1521-0081/75/5/885–958$35.00
PHARMACOLOGICAL REVIEWS

dx.doi.org/10.1124/pharmrev.122.000600
Pharmacol Rev 75:885–958, September 2023

ASSOCIATE EDITOR: CHARLES FRANCE

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

Mauro Maccarrone, Vincenzo Di Marzo, Jürg Gertsch, Uwe Grether, Allyn C. Howlett, Tian Hua, Alexandros Makriyannis, Daniele Piomelli, Natsuo Ueda, and Mario van der Stelt

Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Italy (M.M.); European Center for Brain Research, Santa Lucia Foundation, Rome, Italy (M.M.); Canada Excellence Research Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health, University of Laval, Quebec, Canada (V.D.); Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, Bern, Switzerland (J.G.); Roche Pharma Research & Early Development, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Basel, Switzerland (U.G.); Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina (A.C.H.); iHuman Institute, ShanghaiTech University, Shanghai, China (T.H.); Center for Drug Discovery and Department of Pharmaceutical Sciences, Northeastern University, Boston, Massachusetts (A.M.); Departments of Pharmaceutical Sciences and Biological Chemistry, University of California, Irvine, California (D.P.); Department of Biochemistry, Kagawa University School of Medicine, Miki, Kagawa, Japan (N.U.); Department of Molecular Physiology, Leiden Institute of Chemistry, Leiden University, Leiden, Netherlands (M.S.)

Address correspondence to: Mauro Maccarrone, Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Via Vetoio snc, I-67100 L'Aquila (Italy). E-mail: mauro.maccarrone@univaq.it.

All authors have contributed equally to this study.

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 National Institutes of Health National Institute on Drug Abuse [Grant R01-DA042157]. A.M. was supported by the National Institute on Drug Abuse [Grant P01-DA009158] and by the National Institute on Alcohol Abuse and Alcoholism [Grant U01-AA028963].

No author has an actual or perceived conflict of interest with the contents of this article.

dx.doi.org/10.1124/pharmrev.122.000600.

***Abstract*——The cannabis derivative marijuana is the most widely used recreational drug in the Western world and 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 United States and worldwide. Compelling research evidence and the Food and Drug Administration cannabis-derived cannabidiol approval for severe childhood epilepsy have confirmed the large therapeutic potential of cannabidiol itself, Δ^9-tetrahydrocannabinol 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*-arachidonoyl-ethanolamine); since then, distinct elements of the 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 the ECS as a therapeutic target is presented 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 cannabinoid receptors 1 and 2) and metabolic enzymes (like fatty acid amide hydrolase and monoacylglycerol lipase), will advance our understanding of endocannabinoid signaling and activity at molecular, cellular, and system levels, providing new opportunities to treat patients.**

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) (https://www.unodc.org/wdr2017/field/

ABBREVIATIONS: AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; AEA, *N*-arachidonyl ethanolamine (anandamide); CADD, computer-aided drug discovery; CBD, cannabidiol; CB_1R, cannabinoid receptor 1; CB_2R, 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; 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; 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; PGE_2-G, prostaglandin E_2 glyceryl ester; PGH_2-EA, prostaglandin H_2 ethanolamide; PLC, phospholipase C; PPAR, peroxisome proliferator-activated receptor; SAR, structure-activity relationship; SERI, selective eCB reuptake inhibitor; (*S*)-OOPP, *N*-[(3*S*)-2-oxo-3-oxetanyl]-3-phenylpropanamide; THC, Δ^9-tetrahydrocannabinol; TRP(V), transient receptor potential (vanilloid).

Booklet_1_EXSUM.pdf). Cannabis' increasingly expanding legal status heightens the need for research into its therapeutic potential for a wide range of pathologic 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 nonmedical cannabis use has been associated with short-term and long-term adverse effects, including schizophrenia, alterations 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 Δ^9-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 Δ^8-tetrahydrocannabinol and the propyl derivative Δ^9-tetrahydrocannabivarin), CBD and its analogs (including cannabidivarin), cannabinol and its analogs (including the propyl derivative cannabivarin), and cannabigerol and its analogs are highly abundant. In addition, trace amounts of cannabinodiol, cannabichromene, cannabicyclol, cannabielsoin, and cannabitriol 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.

