosis (Clarivate Cortellis data base cannabidiol, 2022). As reported in Table 2, combinations of CBD and THC have been approved for treating MS-associated spasticity and pain management while glioblastoma trials and studies targeting further indications are ongoing (Clarivate Cortellis data base nabiximols, 2022; Pharmaprojects data base tetrahydrocannabinol plus cannabidiol, 2022). Non-psychoactive dual CB<sub>1</sub>R/CB<sub>2</sub>R agonist Lenabasum (Zurier et al., 1998) is in phase 3 trials for the treatment of systemic sclerosis and dermatomyositis (Adis Insight Web Page Lenabasum, 2022; Corbus Pharmaceuticals, 2022). Most advanced selective CB<sub>2</sub>R agonists are the synthetic cannabinoids olorinab (Adis Insight Web Page olorinab, 2022) and RG7774 (Grether, 2022). Clinical focus of olorinab is on pain related to irritable bowel syndrome, as such or with predominant constipation or diarrhea. RG7774 aims for providing an oral treatment for patients suffering from diabetic retinopathy. AA analog CMX-020 is studied in phase 2 trials for the treatment of pain, osteoarthritis and diabetic neuropathy using both oral and intravenous formulations (ANZCTR Web Page, 2022). Pain, in particular neuropathic pain is also the focus of the selective synthetic CB<sub>2</sub>R agonists CNTX-6016, whose structure has not been yet disclosed (Centrexion Corporation, 2022; ClinicalTrials.gov CNTX-6016, 2022), and NTRX-07 (NeuroTherapia, 2022; Clarivate Cortellis data base NTRX-07, 2022). CNTX-6016 is in phase 2, NTRX-07 in phase 1 trials. Dual CB<sub>1</sub>R/CB<sub>2</sub>R agonist ART-27.13 is in phase 2 trying to
provide treatment options for cachexia and cancer-related anorexia (Artelo Biosciences, 2022). CBD derivative EHP-101, which activates both PPARγ and CB₂R, is aimed at MS and scleroderma patient populations in phase 2 clinical trials (Emerald Health Pharmaceuticals, 2022). Ten additional new chemical entities were investigated in phase 1 and 2 clinical trials for different pain indications (neuropathic, dental, pain associated with osteoarthritis of the knee), postherpetic neuralgia, pruritis, atopic dermatitis, stroke, traumatic brain injury, coronary artery bypass graft and ocular hypertension (Brennecke et al., 2021). Dual CB₁R/CB₂R agonist TAK-937 was terminated due to a narrow safety margin (Clarivate CortellisTM data base TAK-937, 2022), S-777469 due to the lack of a pharmacological effect (Clarivate CortellisTM data base S-777469, 2022; Shionogi, 2022), while for KN 387271 (Clarivate CortellisTM data base KN 387271, 2022) and PRS-211375 13 (Clarivate CortellisTM data base PRS-211375, 2022) business reasons were reported.

It is clear from this summary that there are many therapeutic opportunities for both CB₁R and CB₂R agonists (Pacher and Kunos, 2013), yet the untoward effects of the CB₁R at the CNS have limited the clinical progression of CB₁R agonists that penetrate the blood-brain barrier and preclude their use in the non-hospitalized population. Tissue and cell-type selectivity for therapeutic responses is a challenge, as many cannabinoid and aminoalkylinodle agonists have been relegated to research rather than clinical use. Development of dual-target compounds that act by inhibiting CB₁R-mediated side effects while simultaneously activating CB₂R-mediated beneficial responses is also ongoing. Current development of peripherally-restricted agonists and antagonists that fail to cross the blood-brain barrier will open avenues for treatment of diseases in organs outside of the brain. Research on “biased agonists” that promote cannabinoid receptor conformations that favor G-protein versus β-arrestin signaling is an approach that offers treatment opportunities if one pathway dominates in treatment while the alternative pathway is responsible for side effects. Researchers are screening for allosteric modulators based upon the notion that their effects would be limited to only those receptors simultaneously engaged with an eCB agonist in the disease process. Thus, a positive allosteric modulator could potentiate responses if eCBs are under-stimulating the receptors. In contrast, a negative allosteric modulator would impart non-competitive antagonism in a situation of excessive eCB tone. Research findings not discussed in the present review have recognized the presence of CB₁R and CB₂R receptor heterodimers with a wide range of GPCRs, as well as receptor complexes with other associated proteins. As these studies gain maturity, the understanding of the impact of such receptor combinations within the same cell can open avenues for novel therapeutic compounds. Although the present use of pCB and small molecule agonists is meeting unmet needs of many diseases, particularly those involving inflammation, the future for cannabinoid receptor
pharmacotherapeutics must advance to agonists, antagonists and modulators that exhibit greater selectivity in order to improve treatments and eliminate unwanted side effects.

III. Therapeutic Potential of Metabolic Enzymes of AEA

A. Enzymes of AEA Production

AEA is produced upon demand from the membrane phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine, via two enzyme-mediated reactions (Fig. 3).

1. NAT and iNAT. The first step is the formation of NArPE, which occurs through N-acylation of phosphatidylethanolamine, mediated by Ca\(^{2+}\)-dependent or independent N-acyl transferase (NAT and iNAT). It should be noted that the acyl donor is another phospholipid molecule, such as phosphatidylcholine, rather than acyl-CoA. The presence of N-arachidonoyl-phosphatidylethanolamine in mammalian tissues and the N-acyl-transferase activity responsible for its production were first reported in the late 1990s (Cadas, et al., 1997) and later molecularly identified as cytosolic phospholipase A\(_2\)ε (cPLA\(_2\)ε) (Ogura et al., 2016). Members of the phospholipase A and acyltransferase (PLAAT) family (Jin et al., 2007; Uyama et al., 2012) were identified as Ca\(^{2+}\)-dependent and independent NAT, respectively. cPLA\(_{2}\)ε belongs to the cPLA\(_2\) family with a serine residue as catalytic nucleophile. Since for N-acylation of phosphatidylethanolamine cPLA\(_{2}\)ε selectively abstracts an acyl chain from the sn-1 position of the glycerol backbone of glycerophospholipid, which is abundant in saturated and mono-unsaturated fatty acids rather than poly-unsaturated fatty acids like AA, N-arachidonoyl-phosphatidylethanolamine and AEA account for a small percentage of the N-acyl-phosphatidylethanolamine (NAPE) and fatty acid ethanolamides present in cells. The analysis of cPLA\(_{2}\)ε-deficient mice revealed the central role of this enzyme in the accumulation of NAPEs and N-acylethanolamines in an imiquimod-induced psoriasis model (Liang et al., 2022), as well as in an ex vivo model of brain ischemia (Rahman et al., 2022). The NAPE-forming activity of cPLA\(_{2}\)ε in skin was suggested to be protective against skin inflammation such as psoriasis by producing anti-inflammatory N-acylethanolamines. On the other hand, PLAAT enzymes compose a small protein family with a cysteine residue as catalytic nucleophile (Uyama et al., 2017). Among the five members (1-5) in humans, PLAAT1, 2, and 5 exhibit relatively high NAT activity over the co-existing PLA\(_1\)/A\(_2\) activity (Uyama et al., 2012). Since without any cellular stimulus the NAT activity is easily detected in the cells where recombinant PLAAT is expressed, the role of PLAATs is presumed to maintain the basal levels of NAPEs and N-acylethanolamines in unstimulated cells. However, their contribution to the formation of NAPEs in vivo remains unclarified.
2. NAPE-PLD. NAPE-phospholipase D (PLD) catalyzes the second step of AEA formation (Fig. 3). The enzyme releases AEA and other fatty acid ethanolamides from their corresponding NAPEs in a PLD-type hydrolytic reaction (Okamoto et al., 2004). However, NAPE-PLD is a member of the metallo-β-lactamase superfamily and shows no sequence similarity to classical PLDs converting phosphatidylcholine to phosphatidic acid. Multiple aspartic acid and histidine residues, highly conserved among the family members, are essential for catalytic activity, and metal analysis suggested the presence of Zn$^{2+}$ coordinated by these amino acid residues (Wang et al., 2006). The crystal structure of human NAPE-PLD clarified the formation of homodimers adapted to associate with phospholipids and the presence of a binuclear Zn$^{2+}$ center at the active site (Magotti et al., 2015). Purified recombinant NAPE-PLD selectively hydrolyzes NAPE among various phospholipids (Wang et al., 2006). However, the enzyme does not distinguish N-acyl species in NAPE, explaining why the composition of naturally occurring fatty acid ethanolamides is similar to the N-acyl composition of NAPEs. Recently, the role of NAPE-PLD in energy metabolism received much attention. A common NAPE-PLD haplotype was reported to be protective against obesity (Wangensteen et al., 2011). Conditional knockout of adipocyte, intestinal or hepatic NAPE-PLD showed the tendency to induce obesity (Geurts et al., 2015; Everard et al., 2019; Lefort et al., 2020). Moreover LEI-401, the first brain active NAPE-PLD inhibitor, was instrumental to demonstrate the distinctive role of NAPE-PLD in AEA biosynthesis in the brain (Mock et al., 2020). LEI-401 activated the hypothalamus-pituitary-adrenal axis and impaired fear extinction, thereby emulating the effect of a CB1R antagonist and suggesting the presence of an endogenous AEA tone controlling emotional behavior (Mock et al., 2020).

3. Alternative Pathways. The analysis of NAPE-PLD-deficient mice revealed the existence of alternative pathways for fatty acid ethanolamide biosynthesis in brain (Leung et al., 2006; Tsuboi et al., 2011) and peripheral tissues (Inoue et al., 2017). Among the proposed multi-step pathways (Fig. 3), the route via lyso-NAPE and glycerophospho-N-acylethanolamines appears to be the most important, whereby either α/β-hydrolase domain protein 4 (Simon and Cravatt, 2006) or cPLA$_2$γ (Guo et al., 2021) generates glycerophospho-N-acylethanolamines from NAPE via lyso-NAPE in two consecutive esterase reactions. The resultant compound is further hydrolyzed to generate N-acylethanolamines by glycerophosphodiesterase 1 (Simon and Cravatt, 2008) and 4 (Tsuboi et al., 2015; Rahman et al., 2016). The glycerophosphodiesterase family is composed of seven proteins (1-7) in mammals (Yanaka, 2007), and isoforms 4 and 7 also show lyso-PLD activity directly producing N-acylethanolamines from lyso-NAPE (Tsuboi et al., 2015; Rahman et al., 2016). It is not fully elucidated how much these alternative pathways contribute to the generation of AEA.
and other N-acylethanolamines in the tissues of wild-type mice. The physiological significance in human
tissues also remains unclarified.

B. Enzymes of AEA Degradation

The major pathway of AEA degradation is hydrolysis to AA and ethanolamine, which is mediated by
FAAH (Desarnaud et al., 1995; Hillard et al., 1995; Cravatt et al., 1996), two isoforms of which have been
described FAAH-1 and FAAH-2 (Wei et al., 2006). It should be noted that FAAH-2, sharing 20% sequence
identity with FAAH-1, is expressed in humans but not in rodents (Wei et al., 2006), making its complete
understanding difficult. Different from FAAH-1, which is found in the endoplasmic reticulum and the nucleus,
FAAH-2 may be localized to lipid droplets (Kaczocha et al., 2010). NAAA and acid ceramidase also
hydrolyze AEA, albeit with low activity (Ghidini et al., 2021; Tsuboi et al., 2021). In addition to hydrolytic
degradation, AEA can be oxygenated by lipooxygenases (5-, 12-, 15-LOX), COX-2 or CYP450 (Fig. 4), all of
which have been fully characterized as eicosanoid-generating oxygenase enzymes (Rouzer and Marnett,
2011; Fezza et al., 2014; Simard et al., 2022). The physiological significance of these AEA oxygenation
pathways remains unclear.

1. FAAH. FAAH-1, which is often referred to simply as FAAH, is widely distributed in mammalian tissues
with high expression in liver, brain, and small intestine of rats (Katayama et al., 1997). The analysis of FAAH-
1-deficient mice revealed increased endogenous AEA levels and hence the central role of FAAH-1 in AEA
degradation (Cravatt et al., 2001). FAAH deletion reduced pain sensation, and when AEA was administered
FAAH-1-deficient mice exhibited intense hypomotility, antinociception, catalepsy, and hypothermia in a
CB1R-dependent manner. Although FAAH-1 is highly active with AEA, the enzyme shows broad substrate
specificity, hydrolyzing other fatty acid ethanolamides, N-acyl taurines, and primary fatty acid amides such as
oleamide. FAAH-1 can also catalyze the reverse reaction in which AEA is formed from AA and ethanolamine.
However, the equilibrium constant demonstrated the predominance of the hydrolytic action of AEA
(Katayama et al., 1999). FAAH-1 is an integral membrane protein functioning as a serine hydrolase and
belongs to the amidase signature family characterized by the Ser-Ser-Lys catalytic triad (McKinney and
Cravatt, 2005). Rat FAAH was crystallized as a homodimer. In common with bacterial enzymes of the same
family, the structure exhibits a core fold comprised of a twisted β-sheet consisting of 11 mixed strands
surrounded by a number of α-helices (Bracey et al., 2002). Remarkably, the FAAH dimer is stabilized by the
lipid bilayer and shows a higher enzymatic activity within membranes containing cholesterol (Dainese et al.,
2014) according to an allosteric kinetics (Dainese et al., 2020). Additionally, co-localization of cholesterol,
AEA and FAAH in mouse neuroblastoma cells suggests a mechanism by which cholesterol increases the
substrate accessibility of FAAH (Dainese et al., 2014); yet, the pathophysiological implications of these findings remain to be understood. C385A polymorphism of the FAAH-1 gene (rs324420) results in the formation of P129T mutant, which is associated with the reduction of FAAH activity and cellular expression as well as increased risk for substance use disorders (Sipe et al., 2002). This polymorphism also affects susceptibility to various diseases (Hosseinzadeh Anvar and Ahmadalipour, 2022).

2. NAAA. NAAA is a lysosomal hydrolase (Tsuboi et al., 2007a; Ueda et al., 2010) that shows 33% amino acid identity with acid ceramidase, which hydrolyzes ceramide to sphingosine and fatty acid. Similar to other members of the N-terminal nucleophile hydrolase family (Linhorst et al., 2022), NAAA is synthesized as a catalytically inactive precursor and then matured to heterodimer, consisting of α and β subunits, by post-translational autoproteolytic cleavage (Zhao et al., 2007). This reaction proceeds in vitro only at acidic pH, suggesting that the maturation occurs only after its migration to endosomes/lysosomes from the endoplasmic reticulum via the Golgi apparatus. The resultant N-terminal cysteine residue of the β subunit (Cys-126 in human NAAA, Cys-131 in rodents) functions as the catalytic nucleophile. Importantly, this cysteine residue is also indispensable for the autoproteolytic cleavage. The crystal structures of NAAA elucidated that autoproteolysis exposes the buried active site to enable catalysis (Gorelik et al., 2018). NAAA hydrolyzes various fatty acid ethanolamides in vitro but its highest reactivity is for N-palmitoylethanolamine (Ghidini et al., 2021). The fact that NAAA is highly expressed in macrophages (Tsuboi et al., 2007b) and other immune cells (Ribeiro et al., 2015), suggests that this enzyme may regulate fatty acid ethanolamide levels at the site of inflammation. In fact, in dermatitis induced by treatment of mice with 2,4-dinitrofluorobenzene, NAAA-deficient mice showed elevated N-palmitoylethanolamine, but not N-oleoylethanolamine, levels in ear tissue relative to wild-type controls, and exhibited a strong reduction in the inflammatory reaction (Sasso et al., 2018). Furthermore, NAAA deficiency in mice increased N-palmitoylethanolamine and AEA levels in bone marrow and macrophages and AEA levels in lungs (Xie et al., 2022).

C. FAAH Inhibitors

The first potent, selective, and systemically active FAAH inhibitor was the N-biphenylcarbamate derivative URB597, shown in Fig. 23 (Kathuria et al., 2003; Tarzia et al., 2003). This agent acts by forming a carbamoyl adduct with FAAH’s catalytic serine (Mileni et al., 2010) and exhibits robust anxiolytic-like and antidepressant-like properties, which depend on indirect CB1R activation by accumulated anandamide (Kathuria et al., 2003; Gobbi et al., 2005; Bortolato et al., 2007).

Fig. 23. Placeholder
Importantly, unlike direct-acting CB1R agonists such as THC, URB597 is not rewarding to non-human primates, suggesting lack of abuse potential (Justinova et al., 2008). An exploration of its scaffold unexpectedly led to the identification of the first peripherally restricted FAAH inhibitor, URB937 (Fig. 23), which strongly attenuates pain-related responses in animal models (Clapper et al., 2010). The promising pharmacological profile of URB597 prompted efforts by both academe and industry to create more advanced inhibitors. Reviews of this considerable body of work are available (Tuo et al., 2017; Fazio et al., 2020; Piomelli and Mabou Tagne, 2022), but one especially significant chemical class, the piperidine/piperazine-ureas, should be mentioned here. High-throughput screening of a chemical library led scientists at Johnson & Johnson to discover JNJ-1661010 (Fig. 23), which inhibits human FAAH with nanomolar potency (IC$_{50}$ = 33 nM) and through a covalent mechanism (Keith et al., 2008). Further optimization identified the compound JNJ-42165279, a slowly reversible FAAH inhibitor that was selected for clinical testing. Concomitant work at Pfizer produced several nanomolar piperidine/piperazine-urea covalent FAAH inhibitors (Ahn et al., 2007) and eventually led to PF-04457845 (Fig. 23), which was also moved to clinical development. There are several possible therapeutic indications for which FAAH inhibitors have been or are currently being tested, including anxiety disorders, substance use disorders, and pain.

Building on the observation that URB597 exerts profound anxiolytic-like and antidepressant-like effects in mice and rats, animal and human experiments have shown that AEA signaling at CB1R modulates the emotional response to stress via regulation of prefrontal cortical-amygdala circuits (Patel et al., 2017). For example, subjects carrying the loss-of-function faah gene polymorphism C385A (rs324420) display enhanced fronto-amygdalar connectivity and cued fear extinction (Dincheva et al., 2015). This conclusion was later confirmed by several other human experimental medicine studies. For instance, Paulus and coworkers found that JNJ-42165279 (100 mg) dampens amygdala activity during an emotion face-processing task, an effect that is associated positively with plasma AEA concentrations (Paulus et al., 2021). A lower dose of the drug (25 mg) was tested in a multicenter, placebo-controlled phase 2 trial in patients with social anxiety disorder. The study reported statistically detectable signs of efficacy, but the dosage was considered insufficient to fully inhibit FAAH (Schmidt et al., 2021). Additional clinical testing in anxiety and allied conditions is clearly warranted.

The impact of FAAH inhibitors on tobacco and cannabis use disorders exemplifies well the promise offered by these agents but also their complex actions. URB597 was shown to reduce nicotine reward and to prevent reinstatement of nicotine use in animal models (Justinova et al., 2015), an effect that was associated with reduced burst firing of dopamine neurons in the midbrain and dopamine release in the terminal field of...
such neurons (Melis et al., 2004). Unexpectedly, the effects of URB597 on nicotine reward were prevented by PPARα rather than CB1R blockade, leading to suggest that they were mediated by PPARα agonists, such as N-oleoylethanolamine and N-palmitoylethanolamine, rather than by AEA acting at CB1R. With regard to cannabis, a phase 2 clinical trial demonstrated that PF-04457845 is effective in reducing cannabis use and alleviating cannabis withdrawal symptoms in men (D’Souza et al., 2019).

There is strong preclinical evidence indicating that eCBs are critical regulators of pain sensation (for review, see Finn et al., 2021). The analgesic phenotype of individuals carrying loss-of-function FAAH mutations (C385A, faah-out) supports this conclusion (Habib et al., 2019), but the results of clinical trials have been disappointing (Huggins et al., 2012; Wagenlehner et al., 2017). Possible explanations for this discrepancy include species-specific differences, selection of inadequate clinical pain conditions, inconsistencies between preclinical and clinical study design, and lack of predictive validity of current animal models. Other pathologies where FAAH inhibitors might be clinically useful include chronic cough (Wortley et al., 2017) and urinary tract dysfunction (Wagenlehner et al., 2017). Overall, several FAAH inhibitors have been patented for their potential therapeutic use, as summarized in Table 7 (Fazio et al., 2020).

**Table 7. Placeholder**

Several compounds (URB597, PF-04457845, SSR411298, APD8477, V158866, BIA 10-2474 and JNJ-42165279) have been also tested in clinical trials (Table 8). Of note, the FAAH inhibitor BIA 10-2474 led to adverse neurological side effects and the death of one healthy volunteer in a phase 1 clinical trial (Kerbrat et al., 2016). Since the other FAAH inhibitors tested in clinical trials did not elicit any adverse neurological effects and BIA 10-2474 was shown to have multiple off-targets (Van Esbroeck et al., 2017), inhibition of FAAH is considered to be safe.

**Table 8. Placeholder**

**D. NAAA Inhibitors**

The search for potent, selective, and systemically active NAAA inhibitors started in 2009 with the identification of the β-lactone derivative N-[(3S)-2-oxo-3-oxetanyl]-3-phenylpropanamide ((S)-OOPP) shown in Fig. 24, which inhibits rat NAAA with submicromolar potency (IC$_{50}$ = 420 nM on rat NAAA) via a noncompetitive and partially reversible mechanism (Solorzano et al., 2009).

**Fig. 24. Placeholder**

Due to opening of its β-lactone ring, (S)-OOPP undergoes rapid hydrolytic deactivation, which makes it unsuitable for systemic administration. The compound has, however, two interesting properties (Solorzano, 2009). First, it is selective for NAAA over other functionally (FAAH) or structurally (acid ceramidase) related
lipid amidases. Second, its inhibitory effect is stereospecific, allowing researchers to leverage the enantiomer (R)-OOPP (IC$_{50}$ = 6 μM) as a negative control in pharmacological experiments. These experiments showed that incubation with S-OOPP increases N-palmitoylethanolamine levels in RAW264.7 macrophages stimulated with bacterial endotoxin, whereas (R)-OOPP does not (Solorzano et al., 2009). Moreover, subdermal application of (S)-OOPP, but not (R)-OOPP, blocked carrageenan-induced neutrophil infiltration and plasma extravasation in mice, two effects that are prevented by genetic PPARα ablation and are mimicked by administration of PPARα agonists. These findings identified NAAA as a druggable target for the treatment of inflammation and encouraged efforts to discover inhibitors with greater potency and stability.

The first notable outcome of this search was another β-lactone derivative, ARN077 (also known as URB913), in which the amide group of (S)-OOPP is replaced by a carbamate moiety and a syn-methyl group is introduced at the β position of the lactone ring (Fig. 24).

Compared to (S)-OOPP, ARN077 exhibits better chemical stability and greater NAAA inhibitory potency (IC$_{50}$ = 50 nM on rat NAAA) (Ponzano et al., 2013). ARN077 was found to be selective for NAAA when assessed in a broad panel of potential off-targets. Importantly, topical application of ARN077 on the mouse or rat skin attenuated inflammation and pain-related responses (Sasso et al., 2013, 2018). Despite these significant steps forward, the low chemical and enzymatic stability of the β-lactone ring remained a challenge to the systemic use of ARN077 and other chemically related inhibitors. Efforts were thus undertaken to overcome this problem, which led to the discovery of several new classes of NAAA inhibitors, including β-lactam derivatives (e.g., ARN726) (Ribeiro et al., 2015), isothiocyanate derivatives (e.g., AM9023) (Alhouayek et al., 2015), azetidine-nitrile derivatives (Malamas et al., 2020), and benzothiazole derivatives (e.g., ARN19702) (Migliore et al., 2016), shown in Fig. 24. The discovery, inhibitory properties, and mechanism of action of these agents were recently reviewed (Piomelli et al., 2020). Thus far, three main therapeutic indications have emerged for NAAA inhibitors: inflammation, pain, and neuroinflammation/neurodegeneration.

A chemically diverse set of NAAA inhibitors exhibit notable anti-inflammatory properties in animal models. For example, topical application of the β-lactone ARN077 was shown to suppress skin inflammation elicited by exposure to ultraviolet B-radiation in rats or phorbol ester in mice (Sasso et al., 2013). The compound also attenuated itch and skin inflammation in sensitized mice challenged with 2,4-dinitrofluorobenzene (Sasso et al., 2018). Confirming that ARN077 acts by protecting N-palmitoylethanolamine from NAAA-mediated hydrolysis, the effects of ARN077 were accompanied by restoration of normal N-palmitoylethanolamine content in inflamed skin tissue and were dependent on PPARα activation (Sasso et
The striking effects produced by ARN077 on critical mediators of the allergic response (e.g., interleukin 4 and immunoglobulin E) (Sasso et al., 2018) and the efficacy demonstrated by N-palmitoylethanolamine as an adjuvant treatment for eczema (Eberlein et al., 2008) encourage further evaluation of NAAA as a target for the treatment of the atopic diathesis, a disease cluster that includes atopic dermatitis, bronchial asthma, hay fever and allergic rhinitis. Other inflammatory diseases in which NAAA inhibitor might find clinical use, as suggested by animal model studies, include osteoarthritis (Bonezzi et al., 2016; Zhou et al., 2019), and colitis (Alhouayek et al., 2015; Xiu et al., 2020).

In addition to inflammation, NAAA inhibitors may also be effective in the treatment of pain and neuroinflammation/neurodegeneration. For example, the systemically active NAAA inhibitor ARN19702 exhibited a broad antinociceptive profile in mouse models of acute and chronic pain (Fotio et al., 2021) and alleviated symptoms of neuroinflammation in mouse models of multiple sclerosis (Migliore et al., 2016) and Parkinson’s disease (Palese et al., 2022). Similarly, the topically active β-lactone derivative ARN077 alleviated hypersensitivity in mouse and rat models of neuropathic pain (Sasso et al., 2013), while the oxazolidinone imide derivative F96 (Fig. 24) attenuated acetic acid-induced writhing and tactile allodynia evoked by sciatic nerve injury in mice (Yang et al., 2015). No NAAA-targeting compound has yet reached clinical trials.

IV. Therapeutic Potential of Metabolic Enzymes of 2-AG

A. Metabolism of 2-AG

The endocannabinoid 2-AG can be produced via two distinct biological pathways. The metabolic pathway uses sn-2 arachidonoyl-containing triglycerides, which are hydrolyzed by hormone-sensitive lipase, carboxyl esterases or other lipases towards sn-2 arachidonoyl DAGs (Baggelaar et al., 2018). The signaling pathway utilizes phosphatidylinositol-4,5-bisphosphate, which is converted by phospholipase C (PLC) β in the CNS or PLCγ2 in immune cells. The PLC enzymes are activated by Ca²⁺ ions and integrate Gq protein-coupled receptor activation and extracellular Ca²⁺ influx via ionotropic receptors and voltage-gated Ca²⁺-channels, thereby also producing DAGs. The diglycerides activate protein kinase C and are the central precursors for the production of 2-AG in both the metabolic and signaling pathways. The sn-1 acyl group from DAGs is predominantly hydrolyzed by two isoenzymes, diacylglycerol lipase-α and -β (DAGLα and DAGLβ, also termed diacylglyceride lipases), which produce 2-AG and other sn-2 acylglycerides. The DAGLs were discovered by Doherty’s group in 2003 (Bisogno et al., 2003), and the generation of genetically modified animals lacking daglα and daglβ, the genes encoding the DAGL proteins, demonstrated that these enzymes...
are essential for 2-AG production in the brain (Gao et al., 2010; Tanimura et al., 2010). Of note, the DAGLs also terminate protein kinase C signaling by hydrolyzing DAGs, thus these enzymes are an important hub to connect lipid and kinase signaling.

Termination of 2-AG signaling at CB₁R or CB₂R occurs through hydrolysis of the ester bond, thereby generating AA and glycerol. Monoacylglycerol lipase (MAGL, also termed monoglyceride lipase) is the main enzyme responsible for the inactivation of 2-AG in the brain (Dinh et al., 2001), whereas α/β-hydrolase domain protein 6 and 12 may play a role in 2-AG hydrolysis in specific cell types (Marrs et al., 2006; Blankman et al., 2007). In various tissues, including the brain, 2-AG is responsible for the main supply of AA, which is the central precursor for pro-inflammatory signaling lipids, such as the prostaglandins (Nomura et al., 2010). Thus, MAGL is a central node that connects endocannabinoid and eicosanoid signaling. Modulators of 2-AG metabolism are listed in Table 9, and in the sections below their therapeutic potential will be described. For an extensive review on chemical probes of the endocannabinoid system see also (Punt et al., 2022).

Table 9. Placeholder

B. Therapeutic Potential of Diacylglycerol Lipase-α (DAGLα)

DAGLα belongs to the family of serine hydrolases, and is responsible for the production of 2-AG in the CNS (Bisogno et al., 2003), where it is primarily found in the dendrites and soma of neurons and to a lower extent in astrocytes, but not in microglial cells. DAGLα is expressed in various brain regions, such as cortex, hippocampus, cerebellum and striatum, and its activity is highest in the cerebellum (Baggelaar et al., 2017). DAGLα is a 120 kDa integral plasma membrane protein with multiple domains (Fig. 25), and has four transmembrane helices followed by a lipase domain, which contains the catalytic triad Ser, His, Asp (Bisogno et al., 2003).

Figure 25. Placeholder

DAGLα produces 2-AG on demand as a retrograde messenger upon depolarization of the post-synaptic neuron or by stimulation of G_{q/11}-coupled metabotropic receptors, with or without activation of ionotropic receptors at both excitatory and inhibitory synapses (Gao et al., 2010; Tanimura et al., 2010). Animals with constitutive genetic disruption of DAGLα show a variety of neurological phenotypes, including impaired synaptic transmission, disturbed memory and learning, compromised adult neurogenesis (Gao et al., 2010), hypophagia (Powell et al., 2015), enhanced anxiety and fear responses (Jenniches et al., 2015; Shonesy et al., 2014) and susceptibility to spontaneous seizures (Powell et al., 2015). Multiple, selective pharmacological tools have been developed to modulate DAGLα (as well as DAGLβ) activity in an acute and
temporary manner (Baggelaar et al., 2018; Punt et al., 2023). LEI-105, DO34 and DH376 are currently widely used DAGL inhibitors to study the involvement of these enzymes in physiological processes (Baggelaar et al., 2015; Ogasawara et al., 2016). For example, the same inhibitors were instrumental, in conjunction with genetic models, to unequivocally demonstrate that 2-AG production is "on demand", i.e., when and where needed upon stimuli during short term synaptic plasticity, such as depolarization-induced suppression of inhibition (DSI) or excitation (DSE) in hippocampal and cerebellar slices (Baggelaar et al., 2015; Ogasawara et al., 2016). DAGL inhibitors also contributed to our understanding of the role of 2-AG in cocaine seeking (McReynolds et al., 2018), alcohol addiction (Gianessi et al., 2020), food intake (Deng et al., 2017), neuroinflammation (Ogasawara et al., 2016), anxiety and stress (Bluett et al., 2017), learning and memory (Schurman et al., 2019), pain sensation (Wilkerson et al., 2017) and voluntary movement (Farrell et al., 2021). It should be noted that DO34 and DH376, but not LEI-105, also inhibited other serine hydrolases suABHD6. Thus, it is advisable to include DO53 as a negative control in the experimental design when using DO34 or DH376 (Deng et al., 2017).

DAGLα is very well conserved throughout evolution. Human DAGLα has 97% homology with its mouse ortholog, whereas it has only 79% homology to DAGLβ. DAGLα has a long unstructured C-terminal tail, which contains many phosphorylation sites that regulate its activity and subcellular localization through protein-protein interactions. It has been shown that CaMKII phosphorylates Ser782 and Ser808, thereby reducing the enzyme activity (Shonesy et al., 2013). On the other hand, protein kinase A, which is activated by cAMP, has been shown to phosphorylate multiple sites in the C-terminus of DAGLα, including Ser798, thereby activating the enzyme (Shonesy et al., 2020). It has been suggested that the opposing actions of protein kinase A and CaMKII on DAGLα activity may be important in setting the level of tonic 2-AG signaling. Of note, cAMP-induced phosphorylation of Ser738 of DAGLα has been shown to enhance the interaction of DAGLα with ankyrin-G, a scaffolding protein in dendritic spines (Yoon et al., 2021). This led to increased spine size and decreased DAGLα surface diffusion. Repeated strong excitatory dendritic spine stimulation resulted in a feedback signal that promoted the growth of an inhibitory γ-aminobutyric acid bouton onto the same dendrite in a DAGL-dependent manner (Hu et al., 2020). The C-terminus also contains the consensus motif PPxxF, needed to bind the coiled-coil domain of Homer proteins, which are adapter proteins that localize DAGLα close to the post-synaptic density in the vicinity of metabotropic glutamate receptor 5 (Jung et al., 2005). Interestingly, the surface localization of DAGLα was shown to be a dynamic process controlled by protein kinase C. DAGLα co-localized with β-tubulin and cycled between the plasma membrane and endosomal compartments via EEA1- and Rab5-positive early endosomes in a clathrin-independent pathway.
(Zhou et al., 2016). This process could be disrupted by protein kinase C inhibitors, but not by protein kinase A inhibitors. In a mouse model of Fragile X syndrome, which is the most commonly known genetic cause of autism, an aberrant subcellular localization of DAGLα was found to cause a disruption in glutamatergic signaling, thereby impairing long-term depression (Jung et al., 2012). Recently, the first clinical evidence was presented that a daglα variant, which led to a disrupted cellular localization of the protein, was connected to a human genetic disorder. Nine children from eight families with heterozygous de novo truncating variants in the last exon of DAGLα exhibited developmental delay, ataxia and complex oculomotor abnormalities (Bainbridge et al., 2022). Altogether, these observations demonstrate that the post-translational regulation of DAGLα activity and its subcellular localization enable a tight spatiotemporal control on 2-AG-dependent synaptic transmission. Disturbances in the subcellular localization of DAGLα and its activity result in abnormal neurotransmission and neurological disorders. Unfortunately, pharmacological inhibition of DAGLα in the CNS is unlikely to be of therapeutic value due to on-target toxicity.

C. Therapeutic Potential of Diacylglycerol Lipase-β (DAGLβ)

DAGLβ is the main enzyme responsible for the production of 2-AG in immune cells, including microglia which are the brain resident macrophages. DAGLβ is a 70 kDa multidomain, integral membrane serine hydrolase that lacks the unstructured C-terminal tail observed in DAGLα. This suggests that the activity and subcellular localization of DAGLβ is differently regulated. DAGLβ has a similar substrate preference as DAGLα, but it is also capable of hydrolyzing polyunsaturated fatty acid-specific triacylglycerides (Shin et al., 2020). DAGLβ knockout mice show 50% reduction in 2-AG levels in the brain, whereas in the liver a > 90% reduction was observed (Gao et al., 2010). DAGLβ is not involved in the regulation of DSI or DSE in hippocampal or cerebellar slices (Gao et al., 2010), and in the developing brain it is detected in the axonal growth cone of neurons (Bisogno et al., 2003). DAGLβ is transported to the cone via the adaptor protein complex AP-4 (Davies et al., 2022). A patient deficient in AP-4 was shown to accumulate DAGLβ in the trans-Golgi network of cells and AP-4 knockout mice had reduced eCB levels in the brain (Davies et al., 2022). Recently, a specific subset of nigral dopaminergic neurons in the adult brain was found to express DAGLβ. This expression was implicated in the inhibition of γ-aminobutyric acid release from dorsal striatal spiny projection neurons, and supposed to be involved in locomotor skill learning across sessions (Liu et al., 2022). Multiple homozygous loss-of-function mutations in DAGLβ were linked to sporadic, early onset autosomal recessive Parkinsonism in Chinese families (Liu et al., 2022). PLCγ2, for which activating mutations are associated with autoinflammatory disorders and Alzheimer’s disease, has recently been shown to serve as the principal enzyme providing the DAG pool for DAGLβ-MAGL axis in human innate
immune cells and microglia (Jing et al., 2021). Mouse microglia lacking PLCγ2 displayed a suppressed endocannabinoid-eicosanoid cross-talk and an impaired in vivo inflammatory response to lipopolysaccharide that led to reduced CD68-expression, but not to release of proinflammatory cytokines. These findings extend the previous observations that genetic and pharmacological inhibition of DAGLβ exerts anti-inflammatory properties in mouse macrophages and microglia (Hsu et al., 2012; Viader et al., 2016). Overall, it was suggested that selective inhibitors of DAGLβ (and MAGL) may be therapeutically of interest for immune pathologies caused by activation of PLCγ2.

Currently, no selective DAGLβ inhibitors are available. KT-109 was originally reported as a selective DAGLβ inhibitor (Hsu et al., 2012), which displayed analgesic efficacy in an inflammatory and neuropathic pain model (Shin et al., 2018; Wilkerson et al., 2016), as well as in a sickle cell disease model (Khasabova et al., 2023). However, it should be noted that KT109 also inhibits DAGLα to the same extent as DAGLβ (Deng et al., 2017), thus care should be taken in the interpretation of the effects of this compound. As noted above, non-selective dual DAGL inhibitors, such as DO34 and DH376, have anti-neuroinflammatory properties (Ogasawara et al., 2016; Viader et al., 2016). They reduced production of proinflammatory cytokines and prostaglandins in microglia, and impaired lipopolysaccharide-induced hypothermia in mice. In summary, selective compounds are still required to test the therapeutic potential of DAGLβ inhibition in neuroinflammatory diseases and inflammatory pain.

D. Therapeutic Potential of Monoacylglycerol Lipase (MAGL)

MAGL is a membrane-associated serine hydrolase, which was cloned in 1997 (Karlson et al., 1997) and consists of two tissue-specific splice-variants with a molecular weight of 33 kDa and 36 kDa (Karlson et al., 2001). It has the typical catalytic triade Ser122, Asp239 and His269, and uses monoacylglycerols with different chain length and saturation, including 2-AG, as a substrate (Dinh et al., 2002). Oxidation of two non-catalytic cysteines (C201 and C208) reduces its enzymatic activity (Dotsey et al., 2015). MAGL is abundantly expressed in various tissues (e.g., brain, lung, liver, spleen, kidney, heart and intestines), and is active in different brain regions including hippocampus, cerebellum, cortex and striatum (Baggelaar et al., 2017). MAGL is found in neurons and astrocytes, and to a lesser extent in microglia (Viader et al., 2016), and notably is localized at the presynaptic site along with the CB1 receptor and opposed to DAGLα. This lipase terminates the retrograde eCB signaling mediated by 2-AG, and indeed mice lacking the mgll gene that encodes for MAGL show robust elevations of 2-AG in the brain, and less pronounced elevations in liver, spleen and thymus (Long et al., 2009). This observation suggests that other esterases may participate in the hydrolysis of 2-AG at the periphery. MAGL knockout mice also have significantly reduced AA levels in their
brain, which indicates that the DAGL-MAGL axis is responsible for the pool of free AA in the brain (Nomura et al., 2010). Furthermore, MAGL knockout animals have a desensitized CB1R, and show impaired eCB-dependent synaptic plasticity and physical dependence (Schlosburg et al., 2010).

Several in vivo active MAGL inhibitors, including JZL184 and MJN110, have been described in the literature (Long et al., 2009; Niphakis et al., 2012). Together with the MAGL knockout animals, these inhibitors have been instrumental in studies of the therapeutic potential of MAGL inhibition in a broad range of diseases, spanning from cancer (Nomura et al., 2011), Parkinson’s disease (Nomura et al., 2010), Alzheimer’s disease (Chen et al., 2012) and MS (Hernandez-Torres et al., 2012), to inflammatory and neuropathic pain (Hohmann et al., 2005; Kinsey et al., 2009), acute liver injury (Cao et al., 2013), and anxiety and depression (Bluett et al., 2017; Zhang et al., 2015). For recent reviews, see (Gil-Ordonez et al., 2018; Deng et al., 2020; Van Egmond et al., 2021). Of note, chronic, high dosing of MAGL inhibitors caused desensitization and down-regulation of CB1R, and behavioral tolerance to CB1R agonists. A therapeutic window for anti-nociceptive efficacy without CB1R desensitization was observed upon acute and chronic low dosing. In this respect, MAGL inhibition may have therapeutic potential for treating inflammatory and neuropathic pain, as well as neurodegenerative diseases accompanied by neuroinflammation like MS, Alzheimer’s and Parkinson’s diseases. Several pharmaceutical companies, including Johnson & Johnson, Lundbeck, Takeda Pharmaceuticals, Pfizer and Hoffman-LaRoche, have filed patents describing a diverse range of chemotypes of MAGL inhibitors (Bononi et al., 2021). Among these compounds the covalent, irreversible MAGL inhibitor Lu-AG06466, developed by Lundbeck (formerly ABX-1431 from Abide Therapeutics), is the most advanced experimental drug. It has been reported that Lu-AG06466 exerts adverse effects in the CNS and appeared ineffective in a phase 2 clinical trial for Tourette syndrome (Muller-Vahl et al., 2021; Muller-Vahl et al., 2022), yet this compound is currently being investigated in phase 2 trials for other indications, such as post-traumatic stress disorder and spasticity in multiple sclerosis. Clinical trials for Lu-AG06466 listed in ClinicalTrials.gov at the date of this review are shown in Table 10.

Table 10. Placeholder

Overall, it is hypothesized that reversible MAGL inhibitors may avoid some of the adverse effects observed with covalent, irreversible inhibitors (Van Egmond et al., 2021). However, another strategy to avoid CNS-mediated side effects could be to generate peripherally restricted MAGL inhibitors for the potential treatment of cancer, tissue ischemic-reperfusion injury and/or antinociception.

V. Therapeutic Potential of Transmembrane, Intracellular, and Extracellular Transporters
While translational efforts towards the development of ECS modulators have been primarily dedicated to eCB degradation inhibitors, in particular FAAH and MAGL blockers (Blankman and Cravatt, 2013; van Egmond et al., 2021; Fowler, 2021), translating research on inhibitors of eCB cellular uptake or cellular trafficking remains slow. The development of such transport inhibitors has been convoluted by the fact that extra- and intracellular eCB-binding proteins are promiscuous, as well as by the lack of a concrete target responsible for plasma membrane transport, whose identity remains elusive. Nevertheless RT126, a FABP inhibitor that competes with eCB binding for FABP4 and FABP5, and SYT510, a selective eCB reuptake inhibitor (SERI) which increases extracellular eCB levels in the brain by targeting the putative eCB membrane transporter, are under development by the pharmaceutical industry (https://ir.artelobio.com/news-events/press-releases/detail/90/artelo-biosciences-reports-positive-pre-clinical-results, https://www.synendos.com). Unlike active cellular transport mechanisms that are energy-driven, the lipophilic eCBs seem to traffic between membranes and across aqueous barriers through interactions with binding proteins and pass the plasma membrane by energy-independent mechanisms (Fig. 7). Among these, facilitated diffusion is influenced by both the interaction of eCBs with extra- and intracellular binding proteins and their metabolic enzymes. The measurement of facilitated diffusion and plasma membrane lipid transport is challenging and demands special phenotypic assays not easily accessible for routine screening (Oddi et al., 2010; Fowler, 2013; Rau et al., 2016; Reynoso-Moreno et al., 2023). A major challenge has been to differentiate FAAH and AEA uptake inhibitors as these processes are intrinsically coupled (Fowler et al., 2004; Vandevoorde and Fowler, 2005; Hillard et al., 2007). Therefore, only recently selective and potent eCB cellular uptake inhibitors have been developed (Chicca et al., 2017).

In 2009, the identification of intracellular carrier proteins, primarily FABPs, and lipid droplets as potential sequestration domains for AEA provided a new perspective in AEA transport research (Oddi et al., 2008, 2009; Kaczocha et al., 2009). FABPs facilitate the spatial organization of eCBs into domains and enable the trafficking between plasma and intracellular membranes. In this section, an update on previous reviews on the topic (Fowler, 2013; Nicolussi and Gertsch, 2015; Reynoso-Moreno and Gertsch, 2021) is provided, focussing on the molecular pharmacology and possible implications for therapeutic intervention of using the diverse eCB transport inhibitors shown in Fig. 26. Since such inhibitors show CB1R/CB2R-dependent indirect cannabimimetic effects like analgesia, anti-inflammatory and anxiolytic effects, they constitute a new class of pharmacological inhibitors that indirectly activate the ECS, showing a differential effect on the system compared to FAAH and MAGL inhibitors.

Figure 26. Placeholder
A. Endocannabinoid Trafficking and Transport

Although 2-AG is the major eCB in tissues such as the brain, and is generally more soluble in water than AEA (1400 ng/ml versus 250 ng/ml, respectively; see Tetko et al., 2005), most research on eCB transporters has been carried out on AEA. Intriguingly, almost 30 years after the identification of AEA in porcine brain (Devane et al., 1992b), the mechanisms of eCB membrane transport (i.e., release into the extracellular space and cellular reuptake) remain only partially understood. However, different hypothetical models have been proposed and reviewed for AEA uptake and trafficking (Felder et al., 2006; Yates and Barker, 2009; Nicolussi and Gertsch, 2015), as shown in Fig. 7. The currently best substantiated model of facilitated diffusion is discussed here in the context of the emerging specific pharmacological modulators.

In the 1990s, the first reports on the cellular uptake of AEA designated a temperature and time-dependent transport, which was linked to the enzymatic hydrolysis by FAAH in C6 glioma cells, N18TG2 neuroblastoma cells and primary neuronal cells (Deutsch and Chin, 1993; Di Marzo et al., 1994). This cellular uptake process of AEA was rapid (t_{1/2} = 2.5 min), saturable, and importantly, did not compete with closely related N-acylethanolamines such as N-stearoylethanolamine, N-linoleoylethanolamine or N-palmitoylethanolamine, shown in Table 3 (Di Marzo et al., 1994). Since all N-acylethanolamines compete for FAAH hydrolysis, being ideal substrates for this enzyme, the fact that they showed no competition for cellular AEA uptake clearly suggested a mechanism independent of AEA metabolism (Chicca et al., 2012).

The early investigations in the 1990s suggested a carrier-mediated uptake process for AEA that was not dependent on either ATP nor coupled to ion (Na^+, Cl^-, H^+) gradients (Beltramo et al., 1997; Hillard et al., 1997; Hillard and Jarrahian, 2000). The transport process of AEA displayed high-affinity Michaelis-Menten constants in astrocytes (K_m = 0.3 µM), cortical neurons (K_m = 1.2 µM) and human neuroblastoma CHP100 cells (K_m = 0.2 µM) (Beltramo et al., 1997; Maccarrone et al., 1999), with values comparable to those obtained with the transporters of serotonin (K_m = 0.3 - 0.5 µM), dopamine (K_m = 0.9 - 1.2 µM) and noradrenaline (K_m = 0.4 µM) (Masson et al., 1999; Piomelli, 2003). Among more than 25 cell lines, and considering different assay protocols and confounding factors such as sticking of lipids to plastic and vials, the range of the apparent K_m values for AEA uptake diverges dramatically, from 0.1 µM to 45 µM (Felder et al., 2006; Oddi et al., 2010). Although different routes of AEA catabolism exist (Fig. 4), which in principle can influence AEA cellular uptake, their contribution seems insignificant compared to that of FAAH. In an experiment on [³H]AEA uptake competition in U937 cells, different eCBs congeners (AEA, 2-AGE, O-AEA and NADA, shown in Table 3) competed with [³H]AEA uptake, suggesting that a common cellular membrane uptake mechanism seemingly competes for one target related to cellular eCB uptake (Chicca et al., 2012).
Albumin and Hsp70 have been identified as cytosolic AEA-binding proteins in mouse skin keratinocytes using proteomics and functional assays (Oddi et al., 2009). Another candidate in the list of intracellular carrier proteins for AEA is the reported FAAH-like AEA transporter (FLAT) (Fu et al., 2012). The latter was proposed to be a partially cytosolic, catalytically silent variant of the AEA degrading enzyme FAAH. The role and existence of FLAT as an AEA transporter was subsequently questioned, as no expression in either mouse brain, spinal cord or dorsal root ganglia could be detected by independent groups (Leung et al., 2013; Fowler, 2014). Furthermore, a certain enzymatic activity could still be detected in artificial FLAT-transfected HeLa cells (Leung et al., 2013). On the other hand, an inhibitor of FLAT (ARN272) showed promising indirect cannabimimetic effects in a mouse model of nausea and vomiting (O’Brien et al., 2013).

In a docking study, the sterol carrier protein 2 (SCP2) was shown to be yet another potential eCB carrier protein (Liedhegner et al., 2014). Although an increase of AEA accumulation could be detected in SCP2 transfected HEK-293 cells, competition experiments with AM404 and 2-AG did not show a significant difference in their IC₅₀ values between SCP2-expressing and wildtype cells. It was concluded that SCP2 is a low affinity binding protein for AEA and that it might facilitate AEA cellular uptake to a minor degree. A fluorescent probe displacement assay was developed to screen for SCP2 inhibitors, which might help to elucidate the role of SCP2 in eCB transport. Using this assay, the binding affinities of AEA (Ki =0.68 ± 0.05 μM) and 2-AG (Ki=0.37 ± 0.02 μM) to SCP2 were calculated (Hillard et al., 2017). The binding affinities of a library of previously reported SCP2 inhibitors was tested along with a new series of analogs, where SCPI-1 was the most potent probe with a Ki = 1.0 ± 0.1 μM (Hillard et al., 2017). SCP-2/SCP-x gene ablation in FABP1 null (LKO) mice antagonized the impact of LKO and high fat diet on brain AA and, subsequently, on eCB levels, suggesting that both FABP1 and SCP-2 directly or indirectly participate in regulating the ECS (Martin et al., 2019). In principle, any protein with hydrophobic surfaces/cavities may serve as an acceptor for lipids like AEA and other eCBs. This is confirmed by the recent crystal structure of cellular retinol binding protein in complex with 2-AG (Lee et al., 2020). Currently, the pharmacological competition of eCBs at extracellular binding proteins like albumin, Hsp70, SCP2 or extracellular FABPs by synthetic ligands has not been studied in sufficient detail to allow conclusions regardin their druggability, i.e. it remains unclear whether competing for extracellular AEA protein binding would exert robust cannabimimetic effects, as well as diverse CB₁R/CB₂R-dependent pharmacological effects. Therefore, the current focus is on plasma membrane-associated and intracellular processes. Notably, the involvement of FABPs in the transport of eCBs was suggested already in the context of intracellular PPAR activation (Sun et al., 2008). Consequently,
FABP5 and FABP7 were shown to mediate AEA intracellular transport from the plasma membrane to FAAH in COS-7-FAAH-eGFP and N18TG2 neuroblastoma cells (Kaczocha et al., 2009).

As shown in Fig. 1, membrane-derived AEA and 2-AG can initiate cellular signaling at both extracellularly accessible (e.g., CB and other GPCRs) and intracellularly accessible (e.g., TRPVs, TRPs and PPARs) sites (Ross, 2003; Watanabe et al., 2003; Goodfellow and Glass, 2009; Sigel et al., 2011; Baur et al., 2013). Because any eCB agonist needs to be removed from the orthosteric binding site of its receptor targets, the evolution of a membrane protein that facilitates reuptake and CB1R/CB2R clearance would make sense. The interference with the movement of eCBs through competitive inhibition at binding sites or the putative eCB membrane transporter, therefore, has great potential to modulate pathophysiological processes through the ECS, with a range of possible therapeutic applications like FAAH and MAGL inhibitors.

B. Evolution of Pharmacological Inhibitors of AEA Transport

In the 1990s the first AEA uptake inhibitors were synthesized. Based on the observed substrate specificity, initially mainly structural analogs of AEA were synthesized and tested for [3H]AEA uptake inhibition in rat brain neurons and astrocytes (Khanolkar et al., 1996; Beltramo et al., 1997). The first inhibitor of AEA cellular uptake was the N-(4-hydroxyphenyl)-arachidonamide AM404, which exhibited an IC50 value ~1 µM in neurons and an IC50 value ~5 µM in astrocytes (see Table 11) (Beltramo et al., 1997).

This probe, which was later discovered to be a bioactive AA conjugated metabolite of paracetamol (also known as acetaminophen) (Högestätt et al., 2005), was initially reported to be selective towards uptake inhibition over FAAH inhibition (IC50 > 30 µM). AM404 was later suggested to be a competitive inhibitor of AEA uptake, and was found to be transported as a pseudo-substrate of the postulated AEA transporter (Beltramo and Piomelli, 1999). Yet, independent groups showed that AM404 inhibits FAAH with IC50 values close to those obtained for AEA uptake inhibition (Table 11), thus questioning the selectivity of this compound. In addition, AM404 may also interact with other targets of the ECS. The reversed amide analog AM1172 apparently solved the problem of selectivity by being an equally potent inhibitor of AEA uptake as AM404 but resistant to hydrolysis by FAAH (Fegley et al., 2004). Yet, it was subsequently reported that AM1172 also inhibits FAAH (Hillard et al., 2007).

Table 11. Placeholder

The quest for better AEA cellular uptake inhibitors continued with the aim to increase their potency and generate structure-activity relationship studies for the postulated transporter target (Jarrahian et al., 2000; Di Marzo et al., 2004). Since the inhibition of FAAH also leads to inhibition of [3H]AEA uptake, the aspect of selectivity over FAAH became a crucial differentiation criterion (Day et al., 2001; Deutsch et al., 2001).
Besides the well-studied AEA uptake inhibitor VDM11 (De Petrocellis et al., 2000), more selective inhibitors such as the oleic acid derivatives OMDM-1 and OMDM-2 (Ortar et al., 2003) or UCM707 (López-Rodríguez et al., 2001; 2003) were synthesized (Table 11). Despite their initially published selectivity over FAAH, some of these inhibitors were later found to inhibit FAAH with similar or even identical IC_{50} values obtained for AEA uptake inhibition (Fowler et al., 2004; Vandevoorde and Fowler, 2005; Hillard et al., 2007). Unfortunately, almost nothing is known about the pharmacokinetics and tissue distribution of these compounds and pharmacological effects are difficult to attribute to either FAAH inhibition or AEA transport inhibition. UCM707 was investigated in neuronal preparations of FAAH-/− mice and still showed an IC_{50} = 3 ± 1 µM for AEA accumulation (Ortega-Gutiérrez et al., 2004). This finding agreed with its selectivity for AEA uptake inhibition over FAAH (López-Rodríguez et al., 2003). A direct comparison of the data obtained with neuronal cells of FAAH+/+ mice demonstrated that AEA cellular uptake is a facilitated process in which a specific “UCM707-binding protein” was proposed to participate with a relative contribution of at least 30% (Ortega-Gutiérrez et al., 2004). FABP5 as an intracellular eCB carrier protein (Kaczocha et al., 2012; Sanson et al., 2014) was therefore a possible candidate. However, the affinity of UCM707 to FAPB5 was measured (Table 11) and resulted in a Ki = 25.8 µM (19.5 – 44.7 µM) (Nicolussi, 2014). This low affinity interaction of UCM707 with FABP5 clearly does not match the determined IC_{50} value for AEA cellular uptake. Moreover, given that UCM707 still works in FAAH-lacking cells and synergizes with FAAH inhibitors for AEA uptake inhibition and inhibits AEA efflux (Chicca et al., 2012), the possibility that UCM707 targets a membrane transport mechanism is still valid. Unsurprisingly, the highly potent FAAH inhibitors LY2183240 and URB597 (Table 11) resulted in pronounced AEA cellular uptake inhibition in different cell types (Mor et al., 2004; Moore et al., 2005; Dickason-Chesterfield et al., 2006), and were essentially representative of all FAAH inhibitors. The unexpected and paradoxical inhibition of passive diffusion by small organic molecules, as the primary evidence of the carrier-mediated model, was readily refuted because inhibitors like AM404 did not inhibit AEA cellular uptake at short incubation times (< 40 s) and inhibited FAAH (Glaser et al., 2003). Ligresti and colleagues convincingly showed saturable AEA uptake within 90 s not only in RBL-2H3 and C6 glioma cell lines, but also in mouse brain synaptosomes from FAAH-/− mice (Ligresti et al., 2004). In the study by Glaser and colleagues (Glaser et al., 2003) where simple diffusion of AEA was measured, very high non-physiological AEA concentrations (1 - 100 µM) were used, which may easily mask the transport kinetics seen with concentrations of 50 - 500 nM (as a note, at ≥ 1 µM AEA simple diffusion kinetics can be measured). Yet, such high AEA concentrations are not found in tissues and much less is needed for receptor activation. Eli Lilly developed the highly potent tetrazole inhibitor called LY2183240 with an astonishing IC_{50} = 270 ± 29
pM for AEA cellular uptake in RBL-2H3 cells (Moore et al., 2005; Ortar et al., 2008) (Table 11). Using the modified radiolabeled probe $^{[125]}$I-LY2318912, a high-affinity membrane binding site involved in the transport of AEA could be identified, curiously also in FAAH-lacking HeLa cells (Kd = 7.06 ± 1.69 nM, Bmax = 32.2 ± 2.98 fmol/mg). In human FAAH-transfected HeLa cells neither the binding affinity (Kd) nor the Bmax value changed significantly, indicating that one binding site is independent of FAAH (Moore et al., 2005). Having raised hopes for the molecular identification of the postulated AEA transporter, shortly afterwards LY2183240 was shown to be an ultrapotent, irreversible and nonspecific inhibitor of FAAH, MAGL, and other serine hydrolases (Alexander and Cravatt, 2006).

Additional indirect evidence for the existence of a transporter-mediated (facilitated) AEA uptake mechanism was provided by the demonstration of AEA uptake in synaptosomes from human, mouse and rat brain (Battista et al., 2022), and in neuronal preparations of FAAH knockout mice (Fegley et al., 2004; Ligresti et al., 2004; Ortega-Gutiérrez et al., 2004). Known AEA uptake inhibitors like UCM707 still reduced the accumulation of AEA, but the uptake efficacy was much lower in cells lacking FAAH compared to those from wild-type mice (Fegley et al., 2004; Ligresti et al., 2004; Ortega-Gutiérrez et al., 2004). However, FAAH activity alone did not seem to be causative of all AEA uptake phenomena (Ligresti et al., 2004). In agreement with the view that FAAH is not the only player in AEA transport, cells lacking FAAH like HMC-1 cells (Maccarrone et al., 2000; Nicolussi et al., 2014a) show robust AEA uptake kinetics, although with a lower Vmax than in FAAH-expressing cells. Moreover, an energy-independent and saturable export of $^{[3H]}$AEA was demonstrated in human endothelial cells (Maccarrone et al., 2002). Obviously, hydrolysis by FAAH has no impact on AEA efflux. Additionally, it was demonstrated that the transport inhibitor VDM11 inhibited the release of de novo generated AEA in HEK-293 cells (Ligresti et al., 2004). Taken together, these studies pointed towards a bidirectional membrane transport mechanism for AEA shown by independent groups (Hillard et al., 1997; Maccarrone et al., 2002; Ligresti et al., 2004; Chicca et al., 2012). In this context, the release of AEA and 2-AG was assessed in an electrophysiological study measuring striatal long-term depression in acute brain slice preparation, where postsynaptic blockage of eCB membrane transport using VDM11 achieved a disruption of eCB release (Ronesi et al., 2004). In another study, OMDM-2 and AM404 increased activity-dependent AEA and 2-AG levels in the hypothalamus and inhibited the synaptically-driven spiking activity in postsynaptic neurons upon enhanced retrograde signaling (Di et al., 2005). In urethane-anesthetized rats, VDM11 inhibited the micturition reflex at least in part through CB1R (Honda et al., 2016), suggesting a possible therapeutic role of AEA transport inhibitors in disturbances of the storage function of the bladder or disturbances of the emptying function. The only pharmacological study that uses AEA release...
inhibition as an explanation for the effect was the comparison of OMDM-2 versus the FAAH inhibitor URB597 on social withdrawal in rodents (Seillier and Giuffrida, 2018). Systemic administration of OMDM-2 reduced social interaction, but in contrast to URB597-induced social deficit this effect was not reversed by the TRPV1 antagonist capsazepine. Conversely, the CB₁R antagonist AM251, which did not affect URB597-induced social withdrawal, exacerbated OMDM-2 effect (Seillier and Giuffrida, 2018). The infusion of OMDM-2 and VMD11 in both cases reduced the extracellular levels of dopamine collected from nucleus accumbens, and suggested a role for AEA transport in sleep modulation (Murillo-Rodriguez et al., 2013). Interestingly, AM404 but not VDM11 reduced the acute freezing response in male mice in a strong auditory-cued fear memory via CB₁R- and TRPV1-mediated mechanisms (Llorente-Berzal et al., 2015). Finally, in non-human primates, AM404 reinforced anandamide or cocaine self-administration behavior and induced reinstatement of drug-seeking behavior in abstinent monkeys by a CB₁R-dependent mechanism (Schindler et al., 2016).

C. Preclinical Development of Selective eCB Reuptake Inhibitors

The N-isobutylamido guineensine from Piper species (Table 11) was identified as a nanomolar and strongly selective inhibitor of AEA cellular uptake over FAAH inhibition and other ECS targets (Nicolussi et al., 2014a). Guineensine did not show a relevant inhibition of FAAH activity (IC₅₀ > 50 µM) or FABP5 binding (Ki > 100 µM), and dose-dependently induced cannabimimetic effects in BALB/c mice shown by strong catalepsy, hypothermia, reduced locomotion, and analgesia in the hot plate test. The catalepsy and analgesia were blocked by the CB₁R antagonist rimonabant (SR141716A) (Reynoso-Moreno et al., 2017). The pharmacological evidence of indirect cannabimimetic effects strongly suggests that guineensine also targets eCB cellular reuptake in vivo (Reynoso-Moreno et al., 2017). An efficient total synthesis of guineensine was published which may facilitate the provision of this rare natural product for research (Bartholomäus et al., 2019). Another compound in the list of plant-derived natural AEA uptake inhibitors is the N-benzyl-(9Z,12Z)-octadecadieneamide (macamide 7, shown in Table 11), which exhibited a nanomolar IC₅₀ value for AEA uptake inhibition but also inhibits FAAH at low micromolar concentrations (Hajdu et al., 2014). Furthermore, an analog of the natural product farinosone-C (BSL-34, Table 11) was found to be a more selective inhibitor of AEA uptake (IC₅₀ = 232 nM) over FAAH inhibition (IC₅₀ >10 µM), with close structural similarity to OMDM-2 (Burch et al., 2014).

Building on previous work on N-alkyl-2,4-dodecadienamides from Echinacea purpurea, which have been shown to interact with the ECS (Raduner et al., 2007; Chicca et al., 2009), a series of derivatives and analogs were synthesized. Diverse N-alkylcarbamates were also synthesized and tested in U937 cells for their ability to inhibit AEA hydrolysis and uptake, showing ultrapotent FAAH inhibition that led to hyperpotent
AEA uptake inhibition (Nicolussi et al., 2014b). Interestingly, some of these N-alkylcarbamates (e.g., WOBE492 and WOBE498) showed a FAAH-independent AEA uptake inhibition in HMC-1 cells with IC\textsubscript{50} values below 300 nM (Nicolussi et al., 2014b). This study led to the identification of (2E,4E)-N-[2-(3,4-dimethoxyphenyl)ethyl]-dodeca-2,4-dienamide (WOBE437, shown in Table 11) as a highly potent and selective eCB uptake inhibitor, which was extensively profiled (Chicca et al., 2017). For instance, WOBE437 inhibits AEA and 2-AG uptake in U937 cells with IC\textsubscript{50} values of 10 ± 8 nM and 283 ± 121 nM, respectively (Chicca et al., 2017). Furthermore, WOBE437 was tested in Neuro2a mouse neuroblastoma cells, primary rat cortical neurons and FAAH-deficient HMC-1 cells, showing differential but significant inhibition of AEA uptake in all the cell lines. WOBE437 did not inhibit FAAH, MAGL, α/β-hydrolase domain proteins 6 and 12 or COX-2, nor did it show a significant interaction with CB\textsubscript{1}R/CB\textsubscript{2}R, FABP5 or any of 45 relevant CNS-related receptors/transporters/ion channels/enzymes tested (Chicca et al., 2017). Moreover, in C57BL6/J male mice WOBE437 was found to be orally bioavailable (Reynoso-Moreno et al., 2018), and in a clinically relevant mouse model of MS like experimental autoimmune encephalomyelitis, it significantly reduced disease severity and accelerated recovery through CB\textsubscript{1}R/CB\textsubscript{2}R-dependent mechanisms (Reynoso-Moreno et al., 2021). A structure-activity relationship study on the WOBE437 scaffold for cellular AEA uptake inhibition was recently published (Mäder et al., 2021). However, using a clickable analog of the WOBE437-derived photoaffinity probe RX-055 (Table 11), saccharopine dehydrogenase-like oxidoreductase, vesicle amine transport 1, and ferrochelatase were identified as low affinity (10 µM) off-targets of WOBE437 in Neuro-2a cells (Gagestein et al., 2022), calling for attention on the therapeutic exploitation of this inhibitor at higher doses.

Currently, a new class of SERIs, the thiazolidinones (Table 11), are being developed for the treatment of psychiatric or neurological disorders and inflammation at Synendos Therapeutics, though their target protein has not yet been published.

D. Preclinical Development of FABP Inhibitors

In liver, it has been shown that FABP1 not only acts as an eCB and pCB binding protein but also regulates hepatic eCB levels (Huang et al., 2016). Studies using FABP1 knockout mice revealed a markedly diminished impact of high fat diet on brain eCB levels, especially in male mice, suggesting the involvement of FABP1 in the biosynthesis of these lipids (Martin et al., 2017). FABPs seem to be generally involved in modulating AEA trafficking as the overexpression of FABP5 and FABP7 in COS-7-FAAH-eGFP cells increased AEA uptake and hydrolysis by 32% and 35%, respectively (Kaczocha et al., 2009). N18TG2 cells showed an increase of 36% upon FABP5 and 42% upon FABP7 overexpression. In the same cells,
reduction of AEA uptake and hydrolysis could be monitored after pre-incubation with the FABP4/5 inhibitor BMS309403 (Table 12). While BMS309403 exhibited a Ki = 350 ± 3 nM for FABP5 binding, a concentration of 100 µM of this probe was needed to reach ~50% inhibition of cellular AEA uptake (Sulsky et al., 2007; Furuhashi and Hotamisligil, 2008; Kaczocha et al., 2009). FABP5 was suggested as the main target of the AEA uptake inhibitors OMDM-2, VDM11 and AM404, because these blockers showed binding affinities to FABP5 comparable to the published Ki values for AEA-FABP5 binding (Kaczocha et al., 2012; Nicolussi et al., 2014b).

Table 12. Placeholder

Surprisingly, AA also showed a strong affinity to FABP5 (Kaczocha et al., 2012). It is generally accepted that AA does not affect AEA cellular uptake up to a concentration of 100 µM (Beltramo et al., 1997; Hillard et al., 1997; Piomelli et al., 1999), a finding that would challenge the role of FABP5 in AEA transport. The development of potent and specific FABP5 inhibitors with the aim to modulate AEA cellular transport is ongoing (Berger et al., 2012; Zhou et al., 2019) and a first in vivo evaluation of SBFI-26, one of these compounds shown in Table 12, was reported (Kaczocha et al., 2014). SBFI-26 is an α-truxillic acid 1-naphthyl monoester, originally identified using a computational docking protocol, and synthesized as a mixture of both the (S) and (R) enantiomers (Berger et al., 2012). SBFI-26 produced antinociceptive and anti-inflammatory effects in mice and inhibited the activities of FABP5 and FABP7 with Ki values of 0.9 µM and 0.4 µM, respectively (Berger et al., 2012; Kaczocha et al., 2014). In FABP5, SBFI-26 was unexpectedly found to bind at the substrate entry portal region in addition to binding at the canonical ligand-binding pocket (Hsu et al., 2017). However, it is noted that the high concentrations needed in vitro for AEA cellular uptake inhibition experiments do not match the reported FABP5 affinity of SBFI-26. In rodents, SBFI-16 showed peripheral and supraspinal analgesic effects (Peng et al., 2017) and abrogated pulmonary artery remodelling in pulmonary hypertension secondary to left heart disease and improved cardiac function (Lei et al., 2022). Yet, the involvement of the ECS in these effects was not elucidated. As already pointed out above, the Ki values obtained for AEA binding to FABP5 are not in agreement with the Km values in many cells that show AEA transport. Recently, it was demonstrated that FABP5 both promotes the hydrolysis of AEA to AA and thus reduces brain eCB levels, and directly shuttles AA to the nucleus where it delivers it to PPARβ/δ, enabling its activation (Yu et al., 2014). Interestingly, in adult neurons neither FABP5 nor FABP7 seems to be expressed in significant amounts (Liedhegner et al., 2014).

The first evidence for intracellular carriers of 2-AG was provided by two independent groups. The known cytosolic FABP5, which is an AEA carrier and binds numerous highly abundant fatty acids, was shown also
to bind 2-AG. Using fluorescence polarization and a labelled fatty acid probe which was displaced from FABP5, a Ki = 8.7 µM was determined (Nicolussi, et al., 2014b). Simultaneously, a crystallographic study of FABP5 as an intracellular carrier protein of eCBs confirmed the binding data (Sanson et al., 2014). Of note, the Kd for 2-AG binding to FABP5 more closely matches the Km for 2-AG transport than in the case of AEA.

It was recently reported that FABP5 could act as a synaptic (i.e., extracellular) transporter of 2-AG and control the retrograde signaling by this eCB (Haj-Dahmane et al., 2018). Using dorsal raphe neurons incubated with SBFI-26 (a FABP5 and FABP7 inhibitor) or from FABP5-/- mice, it was shown that FABP5 inhibition or absence prevented DSE, which under normal conditions occurs after depolarization of postsynaptic neurons and phasic 2-AG release, followed by presynaptic CB1R activation, reduction of glutamate release and a reduction in excitatory postsynaptic currents. Furthermore, FABP5 inhibition or absence prevented the increase seen in excitatory postsynaptic currents after incubation with AM251 (a CB1R antagonist/inverse agonist), showing that by acting as a carrier FABP5 modulates the effect of phasic and tonic levels of 2-AG in the control of retrograde signaling. Additionally, in a co-culture of primary hippocampal astrocytes and neurons, it was shown that FABP5 is secreted by astrocytes to the extracellular media in a time-depended manner, supporting its role as an extracellular synaptic transporter of 2-AG. In FABP5-/- neurons, no changes were observed in the protein expression of CB1R, DAGLα (the neuronal isoform) or MAGL, concluding that there are no changes in neither CB1R activation nor 2-AG metabolism. Although non-significant changes in 2-AG levels were observed after incubation with SBFI-26, the opposite occurred in FABP5-/- neurons, which showed a significant increase. Furthermore, dorsal raphe neurons incubated with SBFI-26 showed an increase in AEA levels, which agrees to previous reports (Kaczocha et al., 2009; 2014); however, there were no changes in AEA levels measured in FABP5-/- neurons compared to wild-types. These contradictions raise a question regarding other possible changes in the metabolic pathways of 2-AG and AEA, respectively, that might not be observed at the protein level. As shown recently, deletion of FABP5 impaired tonic 2-AG and AEA signaling at striatal γ-aminobutyric acid synapses of medium spiny neurons, and blunted phasic 2-AG mediated short-term synaptic plasticity without altering CB1R expression or function (Fauzan et al., 2022). Based on the expression of FABP5 in TRPV1-positive nociceptors, a conditional knockout strategy was employed that showed that deletion of FABP5 specifically in nociceptors augments AEA levels, resulting in antinociceptive effects mediated by CB1R (Bogdan et al., 2022). Given that the concentration of free fatty acids including AA may be much higher in the synaptic cleft and in neuronal membranes, it is intriguing that FABP5 binds 2-AG in a physiological environment, which is found at significantly lower concentrations and competes for the same binding site as AA in this protein.
especially because there are not multiple lipid binding sites in FABP5. Overall, it cannot be ruled out that the effects observed with FABP KO mice may also be related to AA metabolism.

E. Translational Implications of eCB Transport Inhibitors

The different models of eCB cellular uptake and trafficking offer different druggable sites, that are schematically depicted in Fig. 26. The identification of intracellular carrier proteins for AEA has clearly provided a missing link to explain how eCBs are able to cross the cytosol, which constitutes a hydrophilic barrier for these lipophilic compounds (Hillard and Jarrahian, 2003; Fegley et al., 2004; Glaser et al., 2005; Hillard et al., 2007; Kaczocha et al., 2009; Oddi et al., 2009; Fowler, 2012; 2013).

To date, different AEA transport inhibitors and detailed pharmacological assessment of their in vivo effects have led to a better understanding of the druggability of such processes within the ECS. However, only few inhibitors used in pharmacological experiments have been studied for their bioavailability, tissue distribution and overall pharmacokinetics, thus in vivo effects of such inhibitors are difficult to interpret. As yet, only FABP5 inhibitors AT26 and SYT510 have shown efficacy in models of pain, anxiety, and inflammation through mechanisms involving the ECS, and are drug candidates in a late preclinical stage (Table 13).

Table 13. Placeholder

The new generation of selective inhibitors for AEA uptake (WOB E437, RX-055, guineensine) also blocks 2-AG uptake but does not interact with any of the known metabolic enzymes or AEA binding proteins, suggesting an additional common target that is competitive with eCB membrane transport. This observation has inspired the development of thiazolidinones like SYT510 that act as SERIs for the treatment of neuropsychiatric disorders – in a manner that is comparable to MAGL inhibitors - and are in early clinical development (Table 13). Based on the pharmacological profiles of these SERIs it can be expected that they are more specific and do not interfere with metabolic classes. Moreover, unlike FAAH, MAGL and FABP5 inhibitors, SERIs rather selectively inhibit the uptake of both AEA and 2-AG, without modulating other lipids (Chicca et al., 2017). Remarkably, their mild modulation of the eCB tone may be beneficial when it comes to issues related to desensitization of CB1R (Reynoso-Moreno et al., 2021).

VI. Therapeutic Potential of Additional Targets within the “Endocannabinoidome”

A. Definition of the Endocannabinoidome

Two realisations, during the last 20 years, have brought to the attention of the scientific community that the ECS should be considered as part of a much wider signaling system, now referred to as the
endocannabinoidome (eCBome) (Balvers et al., 2009; Piscitelli et al., 2011; Di Marzo, 2018; Cristino et al., 2020): 1) the discovery that several endogenous congeners of AEA and 2-AG, i.e. the N-acyl-ethanolamines and 2-monoacylglycerols respectively, and other eCB analogs like long chain fatty acid derivatives are present in tissues and biological fluids, although they seldom share with eCBs the capability of modulating the activity of CB1R and CB2R (while often being biosynthesized and/or degraded by the same enzymes) (Di Marzo, 2018); and 2) the finding that most plant cannabinoids other than THC, such as cannabidiol, cannabigerol, cannabidivarine and cannabichromene, to name a few, also do not share with THC, AEA and 2-AG their activity at cannabinoid receptors, although they often interact, among others, with several receptors of the above mentioned eCB-like molecules (Di Marzo, 2018). The main components of the eCBome are summarized in Table 14.

Table 14. Placeholder

In particular, beyond N-acyl-ethanolamines and 2-monoacylglycerols, whose existence was known even before the discovery of AEA and 2-AG, several other sub-families of eCB-like molecules have been recently discovered, including: 1) primary amides of long chain fatty acid, of which the sleep inducing factor oleamide is the prototypical member (Langstein et al., 1996); 2) amides between long chain fatty acids and several amino acids, such as N-acyl-taurines, -glycines, and -serines (Huang et al., 2001; Milman et al., 2006; Saghatelian et al., 2006); 3) amides of long chain fatty acids with neurotransmitters and other amines, such as N-acyl-dopamines and -serotonines (Huang et al., 2002; Verhoeckx et al., 2011), and 4) oxidation products (usually, but not necessarily, produced by the action of “arachidonate cascade” enzymes COX-2 and LOXs) of the di- and poly-unsaturated members of the above families (namely N-acyl-ethanolamines and 2-monoacylglycerols), a sub-family of lipids that we can refer to as the “oxyendocannabinoidome” (oxyeCBome) (reviewed in Simard et al., 2022). Therefore, considering that several long chain fatty acids exist, from the C16-containing and completely saturated palmitic acid, to the C22-containing hexa-unsaturated docosahexaenoic acid, it can be reckoned that in principle hundreds of such eCBome mediators exist. However, the actual occurrence of only a few dozens of them has been ascertained so far, through the use of bi-dimensional liquid chromatography mass spectrometry approaches (Piscitelli et al., 2011; Leishman et al., 2016; Kantae et al., 2017; Lacroix et al., 2019).

Importantly, each different member of these sub-families, depending on its fatty acid and amine moieties, can modulate the activity of one or more different receptors which, in a few cases, have been suggested to be also targeted by AEA or 2-AG (Table 14), although often at concentrations higher than those required to activate CB1R and CB2R (Di Marzo, 2018; Gómez-Cañas et al., 2023). In fact, at least three classes of
receptors have been suggested to act as targets for eCBome mediators (Morales and Reggio, 2017; Muller et al., 2019; Lago-Fernandez et al., 2021): 1) GPCRs, like CB1R and CB2R and beyond, and including some orphan GPCRs like GPR18, GPR55, GPR110, GPR119 and GPR130, or GPCRs that are known to be activated or inhibited by other mediators, such as some serotonin receptors; 2) ligand-activated ion channels, such as i) TRP channels of the V1-4, A1 and M8 types, and T-type Ca2+ (Ca3.3) channels, which have been suggested to be modulated also by other lipid mediators, and ii) amino acid neurotransmitter-activated targets, such as γ-aminobutyric acid or glycine receptors; and 3) nuclear PPARs. However, for some eCBome mediators, such as the primary amides, the molecular targets are still unknown, although for oleamide evidence of it being a weak agonist of CB1R (Leggett et al., 2004), and a TRPV2 antagonist (Schiano Moriello et al., 2018) exist. Additionally, some eCB-like molecules have also been shown to produce some of their pharmacological actions by interacting with eCB metabolic enzymes. Whilst stimulation of DAGLs by N-palmitoylethanolamine, which possibly explains why this mediator can enhance 2-AG levels in vitro and in vivo, has been only recently shown (Petrosino et al., 2019), inhibition of FAAH leading to increased levels of AEA and other endogenous substrates for this enzymes (e.g., N-acyl-taurines and, under certain circumstances, 2-AG) has been suggested as the basis of some of the pharmacological effects of other NAEs and unsaturated N-acyl-serotonins, -glycines, and -alanines (Jonsson et al., 2001; Petrosino and Di Marzo, 2017; Bashashati et al., 2017; Ayoub et al., 2020). Interestingly, the capability of inhibiting FAAH is shared also by the non-euphoric cannabinoid, CBD (Watanabe et al., 1996; Bisogno et al., 2001).

As mentioned above, several non-THC cannabinoids, as well as AEA and 2-AG, have also been suggested to influence the activity of the above eCBome receptors, including: 1) orphan GPCRs, particularly for CBD which antagonises GPR55 and seems to modulate some opioid and serotonin receptor subtypes (reviewed by de Almeida et al., 2020); 2) TRPV1-4 or TRPA1 channels, which are activated by several non-euphoric cannabinoids - with TRPV1 now being widely considered also as an alternative physiopathological target for AEA, unsaturated NAEs and 2-AG -, and TRPM8, which is antagonised by all tested cannabinoids (with the only exception of cannabichromene) as well as by both AEA and 2-AG (Zygmunt et al., 1999; De Petrocellis et al., 2007; De Petrocellis et al., 2011; De Petrocellis et al., 2012; Muller et al., 2019); 3) Ca3.3 channels and glycine receptors, which can be variedly inhibited by THC, cannabidiol and several types of eCBome mediators, as well as by AEA and some N-acylethanolamines, whereas some subunits of the γ-aminobutyric acid receptor are instead activated by 2-AG and CBD (Sigel et al., 2011; Baur et al., 2013; Chemin et al., 2014; Bakas et al., 2017; Mirlohi et al., 2022); and 4) PPARs, which can be activated by CBD.
and cannabigerol, particularly in their acidic forms normally found in cannabis flowers, as well as, particularly
in the case of PPARα, by some N-acylethanolamines, 2-palmitoyl-glycerol and N-acyl-glycines (O’Sullivan,
2016; Donvito et al., 2019; D’Aniello et al., 2019; Tutunchi et al., 2020; Depommier et al., 2021; Lago-
Fernandez et al., 2021). These eCBome receptors are schematically depicted in Fig. 27.

Figure 27. Placeholder

In summary, the eCBome and the oxyeCBome potentially include perhaps hundreds of mediators
(several combinations of amides between long chain fatty acids and amino acids or bioactive amines, and
the plethora of oxidation products that can be generated from polyunsaturated eCBome mediators, to name
a few), and dozens of receptors. Of the latter, however, many had been previously described as molecular
targets for other mediators (neurotransmitters, fatty acids, etc.) and cannot be listed as “specific” eCBome
receptors, at least not until their preferential role is ascertained as intermediates of the biological effects of
eCBome mediators which, however, are often very promiscuous in their modulation of the activity of
pharmacologically relevant proteins. While it is of crucial importance to know that AEA and 2-AG are
accompanied in tissues by several congeners and metabolites with similar biochemistry and different
pharmacology, a discussion of the pharmacological and therapeutic importance of these non-CB₁R, non-
CB₂R receptors is too speculative and goes beyond the scope of this article.

Indeed, the existence of the eCBomes both opens new opportunities and raises new challenges for the
development of new therapeutics from the study of the ECS. On the one hand, the recognition that some
plant cannabinoids, which are devoid of the typical psychotropic and unwanted effects of THC, owe some of
their pharmacological effects - and hence potential therapeutic actions - to modulation of the activity of
eCBome receptors beyond CB₁R and CB₂R, widens their potential applications in medicine (Fig. 27). This
same realization is also at the basis of the use of some eCBome mediators, such as N-
palmitoylethanolamine and N-oleoylethanolamine, as either synthetic drugs, nutraceuticals and tissue-
targeted nanoparticles (Bowen et al., 2017; Petrosino and Di Marzo, 2017; Wu et al., 2021), or through diets
rich in their fatty acid precursors (Sihag and Di Marzo, 2022), as potential new therapeutic approaches in
inflammatory and metabolic disorders. On the other hand, the fact that several eCBome mediators that have
non-cannabinoid receptors as their main targets, share with AEA or 2-AG the capability of being inactivated
by FAAH (as in the case of N-acyl-ethanolamines, -glycines and -taurines) or MAGL (as in the case of 2-
monoacylglycerols), respectively, may limit the clinical applicability of FAAH and MAGL inhibitors. In fact,
such drugs might concomitantly elevate the tissue levels, not only of AEA or 2-AG, thus indirectly activating
CB₁ and CB₂ receptors, but also of other eCBome mediators with targets whose functions in disease might

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be also opposite to those exerted by cannabinoid receptors in disease. A typical example of this potential problem might be represented by the failure, to date, of FAAH inhibitors to counteract inflammatory and chronic pain in clinical trials, when the fact that such molecules elevate the levels not only of AEA but also of N-palmitoylethanolamine and N-oleoylethanolamine, which may consequently act at targets such as TRPV1 and GPR55, may explain the lack of efficacy. Likewise, inhibitors of DAGLs, which have been proposed as potential treatment for obesity and metabolic disorders through the impairment of 2-AG biosynthesis and subsequent CB1R activation (Bisogno et al., 2013; Janssen and van der Stelt, 2016), might reduce the levels of 2-monoacylglycerols acting at CB2R, GPR119 and TRPV1 which, unlike CB1R, may have beneficial effects in such pathological conditions (Di Marzo and Silvestri, 2019). In yet other pathological conditions, however, where both cannabinoid and other eCBome receptors play similar functions (e.g., inflammation, neurodegeneration, and mood control), such an intrinsic lack of functional selectivity of inhibitors of eCB metabolism may provide additional advantages. It is, therefore, clear that the use of such inhibitors requires: 1) first the understanding of what eCBome receptors are involved in a given disorder, and 2) in case of conflicting effects being predicted for the blockade of a given enzyme, the development of multi-target drugs should be fostered, capable also of modifying the activity of targets that exert opposing roles in a given disorder (see for review Maione et al., 2013). The eCBome mediators and their synthetic analogs that are under clinical testing are listed in Table 15.

Table 15. Placeholder

B. Interaction with the Gut Microbiome

An additional possibility that is attracting attention is to explore targeted nutritional strategies for the therapeutic manipulation of the eCBome, based on the concept that the content of different eCBome mediators is strongly affected by the presence of their fatty acid precursors in the diet (Castonguay-Paradis et al., 2020). Indeed, an important opportunity opened by the discovery of the eCBome lies in its capability of interacting, much more than the ECS does, with another fundamental player in mammalian physiology and pathology which, like the eCBome, is also strongly influenced by diet, medications and other environmental, lifestyle as well as genetic factors: the gut microbiome (Di Marzo and Silvestri, 2019). Trillions of microorganisms, belonging to thousands of species from several phyla (bacteria, archea, viruses, yeasts, eukaryote parasites), populate the mammalian gut and communicate with, and subsequently regulate, the activity of host cells, mostly through the production of a plethora of chemical signals that are capable of interacting with host targets. In particular, some small molecules typically produced by gut bacteria following the digestion (fermentation) of dietary macro- and micro-nutrients, have been well characterised, and include
among others: 1) short chain fatty acids (SCFAs, derived from the processing of dietary complex carbohydrates); 2) branched chain fatty acids and amino acids (usually derived from the processing of dietary proteins); 3) tryptophan derivatives; and 4) secondary bile acids (Gold and Zhu J, 2022). These molecules usually act at receptors located in host cells (e.g., GPCRs and PPARs, in the case of branched chain fatty acids, and the aryl hydrocarbon receptor, in the case of some tryptophan derivatives), as recently reviewed (Ikeda et al., 2022; Wang et al., 2022). However, only recently it has become evident that some gut bacteria and yeasts can produce eCB-like molecules, such as N-acylated glycines, dopamines, tyramines, phenylethylamines and tryptamines, as well as oxyeCBome mediators capable of binding to the same receptors as the host eCBome mediators (De Petrocellis et al., 2009; Cohen et al., 2017; Chang et al., 2021). This emerging “microbendocannabinoidome”, also summarized in Table 14, enlarges the span of microbe-host communication and expands it to the eCBome, as depicted in Fig. 28. It also adds to previous evidence suggesting that, reciprocally, the ECS modulates the gut microbiome.

Figure 28. Placeholder

This evidence came from experiments carried out in animal models of obesity, gut dysbiosis (i.e., perturbation of gut microbiota composition and function) and ensuing metabolic endotoxaemia using CB1R antagonists and TRPV1 agonists (namely capsaicin) (Cluny et al., 2015; Shen et al., 2017; Mehrpouya-Bahrani et al., 2017), as well as in mice where eCB metabolic enzymes were knocked out (Geurts et al., 2015; Dione et al., 2020). These interventions, together with the expected alterations of eCB and eCBome signaling, were found to lead to concomitant and interrelated modulation of metabolic and inflammatory parameters and gut microbiota composition, with increases of the relative abundance of beneficial gut bacteria species, such as Akkermansia muciniphila. More recently, also some therapeutic effects of pCBs were likewise found to be accompanied by corresponding beneficial actions on the gut microbiome. Clearly, it is difficult to understand solely from these *in vivo* studies whether the effects observed on the gut microbiome were the direct consequence of the pharmacological and genetic manipulation of the eCBome, or only an indirect and host-mediated effect of the latter.

In fact, there is now also accumulating *ex vivo* and *in vitro* evidence that host-derived eCB-like mediators can directly affect the composition and function of the gut microbiome. In particular, both N-acyylethanolamines and 2-monoacylglycerols were found to affect the function (e.g., proliferation, biofilm formation, and virulence) of gut bacteria, clearly through mechanisms and at experimental concentrations that are quite different from those underlying their effects on host cells (Ellermann et al., 2020; Dione et al., 2020; Fornelos et al., 2020; Sionov and Steinberg, 2022). On the other hand, it was also shown that the
manipulation of the gut microbiome, either in germ-free mice or following prolonged treatment of mice with antibiotics, directly affects the expression of eCBome receptors, metabolic enzymes and mediators in the gut and other, more distal host tissues like the brain (Muccioli et al., 2010; Aguilera et al., 2015; Manca et al., 2020a; 2020b). The mechanisms through which this influence is exerted are not yet known, but are likely to be due to the action on host cells of the aforementioned microbiome-derived metabolites (i.e., short chain fatty acids), and may include epigenetic regulation of genes encoding for eCBome proteins, since these changes can be reversed upon reinstatement of the gut microbiome.

Also probiotics are known to affect the intestinal ECS and to potentially owe to this interaction part of their pharmacological actions (Rousseaux et al., 2007; Ringel-Kulka et al., 2014; Rossi et al., 2020; Cuozzo et al., 2021). Yet, it remains unclear whether these effects are due to a direct action of probiotic bacterial species on host cells or to their capability of modulating the gut microbiome. Interestingly A. muciniphila, a proposed gut microbiota-derived probiotic whose administration produces beneficial actions in both animal models of obesity and dysmetabolism and authentic obese subjects with metabolic syndrome (Everard et al., 2013; Plovier et al., 2017; Depommier et al., 2019), was found to increase the intestinal levels of pharmacologically active 2-monoacylglycerols (i.e., 2-AG, 2-palmitoyl-glycerol, and 2-oleoyl-glycerol) in mice with diet-induced obesity (Everard et al., 2013), as well as the circulating levels of 2-palmitoyl-glycerol - a PPARα agonist with potential metabolic beneficial activity - in obese individuals (Depommier et al., 2021). Probiotics can also reverse the gut dysbiosis-induced alterations of N-acyl-serotonin and N-acylethanolamine concentrations in gut (Guida et al., 2018) or adipose tissue (Geurts et al., 2015) respectively, with a corresponding amelioration of dysmetabolism and mood disturbances, respectively; again it is not clear whether or not these effects were exerted directly on eCBome mediator biosynthesis or degradation. Clearly, in vitro studies, using for example co-cultures of commensal bacteria or probiotic species (or their culture media) with mammalian intestinal cells or organoids, are again needed to understand whether or not these in vivo effects are the consequence of direct bacterial interactions with host cells.

VII. Conclusion

Plant-derived and endogenous cannabinoids represent two different but equally complex systems, so that the terms “(phyto)cannabinoids” and “endocannabinoids” are actually used to identify rather heterogeneous groups of lipophilic substances. It is striking how some of these molecules happened to share 3D structures, allowing exogenous pCBs to play so many biological activities in our body, because they mimic eCBs. The additional layer of complexity brought about by these structural similarities makes extremely challenging the
use of pCBs and ECS-oriented drugs as potential therapeutics to combat human diseases, and requires
deeper knowledge of the structural and functional details of their potential targets in the cell. Undoubtedly, a
better understanding of these fine molecular clues will allow to turn pCBs and ECS-oriented drugs from
threats to treasure trove for human health.

Among the various components of the ECS, CB₁R, CB₂R and FAAH have been the most largely exploited
to develop therapeutic drugs for human diseases.

Shortly after the discovery of CB₁R, many therapeutic opportunities have identified for its agonists and
antagonists; yet, improvements in medicinal cannabinoids are continually meeting novel challenges (Pacher
and Kunos, 2013). Selectivity for specific tissue responses is necessary to promote beneficial therapeutic
responses while minimizing side effects. Developing agonists that are highly selective for CB₁R but devoid of
activity at other receptors (e.g., CB₂R, GPR55) continues to remain a challenge. Organ-system selectivity is
a second goal for minimizing CNS actions of CB₁R agonists and antagonists and is being met by the
development of peripherally-restricted ligands that have limited access to brain CB₁R (Amato et al., 2019). A
third approach to selectivity involves tuning the functional outcome of ligands such as “biased agonists” that
would modify the active CB₁R conformation to direct signaling preferentially through either G protein
pathways or β-arrestin pathways, and further to select for individual G protein subtypes (Gs or G₁₂/₁₃ versus
Gi/o) and for β-arrestin 1 versus β-arrestin 2. A fourth mechanism for selectivity is to develop allosteric
modulators whose effects would be limited to only those receptors concurrently being stimulated by an
endogenous agonist. A positive allosteric modulator would augment the response to eCBs and could
potentiate ongoing stimulatory signals, whereas a negative allosteric modulator would be expected to
provide non-competitive inhibition to those receptors receiving an endocannabinoid signal. Additionally,
future goals for antagonists would be development of biased antagonists, negative allosteric modulators, as
well as neutral antagonists that do not affect the basal activity of CB₁R. A still unexplored approach with
therapeutic potential that is closer to the concept of polypharmacology involves specifically inhibiting CB₁R
and activating CB₂R. Thus, the future for CB₁R pharmacotherapeutics can be predicted to move from
phytocannabinoid preparations to agonists and antagonists that exhibit greater selectivity through one of
these strategies.

Also CB₂R is a key element of the ECS. It is highly expressed in immune cells and its activation limits
inflammation and associated tissue injury under multiple pathological conditions. Efficacy in preclinical
models of pain, neurodegenerative, cardiovascular, gastrointestinal, liver, kidney and lung diseases has
been demonstrated. Due to this enormous therapeutic potential, a multitude of CB₂R ligands has been
developed that can be categorized as eCBs and related fatty acid derivatives, pCBs or synthetic CB2R ligands. The majority of these ligands include agonists, modulators, neutral antagonists, inverse agonists, allosteric ligands as well as labelled chemical probes. Altogether, a large, structurally diverse chemical space is covered. Generally, early CB2R modulators are dual CB1R/CB2R agonists that are mostly not quite “drug-like”. In contrast, recent ligands often combine high potency for CB2R with favorable overall ADME profiles including low lipophilicity, aqueous solubility and favourable plasma protein binding, which translate into excellent pharmacokinetic profiles and consequently improved developability. To overcome CB1R-driven psychotropic effects two strategies were followed: limiting exposure toward the periphery or enhancing the selectivity over CB1R through excellent structure activity relationship work in the lead optimization phase thus enabling clinical studies with more than 20 new molecular entities. First trials focused on diseases of the CNS and pain. Most recent ligands and clinical studies focus on peripheral indications with a strong inflammatory/ immunomodulatory and/or fibrotic background. Three phytocannabinoids (THC, nabilone and cannabidiol) have been launched. Most advanced selective CB2R agonists are in phase 2 clinical trials. While no CB2R-related toxicity issues have been reported from clinical studies, the demonstration of target engagement and the identification of best-suited human disease condition(s) for the therapeutic use of CB2R modulators still poses challenges for the development of CB2R-based therapies. The generation of translational animal models and a better understanding of CB2R and the ECS in general will help unlocking the receptor’s full therapeutic potential. Recently discovered high-quality labelled chemical probes have enabled a better understanding of CB2R expression, mechanism of action and translatability of results toward the human situation. The in depth understanding of signaling bias as well as CB2R receptor homo- and heterodimers might translate into different functional properties and ultimately tailor-made CB2R therapeutics. Deeper insights in drug-target binding kinetics, their impact on receptor function and in particular, the recently reported structures of antagonist- and agonist-bound CB2R and knowledge on allosterism will facilitate rational drug design. Together with the huge chemical space available to generate tailor-made CB2R modulators this will hopefully guide us to the discovery of potent, effective, and safe medicines for indications with a dire or even unmet need.

Finally, since the discovery of FAAH and MAGL many compounds have been developed starting from inhibitors of other known serine hydrolases, in order to study enzyme activity, its regulation, and relevance in the pathophysiological process. Over the years, different approaches were used to identify and synthesize new classes of single or dual inhibitors, paying more and more attention to the analysis of their selectivity, potency, and mechanism of action. The development of selective FAAH and MAGL inhibitors remains one of
the major issues in drug discovery, as demonstrated also by the large number of research papers and review articles devoted to this topic. The main interest in this field is to develop a therapeutic alternative to the use of cannabinoid receptor agonists, able to prevent or minimize serious psychotropic side-effects due to direct receptor activation. The possibility to increase eCB tone by reducing degradation, and to apply new multi-target strategies that include additional receptors and enzymes, have boosted FAAH and MAGL inhibition studies to generate therapeutics against peripheral and CNS-related pathologies. However, more details seem to be necessary on 3D structure, catalytic mechanism, and regulation of both hydrolases, to design effective inhibitors devoid of off-target activity. Novel in silico approaches like computer-aided drug discovery (CADD) may be useful to reach this goal. In this context, it has to be recalled the tragic use of BIA10-2474, a purported FAAH inhibitor that killed one volunteer and led four others to hospitalization in phase I clinical trials because of serious adverse neurological events (Kerbrat et al., 2016). This BIA 10-2474 disaster clearly reminds us that accurate preclinical characterization of the biochemical profile of any new chemical entity must be performed, before claiming that a new selective drug has been discovered, and especially before allowing its use in clinical trials.

Overall, the same potentialities and limitations revealed by accumulated evidence in so many studies on CB₁R, CB₂R, FAAH and MAGL are likely to apply also to the more recently characterized elements of the ECS, and hopefully will be instructive to avoid making again the same mistakes. In particular, a better appreciation of the 3D structure of the desired targets, also via cryo-electron microscopy (Hua et al., 2020), and the application of powerful in silico tools like CADD, virtual screening (Stasiulewicz et al., 2022) and machine learning (Atz et al., 2023) hold promise to shorten the path from the bench to the patient’s bed in drug discovery programmes oriented toward the ECS. This knowledge of structural details will also help to decipher complex interactions between pCBs/eCBs and other bioactive lipids (e.g., eicosanoids and specialized pro-resolving mediators) that are receiving increasing attention for their therapeutic potential to treat human diseases (Maccarrone, 2023).
Acknowledgments

M.M. wishes to express gratitude to all colleagues who contributed over the last 30 years to his studies on the ECS and its implications for human health and disease. M.M. also thanks Dr. Cinzia Rapino (University of Teramo, Teramo, Italy) and Dr. Marina Fava (Campus Bio-Medico University of Rome, Rome, Italy) for their help in preparing tables 1-3, and figures 1-7, respectively. A.M. thanks Dr. Christos Iliopoulos Tsoutsouvas (Northeastern University, Boston, MA, USA) for his support in collecting information on CB1R and preparing table 4 and figures 8-9. D.P. thanks Dr. Yannick Fotio (University of California, Irvine, CA, USA) for his support in preparing figures 23 and 24. M.vd S. thanks Dr. Na Zhu and Dr. Anthe Janssen (Leiden University, Leiden, Netherlands) for preparing the schemes in tables 1, 3, 7, 9, and for figure 25.

Authorship Contributions

Conceived and supervised the project: Maccarrone. Wrote or contributed to the writing of the manuscript: Maccarrone, Di Marzo, Gertsch, Grether, Howlett, Hua, Makriyannis, Piomelli, Ueda, and Van der Stelt.
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antagonist AM4113 suppresses food intake and food-reinforced behavior but does not induce signs of nausea in rats. *Neuropsychopharmacol* **33**:946-955.


Footnotes

Financial support - For this investigation M.M. was partly supported by the Italian Ministry of Health through the competitive Ricerca Finalizzata 2018 [Grant RF-2018-12365391]. A.C.H. was supported by the National Institute in Drug Abuse [Grant R01-DA-042157]. A.M. was supported by the National Institute in Drug Abuse [Grant P01-DA-009158] and by the National Institute on Alcohol Abuse and Alcoholism [Grant U01-DA-028963].

Conflict of interest statement – No author has an actual or perceived conflict of interest with the contents of this article.
### TABLE 1

Major phytocannabinoids (pCBs)

<table>
<thead>
<tr>
<th>Name (abbreviation)</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>More Abundant pCBs</strong></td>
<td></td>
</tr>
<tr>
<td>Δ⁹-Tetrahydrocannabinol (THC)</td>
<td>![THC structure]</td>
</tr>
<tr>
<td>Cannabidiol (CBD)</td>
<td>![CBD structure]</td>
</tr>
<tr>
<td>Cannabinol (CBN)</td>
<td>![CBN structure]</td>
</tr>
<tr>
<td>Cannabigerol (CBG)</td>
<td>![CBG structure]</td>
</tr>
</tbody>
</table>
Cannabivarmin (CBV)

Cannabidivarmin (CBDV)

Δ9-Tetrahydrocannabivarmin (THCV)

Less Abundant pCBs

Cannabichromene (CBC)
Cannabinol (CBND)

Cannabicyclol (CBL)

Cannabielsoin (CBE)

Cannabinol (CBT)
### TABLE 2
Approved and potential indications for THC and CBD

<table>
<thead>
<tr>
<th>Cannabinoid</th>
<th>Approved (A) and Potential (B) Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>THC</strong></td>
<td>A) Chemotherapy-induced nausea and vomiting; appetite stimulant (HIV/AIDS).</td>
</tr>
<tr>
<td></td>
<td>B) Spasticity in MS; neuropathic pain in MS; cancer pain unresponsive to opioids; other pain conditions (i.e., postherpetic neuralgia, postoperative pain); intraocular pressure in glaucoma; depression; anxiety/sleep disorder; psychosis; tics of Tourette syndrome; tremor/bladder dysfunction in MS; dyskinesias in HD; levodopa-induced dyskinesias in PD; cervical dystonia; epilepsy and AD.</td>
</tr>
<tr>
<td><strong>CBD</strong></td>
<td>B) Childhood epilepsy; tuberous sclerosis complex seizure; Lennox-Gastaut syndrome; Dravet syndrome and infantile spasms.</td>
</tr>
<tr>
<td><strong>THC/CBD</strong></td>
<td>A) Spasticity in MS.</td>
</tr>
<tr>
<td></td>
<td>B) Paraplegia and spasticity in amyotrophic lateral sclerosis; cancer pain unresponsive to opioids; other pain conditions (i.e., postherpetic neuralgia, postoperative pain); intraocular pressure in glaucoma; depression; anxiety/sleep disorder; psychosis; tics of Tourette syndrome; tremor/bladder dysfunction due to MS; dyskinesias in HD; levodopa-induced dyskinesias in PD; cervical dystonia; epilepsy and AD.</td>
</tr>
</tbody>
</table>

AD, Alzheimer’s disease; CBD: cannabidiol; HD, Huntington’s disease; MS, multiple sclerosis; THC, Δ⁹-tetrahydrocannabinol.
### TABLE 3
Major endocannabinoids and congeners

<table>
<thead>
<tr>
<th>Name (abbreviation)</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major ω-6 eCBs</strong></td>
<td></td>
</tr>
<tr>
<td>N-Arachidonylethanolamine</td>
<td><img src="image" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>
  (Anandamide, AEA) |
| 2-Arachidonoylglycerol | ![Chemical Structure](image) |
  (2-AG) |
| 2-Arachidonoylglycerol | ![Chemical Structure](image) |
  (Noladin) Ether (2-AGE) |
| Virodhamine | ![Chemical Structure](image) |
  (O-Arachidonylethanolamine, O-AEA) |
| **Major ω-3 eCBs**  |                    |
| N-Eicosapentaenoylthanolamine | ![Chemical Structure](image) |
  (EPEA) |
| N-Docosahexaenoylthanolamine | ![Chemical Structure](image) |
  (DHEA) |
| **Major eCB-like Compounds** | |
| N-Palmitoylethanolamine | ![Chemical Structure](image) |
  (PEA) |
<table>
<thead>
<tr>
<th>Major eCB-Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N\text{-Oleoyl} \text{ethanolamine} ) (OEA)</td>
</tr>
<tr>
<td>( N\text{-Stearoyl} \text{ethanolamine} ) (SEA)</td>
</tr>
<tr>
<td>( N\text{-Linoleoyl} \text{ethanolamine} ) (LEA)</td>
</tr>
<tr>
<td>( 2\text{-Oleoylglycerol} ) (2-OG)</td>
</tr>
<tr>
<td>( N\text{-Arachidonoyl dopamine} ) (NADA)</td>
</tr>
<tr>
<td>( N\text{-Arachidonoyl glycine} ) (NAGly)</td>
</tr>
<tr>
<td>( N\text{-Arachidonoyl serine} ) (ARA-S)</td>
</tr>
<tr>
<td>( N\text{-Oleoyl glycine} ) (OIGly)</td>
</tr>
<tr>
<td>( N\text{-Oleoyl alanine} ) (OIALa)</td>
</tr>
</tbody>
</table>
### TABLE 4
Diseases/symptoms for treatment with CB₁R agonists and antagonists registered with ClinicalTrials.gov

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Completed Clinical Trials</th>
<th>Ongoing Clinical Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dronabinol</td>
<td>Chronic pain (with opioid treatment)</td>
<td>Osteoarthritis pain</td>
</tr>
<tr>
<td>Marinol</td>
<td>Fibromyalgia, back pain</td>
<td>Diabetic neuropathy</td>
</tr>
<tr>
<td>Phytocannabinoid</td>
<td>Migraine pain</td>
<td>Knee arthroplasty</td>
</tr>
<tr>
<td>Synthetically-produced</td>
<td>Neuropathic, low back pain</td>
<td>Arthroscopic surgery</td>
</tr>
<tr>
<td>Δ⁹-tetrahydrocannabinol (THC)</td>
<td>Cervical dystonia</td>
<td>Sleep and pain in MS</td>
</tr>
<tr>
<td>CB₁R/CB₂R partial agonist</td>
<td>Chest Pain</td>
<td>PostSurgical pain-lumbar fusion</td>
</tr>
<tr>
<td></td>
<td>Neuropathic pain in MS</td>
<td>PostSurgical pain-knee replacement</td>
</tr>
<tr>
<td></td>
<td>Cramps in ALS</td>
<td>Pain in opioid-maintained pts</td>
</tr>
<tr>
<td></td>
<td>Irritable bowel syndrome</td>
<td>Alzheimer’s agitation</td>
</tr>
<tr>
<td></td>
<td>Complex regional pain syndromes</td>
<td>Bipolar disorder</td>
</tr>
<tr>
<td></td>
<td>Cannabis dependence</td>
<td>Sleep</td>
</tr>
<tr>
<td></td>
<td>Cannabis Use disorder</td>
<td>PostTraumatic Stress Disorder</td>
</tr>
<tr>
<td></td>
<td>Marijuana withdrawal</td>
<td>Trauma intrusive memories</td>
</tr>
<tr>
<td></td>
<td>Trichotillomania related behaviors</td>
<td>Glaucoma hemodynamics</td>
</tr>
<tr>
<td></td>
<td>PostTraumatic Stress Disorder</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Obstructive sleep apnea</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PostSurgical N/V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-retroviral therapy N/V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain neoplasms N/V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Schizophrenia</td>
<td></td>
</tr>
<tr>
<td>Dronabinol derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BX-1 oral solution</td>
<td>Spasticity</td>
<td>Chemo N/V, pain in pancreatic CA</td>
</tr>
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*Pharmacological Reviews*

**Pharmrev Fast Forward. Published on 10 May 2023 as DOI 10.1124/pharmrev.122.000600 This article has not been copyedited and formatted. The final version may differ from this version.**
<table>
<thead>
<tr>
<th>Product</th>
<th>Condition</th>
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</thead>
<tbody>
<tr>
<td>Syndros (dronabinol)</td>
<td>Bone pain metastatic breast CA</td>
</tr>
<tr>
<td>Namisol THC</td>
<td>PostSurgical abdominal pain</td>
</tr>
<tr>
<td>Namisol THC</td>
<td>Pancreatitis abdominal pain</td>
</tr>
<tr>
<td>Namisol THC</td>
<td>Dementia- Alzheimers</td>
</tr>
<tr>
<td>Namisol THC</td>
<td>Dementia w/ neuropsych symptoms</td>
</tr>
<tr>
<td>THC olive oil</td>
<td>PostTraumatic Stress Disorder</td>
</tr>
<tr>
<td>THC olive oil</td>
<td>Fibromyalgia-pain</td>
</tr>
<tr>
<td>SCI-110 THC + PEA</td>
<td>Tourette syndrome</td>
</tr>
<tr>
<td>THX-110 THC + PEA</td>
<td>Tourette syndrome</td>
</tr>
<tr>
<td>dronabinol + naltrexone</td>
<td>Opioid dependence</td>
</tr>
<tr>
<td>Nabilone</td>
<td>Cesamet</td>
</tr>
<tr>
<td></td>
<td>Phantom limb pain</td>
</tr>
<tr>
<td>Synthetic THC analog</td>
<td>Fibromyalgia</td>
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<tr>
<td>CB₁R/CB₂R agonist</td>
<td>Failed back surgery pain</td>
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<tr>
<td></td>
<td>Inflammatory bowel pain</td>
</tr>
<tr>
<td></td>
<td>Spinal neuropathic pain</td>
</tr>
<tr>
<td></td>
<td>Pain and insomnia</td>
</tr>
<tr>
<td></td>
<td>Diabetic neuropathies</td>
</tr>
<tr>
<td></td>
<td>Developmental cognitive disability</td>
</tr>
<tr>
<td></td>
<td>Spinal injury muscle</td>
</tr>
<tr>
<td></td>
<td>Obsessive Compulsive Disorder</td>
</tr>
<tr>
<td></td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td></td>
<td>Alzheimer’s Disease agitation</td>
</tr>
<tr>
<td></td>
<td>Post-Surgical N/V</td>
</tr>
<tr>
<td></td>
<td>Cancer anorexia/cachexia</td>
</tr>
<tr>
<td></td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td></td>
<td>Parkinson’s nonmotor symptoms</td>
</tr>
<tr>
<td></td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>PP-01</td>
<td>Cannabis withdrawal</td>
</tr>
<tr>
<td>Nabilone+Gabapentin</td>
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<tr>
<td>Nabiximols</td>
<td>Sativex</td>
</tr>
<tr>
<td></td>
<td>Chemotherapy neuropathic pain</td>
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<td></td>
<td>Diabetic neuropathy</td>
</tr>
<tr>
<td></td>
<td>Phytocannabinoid</td>
</tr>
<tr>
<td></td>
<td>Advanced malignancy pain</td>
</tr>
<tr>
<td></td>
<td>MS spasticity and pain</td>
</tr>
<tr>
<td></td>
<td>Purified Plant Extract</td>
</tr>
<tr>
<td></td>
<td>Pain</td>
</tr>
<tr>
<td></td>
<td>THC:CBD (1:1)</td>
</tr>
<tr>
<td></td>
<td>Tourette syndrome</td>
</tr>
<tr>
<td>THC: CB₁R/CB₂R agonist</td>
<td>Attention Deficit-Hyperactivity Disorder</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>CBD: CB₁ NAM (Negative Allosteric Modulator)</td>
<td>Cannabis dependence</td>
</tr>
</tbody>
</table>

**Mixed THC:CBD**

<table>
<thead>
<tr>
<th>THC:CBD 1:1</th>
<th>Endometriosis pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>THC:CBD 1:1, 1:2</td>
<td>Chronic pain</td>
</tr>
<tr>
<td>THC:CBD1:10</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>THC:CBD 1:50</td>
<td>Childhood epilepsy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NanaBis Oro-MucSpray</th>
<th>Cancer pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>NanaBis Oro-MucSpray</td>
<td>Chronic widespread pain</td>
</tr>
<tr>
<td>THC or THC:CBD 1:10</td>
<td>Chronic spine back and neck pain</td>
</tr>
<tr>
<td>LGP1-20 THC:CBD (1:20)</td>
<td>Adolescent migraines</td>
</tr>
<tr>
<td>FibroCann Solution</td>
<td>Fibromyalgia</td>
</tr>
<tr>
<td>Pure Green SL Tablets</td>
<td>Osteoarthritis pain</td>
</tr>
<tr>
<td>MPL-001 THC:CBD 1:25</td>
<td>PostSurgical osteoarthritis pain</td>
</tr>
<tr>
<td>TN-TC11G THC:CBD1:1</td>
<td>Glioblastoma (w/standard of care)</td>
</tr>
<tr>
<td>TIL-T150 THC:CBD 1:5,1:25</td>
<td>Depression, insomnia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pure Femme SLTab 1:30 + PEA + terpenes</th>
<th>Menstrual symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>THC or CBD</td>
<td>HIV cognition</td>
</tr>
<tr>
<td>THC + CBD + CBG</td>
<td>Chronic migraine</td>
</tr>
</tbody>
</table>

**Pro-drug paracetamol** *(or acetaminophen)*

<table>
<thead>
<tr>
<th>Biometabolite is AM404</th>
<th>Pruritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB₁R/CB₂R agonist</td>
<td>Pre-Surgical analgesia</td>
</tr>
</tbody>
</table>

| Pain in tonsillectomies | |

**SR141716**

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<table>
<thead>
<tr>
<th>Rimonabant</th>
<th>Carotid atherosclerosis</th>
<th>Recovery spinal cord injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acomplia, Zimulti</td>
<td>Cannabis dependence</td>
<td></td>
</tr>
<tr>
<td>CB₁R antagonist/inverse agonist</td>
<td>Diabetes w/ metformin</td>
<td>Obesity, weight loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metabolic syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduce alcohol consumption</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fatty liver-NASH in T2D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smoking cessation</td>
</tr>
<tr>
<td>MK-0364</td>
<td></td>
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</tr>
<tr>
<td>Taranabant</td>
<td>Obesity</td>
<td></td>
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<tr>
<td>CB₁R antagonist/inverse agonist</td>
<td>Smoking cessation</td>
<td></td>
</tr>
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<td>Fatty liver-NASH in T2D</td>
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<td>CP-945598</td>
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<tr>
<td>Otenabant</td>
<td>Non-alcoholic steato-hepatitis</td>
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<tr>
<td>CB₁R antagonist/inverse agonist</td>
<td>Obesity</td>
<td></td>
</tr>
<tr>
<td>SLV319</td>
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<td></td>
</tr>
<tr>
<td>Ibipinabant</td>
<td>Obesity</td>
<td></td>
</tr>
<tr>
<td>CB₁R antagonist/inverse agonist</td>
<td>Smoking cessation</td>
<td></td>
</tr>
<tr>
<td>SR147778</td>
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<td></td>
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<tr>
<td>Surinabant</td>
<td>Obesity</td>
<td></td>
</tr>
<tr>
<td>CB₁R antagonist/inverse agonist</td>
<td>Smoking cessation</td>
<td></td>
</tr>
<tr>
<td>ANEB-001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB₁R antagonist/inverse agonist</td>
<td>Acute cannabis intoxication</td>
<td></td>
</tr>
<tr>
<td>GFB-024</td>
<td></td>
<td>Diabetic nephropathies</td>
</tr>
</tbody>
</table>
inverse agonist
monoclonal Ab

Nimacimab

Peripherally acting CB₁R  Diabetic gastroparesis
antagonist/inverse
agonist monoclonal Ab
**TABLE 5**

Diseases/symptoms for treatment with CB2R agonists and antagonists registered with ClinicalTrials.gov

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Completed Clinical Trials</th>
<th>Ongoing Clinical Trials</th>
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</thead>
<tbody>
<tr>
<td><strong>Class/Efficacy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dronabinol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dronabinol derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nabilone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nabiximols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed THC:CBD</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cannabidiol</strong></td>
<td></td>
<td></td>
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<tr>
<td>Epidiolex</td>
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<td></td>
</tr>
<tr>
<td>CB1R/CB2R ligand</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate Cancer</td>
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<td>Typical Absence Seizures</td>
</tr>
<tr>
<td>Cannabis Use Disorder</td>
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<td>Autism</td>
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<tr>
<td>Opioid Withdrawal</td>
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<td>Fibromyalgia</td>
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<tr>
<td>Musculoskeletal Pain</td>
<td></td>
<td>Aromatase Inhibitor-Associated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arthralgias</td>
</tr>
<tr>
<td>Alcohol Use Disorder</td>
<td></td>
<td>Back Pain</td>
</tr>
<tr>
<td>Post Traumatic Stress Disorder</td>
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<td>Depressive Symptoms</td>
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<tr>
<td>Inflammatory Bowel Disease</td>
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<td>Electrical Status Epilepticus of Slow-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wave Sleep</td>
</tr>
<tr>
<td>Knee Osteoarthritis</td>
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<td>Dental Pain</td>
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<tr>
<td>Parkinson’s Disease</td>
<td></td>
<td>Behavioural Problems in Children</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and Adolescents With Intellectual</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Disability</td>
</tr>
<tr>
<td>Opiate Addiction</td>
<td></td>
<td>Knee Arthritis</td>
</tr>
<tr>
<td>Epilepsy</td>
<td></td>
<td>Chemotherapy-Induced Peripheral</td>
</tr>
<tr>
<td>Disease/Medical Condition</td>
<td>Medical Condition</td>
<td></td>
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<tr>
<td>------------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------</td>
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</tr>
<tr>
<td>Neuropathy</td>
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<td></td>
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<tr>
<td>Seizures</td>
<td>Bipolar Disorder</td>
<td></td>
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<tr>
<td>Covid19</td>
<td>Hypertension</td>
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<tr>
<td>Burn Out</td>
<td>Anxiety and Fear</td>
<td></td>
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<tr>
<td>Chronic Periodontitis</td>
<td>Chronic Pain</td>
<td></td>
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<tr>
<td>Urinary Stone</td>
<td>Early Psychosis</td>
<td></td>
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<td>Schizophrenia</td>
<td>Posttraumatic Stress Disorder</td>
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<tr>
<td>Blepharospasm</td>
<td>Anorexia Nervosa</td>
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<tr>
<td>Cocaine Craving/Dependence</td>
<td>Gastroparesis and Functional Dyspepsia</td>
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<tr>
<td>Generalized Anxiety Disorder</td>
<td>Anxiety in Advanced Breast Cancer</td>
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<tr>
<td>Lennox-Gastaut Syndrome</td>
<td>Traumatic Brain Injury</td>
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<tr>
<td>Dravet Syndrome</td>
<td>Tobacco Cessation</td>
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<tr>
<td>Psychotic Disorders</td>
<td>Social Anxiety Disorder</td>
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<tr>
<td>Infantile Spasms</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>Fragile X Syndrome</td>
<td>Diabetes</td>
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<tr>
<td>Tuberous Sclerosis Complex</td>
<td>Chronic Pain</td>
<td></td>
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<tr>
<td>Psoriatic Arthritis</td>
<td>Endometriosis Pain</td>
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<tr>
<td>Hand Osteoarthritis</td>
<td>Social Anxiety Disorder</td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>Radiculopathy</td>
<td></td>
</tr>
<tr>
<td>Diabetic Neuropathies</td>
<td>Sleep Disturbance</td>
<td></td>
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<tr>
<td>Ulcerative Colitis</td>
<td>Insomnia</td>
<td></td>
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<tr>
<td>Fatty Liver</td>
<td>Prevention aGVHD</td>
<td></td>
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<tr>
<td>Prader-Willi Syndrome</td>
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<td></td>
</tr>
<tr>
<td>Musculoskeletal Pain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Lenabasum**

- CB₂R/CB₁R agonist
- Cystic fibrosis
- Dermatomyositis
- Systemic Lupus Erythematosus

**Olorinab**
<table>
<thead>
<tr>
<th>CB₂R agonist</th>
<th>Crohn's Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abdominal Pain</td>
</tr>
</tbody>
</table>

**RG7774**

<table>
<thead>
<tr>
<th>CB₂R agonist</th>
<th>Diabetic Retinopathy</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>

**CNTX-6016**

<table>
<thead>
<tr>
<th>CB₂R agonist</th>
<th>Chronic Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pain</td>
</tr>
<tr>
<td></td>
<td>Nociceptive Pain</td>
</tr>
<tr>
<td></td>
<td>Pain</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CB₂R agonist</th>
<th>Diffuse Cutaneous Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ agonist</td>
<td>Sclerosis</td>
</tr>
</tbody>
</table>

*Studies with the status "not yet recruiting, recruiting", "enrolling by invitation", "active, not recruiting" and "completed" were included in this table.

*See Table 4 for respective CB₁R data.*
### Table 6

<table>
<thead>
<tr>
<th>Drug</th>
<th>Chemical Class</th>
<th>Mode of Action</th>
<th>CB₂R/CB₁R in vitro Pharmacology</th>
<th>Indication(s)</th>
<th>Highest Phase of Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dronabinol</td>
<td>Classical cannabinoid</td>
<td>CB₂R/CB₁R agonist</td>
<td>(pK_i = 8.16/8.48^a)</td>
<td>Appetite loss, CINV, anorexia, cancer pain</td>
<td>Launched</td>
</tr>
<tr>
<td>(THC, Syndros, Marinol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nabilone</td>
<td>Classical cannabinoid</td>
<td>CB₂R/CB₁R agonist</td>
<td>(K_i = 1.84/2.19 \text{nM})</td>
<td>CINV</td>
<td>Launched</td>
</tr>
<tr>
<td>(Cesamet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lenabasum</td>
<td>Classical cannabinoid</td>
<td>CB₂R/CB₁R agonist</td>
<td>(K_i = 51/628 \text{ nM})</td>
<td>CF, SLE, RA, systemic sclerosis, dermatomyositis</td>
<td>Phase 3</td>
</tr>
<tr>
<td>(Ajulemic acid)</td>
<td></td>
<td></td>
<td></td>
<td>(systemic sclerosis since 2017; dermatomyositis since 2018)</td>
<td></td>
</tr>
<tr>
<td>Olorinab</td>
<td>Tricyclic 3,5,5-fused pyrazole 3-carboxamide</td>
<td>CB₂ R agonist</td>
<td>(EC_{50}=6.2/&gt;10^4 \text{nM})</td>
<td>IBS related pain, IBS with predominant constipation or diarrhoea</td>
<td>Phase 2b (since 2017)</td>
</tr>
<tr>
<td>(ADP-371)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CMX-020</td>
<td>Arachidonic acid analog</td>
<td>CB₂R/CB₁R agonist, TRPV1 agonist</td>
<td>(K_i = 150/21 \text{ nM})</td>
<td>Pain, OA, DnP</td>
<td>Phase 2 (since 2015)</td>
</tr>
<tr>
<td>RG7774</td>
<td>Triazolopyrimidine</td>
<td>CB₂ R agonist</td>
<td>(EC_{50}=1/&gt;10^4 \text{nM})</td>
<td>DR</td>
<td>Phase 2 (since 2020)</td>
</tr>
<tr>
<td>CNTX-6016</td>
<td>Piperidine based ligand</td>
<td>CB₂ R agonist</td>
<td>--</td>
<td>Pain Np, DnP</td>
<td>Phase 2 (since 2020)</td>
</tr>
<tr>
<td>ART-27.13</td>
<td>Benzimidazole</td>
<td>CB₂R/CB₁R agonist</td>
<td>(K_i = 0.9/12 \text{ nM})</td>
<td>Pain, cachexia, CINV</td>
<td>Phase 2 (since 2021)</td>
</tr>
<tr>
<td>(AZD-1940)</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>EHP-101</td>
<td>Cannabidiol</td>
<td>CB₂ R agonist,</td>
<td>(K_i = 170/&gt;4x10^4 \text{nM})</td>
<td>MS, ScD</td>
<td>Phase 2 (since 2020)</td>
</tr>
<tr>
<td>(VCE-004.8) derivative</td>
<td>PPARγ agonist</td>
<td>$K_i=22/10^4$ nM</td>
<td>AD, Np pain, cognitive disorder</td>
<td>2020</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>---------------</td>
<td>------------------</td>
<td>---------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>NTRX-07 (MDA-7)</td>
<td>CB₂R agonist</td>
<td></td>
<td>Phase 1 (since 2019)</td>
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<tr>
<td>2,3-Dihydro-1-benzofuran</td>
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<td></td>
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</tr>
</tbody>
</table>

*Consensus human CB₂R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017).

bFunctional activity in β-Arrestin-2 assay on human cannabinoid receptors (Han et al., 2017).

Functional activity in cAMP assay on human cannabinoid receptors (Grether, 2022).

CINV, chemotherapy induced nausea and vomiting; LGS, Lennox Gastaut syndrome; CF, cystic fibrosis; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; IBS, irritable bowel syndrome; OA, osteoarthritis; DnP, diabetic neuropathy; DR, diabetic retinopathy; Np, neuropathic; MS, multiple sclerosis; ScD, scleroderma; AD, Alzheimer’s disease.
TABLE 7

Potential therapeutic use of patented FAAH inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Potential Therapeutic Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxazole Derivatives</td>
<td>Treatment of different types of pain: post-operative pain, chronic pain, cancer pain,</td>
</tr>
<tr>
<td></td>
<td>cancer chemotherapy, neuralgia, nociception pain, inflammatory pain</td>
</tr>
<tr>
<td>Urea Derivatives</td>
<td>Treatment of depression, analgesia, and cannabis use disorders</td>
</tr>
<tr>
<td>Urea/Carbamate</td>
<td>Treatment of pain, inflammation, neuropathy, neurodegenerative diseases, anxiety,</td>
</tr>
<tr>
<td></td>
<td>motor function disorder, infertility, eating disorders, THC-dependence, metabolic</td>
</tr>
<tr>
<td></td>
<td>disorders, movement disorders, chemotherapy-induced nausea and vomiting, and cancer</td>
</tr>
<tr>
<td>ARN2508</td>
<td>Treatment of intestinal inflammation where a pure FAAH inhibitor was weakly active and</td>
</tr>
<tr>
<td></td>
<td>the pure COX inhibitor flurbiprofen aggravated inflammation</td>
</tr>
<tr>
<td></td>
<td>Simultaneous blockade of FAAH and COX-1/COX-2 results in a combination of profound</td>
</tr>
<tr>
<td></td>
<td>anti-inflammatory and tissue protective actions</td>
</tr>
<tr>
<td>Oxazolyl-ketones</td>
<td>[replacement of the phenyl hexyl group of OL-135 with a</td>
</tr>
<tr>
<td></td>
<td>Treatment of anxiety, pain, sleep disorders, eating disorders, inflammation, or movement</td>
</tr>
<tr>
<td></td>
<td>disorders (e.g., in multiple sclerosis)</td>
</tr>
</tbody>
</table>
piperidine ring]

JNJ-42119779 Effective in the spinal nerve ligation (Chung) model of neuropathic pain

JNJ-40413269 Effective in the rat spinal nerve ligation (Chung) model of neuropathic pain

2,3,4-Tetrahydro-2,6-naphthyridines Treatment of pain, anxiety, depression, inflammation, cognitive disorders, weight and eating disorders, Parkinson’s disease, Alzheimer’s disease, spasticity, addiction, glaucoma

For further details see Fazio et al., 2020.
## TABLE 8
Diseases/symptoms for treatment with FAAH inhibitors registered with ClinicalTrials.gov

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Completed Clinical Trials</th>
<th>Ongoing Clinical Trials</th>
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<tbody>
<tr>
<td>Brand Name</td>
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<td>FAAH inhibitors</td>
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<td><strong>PF-04457845</strong></td>
<td>Tourette Syndrome</td>
<td>Cannabis Use Disorder</td>
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<td>Cannabis Withdrawal</td>
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<tr>
<td></td>
<td>Fear Conditioning</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acute pain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chronic pain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Knee Osteoarthritis</td>
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<tr>
<td><strong>URB597</strong></td>
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<td>Schizophrenia</td>
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<tr>
<td><strong>SSR411298</strong></td>
<td>Major depressive disorder</td>
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<tr>
<td></td>
<td>Cancer pain</td>
<td></td>
</tr>
<tr>
<td><strong>APD8477</strong></td>
<td>Peripheral neuropathic pain</td>
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<tr>
<td><strong>V158866</strong></td>
<td>Neuropathic pain</td>
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</tr>
<tr>
<td><strong>JNJ-42165279</strong></td>
<td>Major depressive disorder</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Social anxiety disorder</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autism</td>
<td></td>
</tr>
</tbody>
</table>

*Studies with the status "not yet recruiting, recruiting", "enrolling by invitation", "active, not recruiting" and "completed" were included in this table.*
### TABLE 9

Modulators of 2-AG metabolism

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Phase</th>
<th>Structure</th>
<th>Reference</th>
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<tr>
<td>LEI-105</td>
<td>DAGL</td>
<td>Preclinical</td>
<td><img src="image" alt="Structure LEI-105" /></td>
<td>Baggelaar et al., 2015</td>
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<tr>
<td>DO34</td>
<td>DAGL</td>
<td>Preclinical</td>
<td><img src="image" alt="Structure DO34" /></td>
<td>Ogasawara et al., 2016</td>
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<tr>
<td>DH376</td>
<td>DAGL</td>
<td>Preclinical</td>
<td><img src="image" alt="Structure DH376" /></td>
<td>Ogasawara et al., 2016</td>
</tr>
<tr>
<td>DO53</td>
<td>Negative control</td>
<td>Preclinical</td>
<td><img src="image" alt="Structure DO53" /></td>
<td>Ogasawara et al., 2016</td>
</tr>
<tr>
<td>KT109</td>
<td>DAGL</td>
<td>Preclinical</td>
<td><img src="image" alt="Structure KT109" /></td>
<td>Hsu et al., 2012</td>
</tr>
<tr>
<td>JZL184</td>
<td>MAGL</td>
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<td><img src="image" alt="Structure JZL184" /></td>
<td>Long et al., 2009</td>
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<tr>
<td>MJN110</td>
<td>MAGL</td>
<td>Preclinical</td>
<td><img src="image" alt="Structure MJN110" /></td>
<td>Niphakis et al., 2012</td>
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<td>ABX-1431</td>
<td>MAGL</td>
<td>Phase 2</td>
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<td>Cisar et al., 2018</td>
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<tr>
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<td>(Lu-AG06466)</td>
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</tr>
<tr>
<td>Identifier</td>
<td>Status</td>
<td>Condition</td>
<td>Title</td>
<td></td>
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<td>--------------</td>
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<tr>
<td>NCT04597450</td>
<td>Ongoing</td>
<td>PTSD</td>
<td>Lu-AG06466 in Participants With Post Traumatic Stress Disorder</td>
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<tr>
<td>NCT04990219</td>
<td>Ongoing</td>
<td>Multiple Sclerosis</td>
<td>A Study of Lu-AG06466 for the Treatment of Spasticity in Participants With Multiple Sclerosis</td>
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<tr>
<td>NCT05028673</td>
<td>Completed</td>
<td>Healthy</td>
<td>A Study to Evaluate a New Tablet Formulation of Lu-AG06466 in Healthy Participants</td>
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<td>FABP5 Ki value (µM)</td>
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<td>-----------------------------</td>
<td>---------------------</td>
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<td></td>
<td>( K_m )</td>
<td>( V_{max} )</td>
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<td></td>
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<td></td>
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### Farinosone-C derivative (BSL-34)

**Burch et al., 2014**

<table>
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<th>EC50</th>
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<td>1.0 µM</td>
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<td>Rat cortical astrocytes</td>
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<td>Astrocytoma</td>
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<tr>
<td></td>
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<td>10.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>RBL-2H3</td>
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<td>4</td>
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</tr>
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<td>6</td>
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<td></td>
<td>RBL-2H3</td>
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<td></td>
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**Beltramo et al., 1997; Maccarrone et al., 1999; Piomelli et al., 1999; De Petrocellis et al., 2000; Rakhashan et al., 2000; Jarrahian et al., 2000; Deutsch et al., 2001; Porter et al., 2002; Glaser et al., 2003; López-Rodrı́guez et al., 2003; Fowler et al., 2004; Vandevooerde and Fowler, 2005; Dickason-Chesterfield et al., 2006; Hillard et al., 2007; Nicolussi et al., 2014b**

- **Km = 1.2 µM, Vmax = 14.7 ± 0.6 pmol/min/mg in neurons**
- **Km = 0.32 ± 0.1 µM, Vmax = 171 pmol/min/mg in astrocytes**
- **Km = 0.6 ± 0.1 µM, Vmax = 14.7 ± 1.5 pmol/min/mg in astrocytoma**
- **Km = 0.7 ± 0.1 µM, Vmax = 0.39 fmol/min/cell in C9 glioma cells**
- **Km = 11.4 ± 2.3 µM, Vmax = 17.5 ± 1.5 pmol/min/mg in RBL-2H3 cells**
- **Km = 0.13 ± 0.01 µM, Vmax = 140 ± 15 pmol/min/mg in U937 cells**
- **Cerebellar**
  - **Km = 1.2 µM, Vmax = 14.7 ± 0.6 pmol/min/mg in granular C9 glioma cells**
  - **Km = 11.4 ± 2.3 µM, Vmax = 17.5 ± 1.5 pmol/min/mg in RBL-2H3 cells**
  - **Km = 0.13 ± 0.01 µM, Vmax = 140 ± 15 pmol/min/mg in U937 cells**
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<th>IC₅₀</th>
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<td>C6 glioma</td>
<td>1.2</td>
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<td>U937</td>
<td>5.2</td>
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<td></td>
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<td>3.1</td>
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<td></td>
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<td>4.9</td>
</tr>
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<td></td>
<td></td>
<td>granular</td>
<td>&gt;50</td>
</tr>
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<td></td>
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<td></td>
<td>HeLa*</td>
<td>&gt;100</td>
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<td>41</td>
<td>WT cortical</td>
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<td></td>
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<td>25</td>
<td>neurons</td>
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UCM707

López-Rodríguez et al., 2003; Fegley et al., 2004; Fowler et al., 2004; Dickason-Chesterfield et al., 2006, Kaczocha et al., 2006; Hillard et al., 2007, Chicca et al., 2012, Nicolussi et al., 2014b

<table>
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<td>56.4</td>
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<td>Cerebellar granular neurons</td>
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**LY2183240**

Moore et al., 2005; Alexander & Cravatt, 2006; Dickason-Chesterfield et al., 2006; Ortar et al., 2008; Nicolussi, 2014; Nicolussi et al., 2014b

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<th>Vmax</th>
<th>RBL-2H3</th>
<th>U937</th>
<th>HMC-1*</th>
<th>RBL-2H3 cells</th>
<th>HeLa*</th>
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**WOBE492**

Chicca et al., 2017

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**WOBE498**

Chicca et al., 2017

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*Cells lacking FAAH-activity.

ND, not determined.
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<th>FAAH IC₅₀ Value</th>
<th>FAAH Kᵢ Value</th>
<th>FABP5 IC₅₀ Value</th>
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<td>Cell Type</td>
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<td>Value (µM)</td>
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<td>COS7-FAAH-eGFP</td>
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<td>48%</td>
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<td></td>
<td>in h.]</td>
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<td>[30% inh.]</td>
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<td>~20</td>
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<td>[40% inh.]</td>
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<td>Berger et al., 2012; Zhou et al., 2019</td>
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*Cells lacking FAAH-activity.
TABLE 13
Drug candidates that target eCB transport in late preclinical stage

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<th>Name</th>
<th>Company</th>
<th>Indication(s)</th>
<th>Target</th>
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<td>RT26</td>
<td>Artelo Biosciences</td>
<td>Prostate cancer</td>
<td>FABP5 inhibitor</td>
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<td></td>
<td></td>
<td>Chemotherapy-Induced</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peripheral Neuropathy</td>
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</tr>
<tr>
<td>SYT510</td>
<td>Synendos Therapeutics</td>
<td>Neuropsychiatric Disorders</td>
<td>SERI, undisclosed target</td>
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</table>

SERI, selective eCB reuptake inhibitor.
TABLE 14

Lipid signals from the eCBomes, their targets and metabolic enzymes

<table>
<thead>
<tr>
<th>Family</th>
<th>Most Studied</th>
<th>Subfamily (If Applicable)</th>
<th>Most Studied And/Or Most Tissue Abundant Members</th>
<th>Best Established Molecular Target(s)</th>
<th>Best Established Anabolic Enzyme(s)</th>
<th>Best Established Catabolic Enzyme(s)</th>
<th>Main Potential Therapeutic Applications (Based On The Best Established Pharmacological Actions)</th>
<th>References</th>
</tr>
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<tbody>
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<td>NAEs</td>
<td>N-Arachidonoyl-ethanolamine</td>
<td>(AEA and its Congeners)</td>
<td>N-Arachidonoyl-ethanolamine</td>
<td>CB1R (↑), CB2R (↑), TRPV1 (↑), TRPM8 (↓), Cav3.3 (↓)</td>
<td>NAPE-PLD, ABHD4+GDE</td>
<td>NAPE-PLD, ABHD4+GDE</td>
<td>FAAH-1</td>
<td>Chronic and inflammatory pain; obesity, NASH and type 2 diabetes</td>
</tr>
<tr>
<td></td>
<td>N-Docosa-hexaenoyl-ethanolamine</td>
<td></td>
<td>N-Docosa-hexaenoyl-ethanolamine</td>
<td>GPR110 (↑), CB1R (↑)</td>
<td>DAGLα, DAGLβ</td>
<td>DAGLα</td>
<td>FAAH-1</td>
<td>Inflammation; neurodegenerative disorders; cancer.</td>
</tr>
<tr>
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<td>N-Oleoyl-ethanolamine</td>
<td></td>
<td>N-Oleoyl-ethanolamine</td>
<td>PPARα (↑), TRPV1 (↑), GPR119 (↑)</td>
<td>FAAH-1, FAAH-2</td>
<td>FAAH-1, FAAH-2</td>
<td>Obesity, type 2 diabetes, steatosis and related disorders</td>
<td>Bowen et al., 2017</td>
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<tr>
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<td>N-Palmitoyl-ethanolamine</td>
<td></td>
<td>N-Palmitoyl-ethanolamine</td>
<td>PPARα (↑), GPR55 (↑)</td>
<td>FAAH-1, FAAH-2</td>
<td>FAAH-1, FAAH-2</td>
<td>Obesity, type 2 diabetes, steatosis and related disorders</td>
<td>Bow</td>
</tr>
<tr>
<td>MAGs</td>
<td>2-Arachidonoyl-glycerol</td>
<td>(2-AG and its Congeners)</td>
<td>2-Arachidonoyl-glycerol</td>
<td>CB1R (↑), CB2R (↑), TRPV1 (↑), TRPM8 (↓)</td>
<td>DAGLα, DAGLβ</td>
<td>DAGLα, DAGLβ</td>
<td>FAAH-1</td>
<td>Chronic and inflammatory pain; anxiety, depression; (neuro)inflammatory disorders</td>
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</tbody>
</table>

**eCBome**

Obesity and type 2 diabetes
<table>
<thead>
<tr>
<th>Primary Amides</th>
<th>2-Oleoyl- and 2-Linoleoyl-glycerol</th>
<th>GPR119(↑), TRPV1(↑)</th>
<th>Type 2 diabetes</th>
<th>Hansen and Vana, 2019</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1- or 2-Palmitoyl-glycerol</td>
<td>PPARα(↑)</td>
<td>Obesity, type 2 diabetes, steatosis and related disorders</td>
<td>Depommier et al., 2020</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N-Acyl-amino acids (Lipo-aminoacids)</th>
<th>N-Oleoyl-glycine</th>
<th>PPARα(↑), GLYATL3 PAM</th>
<th>Brain trauma and its consequences; neuroprotection; nicotine and opiate addiction; pain</th>
<th>Huang et al., 2001; Foster et al., 2019; Donvito et al., 2019; Piscitelli et al., 2020</th>
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<tr>
<td>N-Acyl-alanines</td>
<td>N-Oleoyl-alanine</td>
<td>PPARα(↑), FAAH-1(↓)</td>
<td>Nicotine and opiate addiction</td>
<td>Ayoub et al., 2020</td>
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<tr>
<td>N-Acyl-serines</td>
<td>N-Oleoyl-serine</td>
<td>Cav3.3(↓)</td>
<td>Osteoporosis</td>
<td>Milman et al., 2006</td>
</tr>
<tr>
<td>N-Acyl-taurines</td>
<td>N-Oleoyl- and N-Arachidonoyl-taurine</td>
<td>TRPV1(↑), TRPV4(↑), Cav3.3(↓)</td>
<td>FAAH-1</td>
<td>Skin wound healing; type 2 diabetes and dyslipidemia</td>
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<td>N-Acyl-dopamines</td>
<td>N-Arachidonoyl-dopamine</td>
<td>TRPV1(↑), CB,R(↑), FAAH(↓), Cav3.3(↓)</td>
<td>COMT, Cyp, Pain; cancer; nausea; neuroinflammatory and neurodegenerative disorders</td>
<td>De Petrocellis and Di Marzo, 2014</td>
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<tr>
<td>N-Acyl-serotonins</td>
<td>N-Arachidonoyl-serotonin</td>
<td>TRPV1(↑), FAAH(↓), Cav3.3(↓)</td>
<td>Cyp</td>
<td>Chronic and inflammatory pain; anxiety; depression; (neuro)inflammatory disorders; epilepsy; IBS</td>
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<td>COX-2 Derivatives</td>
<td>Prostaglandin-glycerol esters</td>
<td>Prostaglandin E2-glycerol ester</td>
<td>COX-2 Derivatives</td>
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<tr>
<td>Prostamides</td>
<td>P2Y6↑</td>
<td>COX-2</td>
<td>MAGL</td>
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<tr>
<td>Prostamide F2α</td>
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<td>+PGES</td>
<td>Hippocampal</td>
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<td>hetromer (↑)</td>
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<td>excitotoxicity</td>
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<tr>
<td>+PGFS</td>
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<tr>
<td>Pain</td>
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<tr>
<td>15-LOX Linoleylethanolamide</td>
<td>13-hydroxy-Octadecaenoyl-ethanolamide</td>
<td>TRPV1↑</td>
<td>15-LOX</td>
<td>?</td>
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<td>15-LOX Derivatives</td>
<td></td>
<td></td>
<td>Simard et al., 2022</td>
<td></td>
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<tr>
<td>Cyp Epoxysaturated ethanolamides</td>
<td>5,6-Epoxy-eicosatrienoyl-ethanolamide</td>
<td>TRPV4↑</td>
<td>Cyp</td>
<td>Cardiovascular disorders;</td>
</tr>
<tr>
<td>Cyp Derivatives</td>
<td></td>
<td></td>
<td>Snider et al., 2009</td>
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</table>
| Microbiota-derived N-Acyl-amides | Glycine and Alanine | Commendamide, N-Myristoyl-alanine | Bacterial N-Acyl-
|                        |                            | GPR132↑ |transferase | Cohen et al., 2017 |
|                        |                            |                                 | (choA/glsB) |
|                        |                            |                                 |                   |
| Serinol Derivatives | N-Oleoyl-serinol | GPR119↑ | Bacterial N-Acyl-
|                        |                            |                                 |transferase |
|                        |                            |                                 | (choA/glsB) + |
|                        |                            |                                 | and O-Acyl-
|                        |                            |                                 |transferases |
|                        |                            |                                 | (choB/glsA) |
| N-Acyl-tyramines, N-Acyl-tryptamines, N-Acyl-aminoacids | N-Lauroyl-tryptamine | GPR183↑ | Bacterial N-Acyl-
|                        |                            |                                 |transferase |
|                        |                            |                                 | (choA/glsB) |
|                        |                            |                                 | and O-Acyl-
|                        |                            |                                 |transferases |
|                        |                            |                                 | (choB/glsA) |
|                        |                            |                                 |                   |
|                        | Diacylated Glycine Lipids | Bacterial N-Acyl-
|                        | ?                            |transferase |
|                        | ?                            | (choA/glsB) + |
|                        | ?                            | and O-Acyl-
|                        | ?                            |transferases |
|                        | ?                            | (choB/glsA) |
|                        | Intestinal fitness; eubiosis | Bacterial N-Acyl-
|                        | ?                            |transferase |
|                        | ?                            | (choA/glsB) + |
|                        | ?                            | and O-Acyl-
|                        | ?                            |transferases |
|                        | ?                            | (choB/glsA) |
|                        | Type 2 diabetes | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Immune disorders | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Eubiosis | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Hypertension; inflammation | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Neuroinflammation | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Intestinal fitness; eubiosis | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Type 2 diabetes | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Immune disorders | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Eubiosis | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Intestinal fitness; eubiosis | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Type 2 diabetes | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Immune disorders | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Eubiosis | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Intestinal fitness; eubiosis | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Type 2 diabetes | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Immune disorders | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
|                        | Eubiosis | Bacterial N-Acyl-
|                        | ? |transferase |
|                        | ? | (choA/glsB) + |
|                        | ? | and O-Acyl-
|                        | ? |transferases |
|                        | ? | (choB/glsA) |
The main potential therapeutic applications of the manipulation of their levels and activity is also shown. Upward and downward arrows denote agonism/activation or antagonism/inhibition. Endocannabinoid-like mediators derived from gut microbiota are also included. Notably, several receptors other than CB1R and CB2R have been found to be also modulated by non-euphoric plant cannabinoids such as cannabidiol, i.e. GPR55, GPR118, GPR132, TRPV1, TRPV2, TRPM8, PPARs and Cav3.3. Potential therapeutic applications obtained from counteracting the action or the formation of the mediators are shown in italics. Abbreviations: ABHD, α/β-hydrolase domain; Alt4-FP, splicing variant 4 of the FP receptor; Cav3.3, T-type Ca\(^{2+}\) channels; COMT, catechol-O-methyl transferase; Covid-19, coronavirus disease type 19; COX-2, cyclooxygenase-2; Cyp, cytochrome p450; DAGL, diacylglycerol lipase; eCBome, endocannabinoidome; FAAH, fatty acid amide hydrolase; FP, prostaglandin F receptor; GDE1, glycerodiesterase type 1; GLYATL3, glycine-\(N\)-Acyltransferase Like 3; GPR, orphan G-protein couple receptor; IBS, irritable bowel syndrome; MAGL, monoacylglycerol lipase; μbeCBome, microbendocannabinoidome; NAPE-PLD, \(N\)-acyl-phosphatidylethanolamines-specific phospholipase D-like enzyme; oxyeCBome, oxyendocannabinoidome; PAM, peptidyl-glycine alpha-amidating monooxygenase; PGES, prostaglandin E synthase; PGFS, prostaglandin F synthase; PPAR, peroxisome proliferator-activated receptor; TRPM8, transient receptor potential melastatin type-8; TRPV1, transient receptor potential vanilloid type-1; TRPV2, transient receptor potential vanilloid type-2.
<table>
<thead>
<tr>
<th>Name</th>
<th>Company (and Commercial Name) when Available</th>
<th>Indication(s)</th>
<th>Main Proposed Target</th>
<th>Clinical Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N-Palmitoylethanolamine (PEA)</strong></td>
<td>Epitech Italy (Normast®, Pelvilen®, Glialia®)</td>
<td>Neuropathic pain from spinal cord injury</td>
<td>PPARα GPR55$ TRPV1# CB2R#</td>
<td>NCT01851499</td>
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<td>Fibromyalgia</td>
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<td>Chronic pelvic pain in endometriosis</td>
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<td>NCT02372903</td>
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<td>Frontotemporal Dementia</td>
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<td>Tourette's syndrome (in combination with dronabinol)</td>
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<td>NCT03066193</td>
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<td><strong>N-Oleylethanolamine (OEA)</strong></td>
<td>University of California Davis, California, United States</td>
<td>Post-prandial inflammation</td>
<td>PPARα TRPV1 GPR119</td>
<td>NCT05017428</td>
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<td>NutriForward LLC (RiduZone, 90% OEA)</td>
<td>Overweight and obesity</td>
<td>NCT04614233</td>
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</table>

<table>
<thead>
<tr>
<th>Bimatoprost (Prostamide F&lt;sub&gt;2α&lt;/sub&gt; analog)</th>
<th>Allergan (Lumigan®)</th>
<th>Intraocular Pressure, Glaucoma, Ocular Hypertension</th>
<th>FP/Alt4-FP heteromer</th>
<th>Several completed studies</th>
</tr>
</thead>
</table>

Eyelash hypotrichosis, alopecia

Reported studies are mostly completed (only in a few cases recruitment is still ongoing), and results of most of them have not been disclosed yet. An exception is the case of bimatoprost, which has proven to be very effective on Eyelash hypotrichosis and promising on various forms of alopecia (Jha et al., 2018). N-Palmitoylethanolamine is often administered as the ultramicronised solid, and/or in combination with other molecules, such as transpolydatin (in Pelvilen®, for pelvic pain) or luteolin (Glialia®, for some CNS disorders) (Petrosino and Di Marzo, 2017). Abbreviations: see Table 14.

Legend: $, still controversial; #, these targets are activated indirectly via elevation of endogenous ligand levels or activity.
**Figure Legends**

**Fig. 1.** Endocannabinoid binding receptors. The two major endocannabinoids anandamide and 2-arachidonoylglycerol bind to and activate metabotropic and ionotropic membrane receptors (with either an intracellular or an extracellular binding site), and nuclear receptors.

**Fig. 2.** Endocannabinoid signaling pathways. Receptor binding by anandamide and 2-arachidonoylglycerol triggers various signal transduction pathways, that activate G proteins, ion channels as well as gene transcription.

**Fig. 3.** Biosynthetic pathways of anandamide. AEA can be synthesized from membrane phospholipid precursors via different routes. The Ca\(^{2+}\)-dependent hydrolysis of NAPE by NAPE-PLD is considered the most relevant among these biosynthetic pathways.

**Fig. 4.** Catabolic pathways of anandamide. AEA can be cleaved to arachidonic acid and ethanolamine by different hydrolytic routes. FAAH-1 is considered the most relevant among these catabolic pathways. Alternatively to hydrolytic routes, AEA can be oxidized by LOXs, COX-2 or cytochrome P450 to generate various eicosanoid-like PG-ethanolamides or hydroxy-AEAs.

**Fig. 5.** Biosynthetic pathways of 2-arachidonoylglycerol. 2-AG can be synthesized from membrane phospholipid precursors via different routes. The Ca\(^{2+}\)- and glutathione-dependent hydrolysis of DAG by DAGLα/β is considered the most relevant among these biosynthetic pathways.

**Fig. 6.** Catabolic pathways of 2-arachidonoylglycerol. 2-AG can be cleaved into arachidonic acid and glycerol by different hydrolytic routes. MAGL is considered the most relevant among these catabolic pathways. Alternatively to hydrolytic routes, 2-AG can be oxidized by LOXs or COX-2 to generate various eicosanoid-like PG-glycerol esters or hydroxy-2-AGs.

**Fig. 7.** Transport of endocannabinoids. A) Anandamide and 2-arachidonoylglycerol can cross the plasma membrane via different mechanisms, that include passive diffusion, exocytosis of microvesicles and a putative membrane transporter. B) Intracellular trafficking of anandamide and 2-arachidonoylglycerol is driven by various carriers that include structurally unrelated proteins like albumin, RBP2, HSP70, FABPs, SCP2 and FLAT.

**Fig. 8.** Chemical structure, CB\(_2\)R binding affinity and selectivity of relevant non-classical cannabinoids.

\(^a\)Consensus human CB\(_2\)R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). \(^b\)CB\(_2\)R selectivity (10\(^a\)(pKi CB\(_2\)R-pKi CB\(_1\)R)).
Fig. 9. Chemical structure, CB2R binding affinity and selectivity of representative aminoalkylindole CB2R ligands. aConsensus human CB2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). bCB2R selectivity (10^(-pKi CB2R-pKi CB1R)).

Fig. 10. A) X-ray structure of CB1R (blue) bound to the antagonist AM6538. B) Cryo-EM structure of CB1R (green) in complex with G proteins (α subunit in yellow, β subunit in blue, γ subunit in purple), and the classical cannabinoid agonist AM841. C) Chemical structures of AM6538 and AM841.

Fig. 11. Comparison of ligand binding modes in CB1R and CB2R. (A) The binding pocket of AM10257 in CB2R crystal structure (PDB code 5ZTY). AM10257 and the key residues are shown in sticks as the following color code: CB2R, brown; AM10257, light coral. (B-D) Binding pose comparison of AM6538 in CB2R (PDB code 5TGZ), and AM10257 in CB2R, using color code as follows: CB1R, slate blue; AM6538, dodger blue; CB2R, brown; AM10257, light coral. (E-F) The binding pocket of AM12033 in CB2R (PDB code 6KPF) and WIN55,212-2 in CB2R (PDB code 6TP0). Ligands and the key residues are shown in sticks as the following color code: AM12033, brown; CB2R (6KPF), dark green; WIN55,212-2, royal blue; CB2R (6TP0), dark salmon. (G) The conformational comparison of “toggle switch” residues Trp2586.48 between AM12033- and WIN55,212-2-bound CB2R. (H-J) Binding pose comparison of THC-like agonist in CB1R (PDB code 6KPG) and CB2R (PDB code 6KPF). THC-like agonists are shown as sticks (H) and surface (I-J), the key residues are shown in sticks as the following color code: CB2R, dark green; AM12033, brown; CB1R, maroon; AM841, dark khaki. (K-M) Binding pose comparison of agonist FUB in CB1R (PDB code 6N4B) and agonist WIN55,212-2 in CB2R (PDB code 6TP0). FUB and WIN55,212-2 are shown as sticks (K) and surface (L-M), the key residues are shown in sticks as the following color code: CB2R, dark salmon; WIN55,212-2, royal blue; CB1R, dark cyan; FUB, orange.

Fig. 12. Conformational changes during CB2R activation. (A-C) The conformational change of key residues between inactive- and active-CB2R. “Toggle switch residue” (A), D3.49R3.50Y3.51 motif (B) and N7.49P7.50xxY7.53 motif (C). (D-F) The overall structure (D), the extracellular region (E) and intracellular region (F) comparison of inactive- (brown) and active-state (dark green) CB2R structures.

Fig. 13. Structures of the clinically tested cannabinoid agonists (A) and the selective CB1R antagonists (B).

Fig. 14. Structures of the CB2R in different states. (A) Crystal structure of antagonist AM10257-bound CB2R (PDB code 5ZTY). (B) Crystal structure of agonist AM12033-bound CB2R (PDB code 6KPC). (C) Cryo-EM structure of AM12033-bound CB2R-Gi complex (PDB code 6KPF). (D) Cryo-EM structure of WIN55,212-2-bound CB2R-Gi complex (PDB code 6TP0), using color code as follows: CB2R-AM10257, brown; CB2R-
AM12033 (PDB code 6KPC), sky blue; CB2R-AM12033 (PDB code 6KPF), green; CB2R-WIN55,212-2, dark salmon; Gαi in CB2R-AM12033, purple; Gβ in CB2R-AM12033, teal; Gγ in CB2R-AM12033, orchid; scFv16 in CB2R-AM12033, cornflower blue; Gαi in CB2R-WIN55,212-2, medium purple; Gβ in CB2R-WIN55,212-2, turquoise; Gγ in CB2R-WIN55,212-2, plum; scFv16 in CB2R-WIN55,212-2, light blue.

**Fig. 15.** Chemical structure and CB2R binding affinity of THC, N-arachidonoylethanolamine and 2-arachidonoyl glycerol. aConsensus human CB2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). bCB2R selectivity (10^(pKi CB2R-pKi CB1R)).

**Fig. 16.** Chemical structure and CB2R binding affinity of noladin ether and synthetic eCB analogs. aBinding to spleen cannabinoid receptor. bWith phenylmethanesulfonyl fluoride.

**Fig. 17.** Chemical structure, CB2R binding affinity and selectivity of representative classical cannabinoids. aConsensus human CB2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). bCB2R selectivity (10^(pKi CB2R-pKi CB1R)).

**Fig. 18.** Chemical structure, CB2R binding affinity or functional activity and selectivity of clinically evaluated bicyclic (het)aryl derived CB2R ligands.

**Fig. 19.** Chemical structure, CB2R binding affinity or functional activity and selectivity of clinically evaluated bicyclic aliphatic (het)aryl arrays.

**Fig. 20.** Chemical structure, CB2R binding affinity or functional activity and selectivity of clinically evaluated CB2R agonists and CB2R inverse agonists SR144528 containing five and six-membered central cores. aConsensus human CB2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). bCB2R selectivity (10^(pKi CB2R-pKi CB1R)).

**Fig. 21.** Chemical structure of validated CB2R allosteric modulators.

**Fig. 22.** Chemical structure, CB2R binding affinity and selectivity of CB2R radioligands, PET tracers, fluorescent and pAfBPP probes.

**Fig. 23:** Chemical structures of representative inhibitors of FAAH.

**Fig. 24:** Chemical structures of representative inhibitors of NAAA.

**Fig. 25.** A) Structured part of the AlphaFold model for human DAGLα, residues 1-681; red: transmembrane domain, blue: catalytic domain, green: regulatory loop. B) Unstructured tail region from the AlphaFold model,
residues 682-1042 highlighting potential phosphorylation sites, as discussed in the text, and Homer binding domain. C) Schematic representation with highlighted regions and relevant serines shown.

**Fig. 26.** Model of eCB membrane transport and trafficking showing druggable targets. Molecular pharmacology and possible implications for therapeutic intervention of using diverse eCB transport inhibitors are shown. See text for details and abbreviations.

**Fig. 27.** The eCBome receptors as a pharmacological substrate for plant-derived cannabinoids and host or commensal bacteria-derived eCBs and eCB-like molecules. The elements of the ECS as part of the eCBome are shown squared in red. Abbreviations: CBD, cannabidiol; THC, Δ⁹-tetrahydrocannabinol; THCV, Δ⁹-tetrahydrocannabidivarin. The chemical structures of commendamide, N-miristoyl-alanine and N-oleoyl-serinol are shown from the top right and down.

**Fig. 28.** Reciprocal modulation of the ECS and the gut microbiome. The emerging microbendocannabinoidome (μbeCBome), also summarized in Table 14, enlarges the span of microbe-host communication and expands it to the eCBome.
CB1R, TRPV1,2,3,4, GPR55, GPR119

Nucleus, TRPA1, TRPM8

Extracellular binding site, Intracellular binding site

Transcription factors, Mitochondrion

2-Arachidonoylglycerol, N-Arachidonoyl ethanolamine (anandamide)

CB1/R: Type 1/2 cannabinoid receptor

GPR: G protein coupled receptor

TRPV: Transient receptor potential vanilloid

TRPA: Transient receptor potential cation channel A

TRPM: Transient receptor potential melastatin

PPAR: Peroxisome proliferator-activated receptor

Figure 1
Figure 2
Figure 3

Phosphatidylethanolamine → NH₃⁺ → Phosphatidylcholine

NArPE → NAPE → sPLA2 → ABHD4 → Lyso-NAPE

Glycero-p-AEA → ABHD0 → Glycero-p-AEA

GDE1,4,7 → Lysophospholipase D isoforms 1 and 3

NAPE-PLD → NAPE-PD

PTPN22 → SHIP1

AEA: N-Arachidonoyl ethanolamine
ABHD4: α/β-Hydrolase domain protein 4
GDE1,4,7: Glycerophosphodiesterase isoforms 1, 4 and 7
GDPD1,3: Lysophospholipase D isoforms 1 and 3
NAPE-PLD: N-acyl phosphatidylethanolamines-specific phospholipase
NArPE: N-arachidonoyl phosphatidylethanolamine
(i)NAT: (Calcium independent) N-acyltransferase
p-AEA: Phospho-AEA
sPLA2: Soluble phospholipase A₂
PLC: Phospholipase C
PLD: Phospholipase D
PTPN22: Protein tyrosine phosphatase non-receptor type 22
SHIP1: SH2 domain-containing polyinositol-5-phosphatase 1
Figure 4

AA: Arachidonic acid
AEA: N-Arachidonoyl ethanolamine
CYP450: Cytochrome P450
EET-EA: Epoxyeicosatrienoyl ethanolamide
EtNH2: Ethanolamine
FAAH-1/2: Fatty acid amide hydrolase 1/2
HETE-EA: Hydroxyeicosatetraenoyl ethanolamide
LOX: Lipoxigenase
NAAA: N-Acylethanolamine acid amide hydrolase
PGH2-EA: Prostaglandin H2 ethanolamide
Figure 5

2-AG-3P: 2-Arachidonoylglycerol-3-phosphate
2-AG: 2-Arachidonoylglycerol
DAG: Diacylglycerol
DAGL: Diacylglycerol lipase
PH: Phosphohydrolase
PLA₁: Phospholipase A₁
PLC: Phospholipase C
Figure 6

2-AG: 2-Arachidonoylglycerol
AA: Arachidonic acid
ABHD: o/b-Hydrolase domain protein
CES: Carboxylesterase
COX: Cyclooxygenase
HETE-G: Hydroxy-eicosatetraenoyleglycerol
LOX: Lipoxigenase
MAGL: Monoacylglycerol lipase
PGE₂-G: Prostaglandin E₂ glyceryl ester
PPT1: Palmitoyl-protein thioesterase 1
A) Transmembrane transport of endocannabinoids

B) Intracellular trafficking of endocannabinoids

Main localization of:

- Albumin: Liver, Serum
- FABP1: Liver
- FLAT: Liver, intestine, pancreas
- FABP2: Intestine
- HSP70: Ubiquitous
- SCP2: Leydig cells
- RBP2: Small intestine
- 2-Arachidonoylglycerol
- N-Arachidonoyl ethanolamine (anandamide)
CB2R \( pK_i = 170 \text{ nM} \)  
CB2R \( K_i = 2'860 \text{ nM} \)  
Ratio CB1R/CB2R >235

CB2R \( pK_i = 7.44 \text{ a} \)  
CB2R selectivity >166 b

CB2R \( pK_i = 8.44 \text{ a} \)  
CB2R selectivity = 0.2 b

CB2R \( K_i = 9 \text{ nM} \)  
Ratio CB1R/CB2R = 37

CB2R \( K_i = 7.22 \text{ a} \)  
CB2R selectivity >278 b

CB2R \( K_i = 2'860 \text{ nM} \)  
Ratio CB1R/CB2R >235

CB2R \( K_i = 170 \text{ nM} \)  
Ratio CB1R/CB2R = 1.5

CB2R selectivity >166 b

CB2R \( K_i = 9 \text{ nM} \)  
Ratio CB1R/CB2R >235

CB2R \( K_i = 170 \text{ nM} \)  
Ratio CB1R/CB2R = 1.5

CB2R selectivity >166 b

Figure 8
CB2R

Ki = 4.4 nM

WIN55212-2

CB2R

pKi = 8.57

CB2R selectivity 1

ratio CB1R/CB2R = 192

(A-7856260)

CB2 R Ki = 4.4 nM
ratio CB1 R/CB2 R = 192

(CB2 R pKi = 8.57)

CB2_R selectivity 1

Figure 9
Figure 11
Figure 12

CB₂R-AM10257 (inactive state)  CB₂R-AM12033 (active state)
$\Delta^2$-THC

$\text{CB}_2 \text{R } pK_i = 8.16 \text{ a}$

$\text{CB}_2 \text{R selectivity} = 0.5 \text{ b}$

$\text{CB}_2 \text{R selectivity} = 1 \text{ b}$

$\text{N-Arachidonylethanolamine}$

$\text{CB}_2 \text{R } pK_i = 6.91 \text{ a}$

$\text{CB}_2 \text{R selectivity} = 1 \text{ b}$

$\text{2-Arachidonoyl glycerol}$

$\text{CB}_2 \text{R } pK_i = 6.94 \text{ a}$

$\text{CB}_2 \text{R selectivity} = 1 \text{ b}$

Figure 15
CB₂R \textit{Ki} = 715 \text{nM}^{a,b}

ratio \text{CB}_1R/\text{CB}_2R = 0.003

CB₂R \textit{Ki} = 150 \text{nM}

ratio \text{CB}_1R/\text{CB}_2R = 0.1

Noladin ether
\text{CB}_2R \textit{Ki} = 3000 \text{nM}

ACPA
\text{CB}_2R \textit{Ki} = 715 \text{nM}^{a,b}

ratio \text{CB}_1R/\text{CB}_2R = 0.003

CMX-020
\text{CB}_2R \textit{Ki} = 150 \text{nM}

ratio \text{CB}_1R/\text{CB}_2R = 0.1

Figure 16
Lenabasum, Ajulemic acid
CB₂R \( K_i = 51 \text{ nM} \)
ratio CB₁R/CB₂R = 12

Nabilone
CB₁R \( K_i = 1.84 \text{ nM} \)
ratio CB₁R/CB₂R = 1.2

JWH133
CB₂R \( pK_i = \leq 5^a \)
CB₂R selectivity > 153 \(^b\)

Figure 17
CB2R EC50 = 1 nM
LY-2828360 CB2R EC50 = 20 nM
ART-27.13 CB1R Kᵢ = 0.9 nM
ratio CB1R/CB2R = 13
ratio CB1R/CB2R > 5'000
ratio CB1R/CB2R > 6'940

Figure 18
CB<sub>2</sub>R Ki<sub>CB</sub> = 0.74 nM

TAK-937

Olorinab

CB<sub>2</sub>R Ki<sub>CB</sub> = 12 nM

Tedalinab

CB<sub>2</sub>R Ki<sub>CB</sub> = 422 nM

EC<sub>50</sub> ratio CB<sub>1</sub>R/CB<sub>2</sub>R = 9

EC<sub>50</sub> ratio CB<sub>1</sub>R/CB<sub>2</sub>R = >1613

EC<sub>50</sub> ratio CB<sub>1</sub>R/CB<sub>2</sub>R = >27

EC<sub>50</sub> ratio CB<sub>1</sub>R/CB<sub>2</sub>R = >80

Figure 1
GW-842166X
CB₂R EC₅₀ = 63 nM
ratio CB₁R/CB₂R > 476

KN 387271
CB₂R Ki = 1.9 nM
ratio CB₁R/CB₂R = 3

S-777469
CB₂R Ki = 36 nM
ratio CB₁R/CB₂R = 128

SR144528
CB₂R pKi = 7.88
CB₂R = selectivity 129

Figure 20
Figure 24
Figure 25
Figure 26
Figure 27

Endocannabinoid system

Activated by:
- AEA (CB1 > CB2 = TRPV1 > PPARγ and perhaps GPR55)
- OEA (PPARα = TRPV1, GPR119)
- LEA (GPR119, TRPV1)
- PEA (PPARα, GPR55)
- DHEA (GPR110)

Inactivated by:
- N-acyl-serotonins (TRPV1)
- N-acyl-ethanolamines, primary amides (TRPV2)
- N-acyl-glycines, N-acyl-dopamines, N-acyl-serotonins (Cov3)

2-AG (CB1 = CB2 >> TRPV1)
- 2-OG (GPR119, TRPV1)
- 2-LG (GPR119, TRPV1)
- N-acyl-taurines (TRPV4, TRPV1)
- N-acyl-glycines (PPARα, perhaps GPR18)
- N-acyl-dopamines (TRPV1 > CB1)
- Oleamide (CB1 >> CB2)

THC

CBD

THCV

Activation
Inhibition
Whole mouse gut microbiota
(from a fecal matter transplant to germ free mice)

Commensal bacteria
involved in IBD (ex vivo)
L. gasseri RJX1262
L. gasseri DSM 20243
E. coli RJX1083
B. fragilis

Commensal bacteria
involved in obesity (ex vivo)
Lactobacillaceae,
Erysipelotrichaceae

Commensal bacteria
A. muciniphila

Probiotics
Lactobacillus sp.

Enteric pathogens
(ex vivo)
Enterobacteriaceae

N-acyl-ethanolamines
Mouse gut

N-acyl-ethanolamines
IBD human and mouse gut

N-acyl-ethanolamines
Female mouse brain

N-acyl-ethanolamines
White adipose tissue of HFD obese mice

CB1 R
Mice with HFD obesity

CB2 R
IBS human gut

TRPV1 channels
« Depressed » mouse hippocampus

2-acyl-glycerols
Male mouse brain

2-Palmitoyl-glycerol
Obese human plasma

2-AG
Mouse gut

N-acyl-serotonins
« Depressed » mouse gut

2-acyl-glycerols
Colon of HFD obese mice

Figure 28