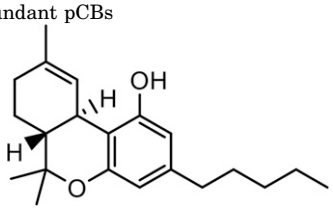
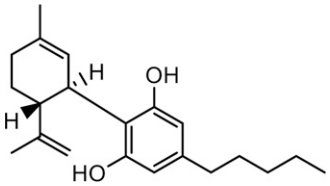
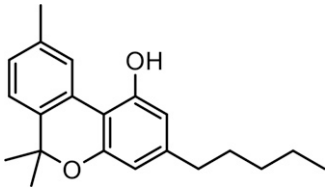
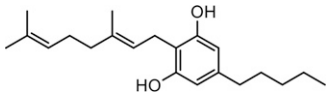
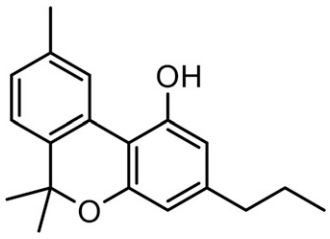
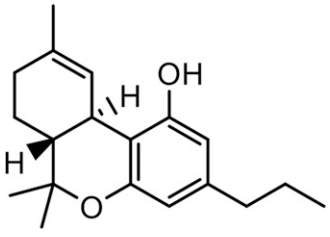
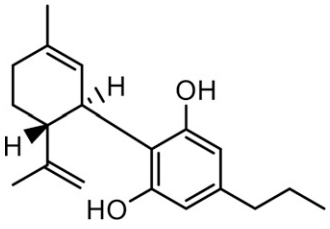
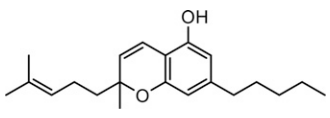
To date, the therapeutic potential of THC and CBD, alone or in combination, seems apparent and has been critically discussed in recent reviews (Maccarrone et al., 2017; Friedman et al., 2019; Pacher et al., 2020; Rock et al., 2021; Stella, 2023). Here, the main applications of THC and CBD for human health are summarized in Table 2.

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, 2023; 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 antihyperalgesic effect (Zhu et al., 2020). Further research has shown that cannabinol 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 anticancer potential (Li et al., 2022). Humans and other mammals do not produce pCBs but can effectively remove them via the cytochrome P450 and glucuronidation pathways in the liver and other organs (Huestis, 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.

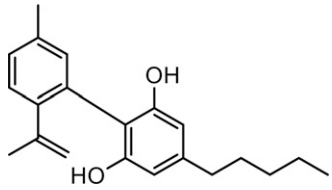
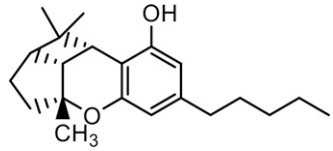
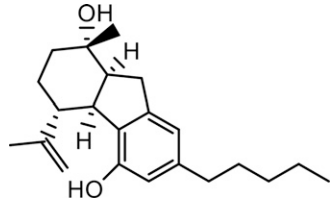
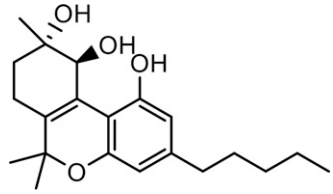
Taken together, the complexity of cannabis extracts seems apparent. However, such a complexity is mirrored, and possibly even exceeded, by that of the ensemble of receptors, enzymes, and transporters of endocannabinoid (eCB) substances that together form the "eCB system" (ECS), recently discussed in comprehensive reviews (Iannotti et al., 2016; Maccarrone, 2017; Baggelaar et al., 2018; Cristino 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 about 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^9-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 [^{3}H]CP55940 to cannabinoid receptors 1 and 2 (CB_1R and CB_2R), stimulation of transient receptor

TABLE 1
Major phytocannabinoids (pCBs)

Name (abbreviation)	Chemical Structure
More Abundant pCBs	
Δ^9-Tetrahydrocannabinol (THC)	
Cannabidiol (CBD)	
Cannabinol (CBN)	
Cannabigerol (CBG)	
Cannabivarin (CBV)	
Cannabidivarin (CBDV)	
Δ^9-Tetrahydrocannabivarin (THCV)	
Less Abundant pCBs	
Cannabichromene (CBC)	

(*continued*)

TABLE 1—*Continued*

Name (abbreviation)	Chemical Structure
Cannabinodiol (CBND)	
Cannabicyclol (CBL)	
Cannabielsoin (CBE)	
Cannabitriol (CBT)	

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 CB_1R and CB_2R (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 1940s (Adams et al., 1948) and its complete structural elucidation 20 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

TABLE 2
Approved and potential indications for THC and CBD

Cannabinoid	Approved (A) and Potential (B) Indications
THC	(A) Chemotherapy-induced nausea and vomiting; appetite stimulant (HIV/AIDS). (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.
CBD	(B) Childhood epilepsy; tuberous sclerosis complex seizure; Lennox-Gastaut syndrome; Dravet syndrome and infantile spasms.
THC/CBD	(A) Spasticity in MS. (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.

AD, Alzheimer's disease; CBD, cannabidiol; HD, Huntington's disease; MS, multiple sclerosis; PD, Parkinson's disease; THC, Δ^9-tetrahydrocannabinol.

et al., 1992a). The pharmacological characterization eventually led to the molecular cloning of the CB_1R from rat (Matsuda et al., 1990) and human (Gerard et al., 1990, 1991) orphan G protein-coupled receptor (GPCR) clones. CB_1R 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 afterward a second molecular target of THC was found and named CB_2R (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 the report of the International Union of Pharmacology Cannabinoid Receptor Nomenclature Committee (Howlett et al., 2002).

The identification of CB_1R, the most abundant GPCR in the mammalian brain, and of CB_2R 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 CB_1R and CB_2R 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.

Other potential members of the eCB family have been discovered, including: (1) ω-6 fatty acid-derived eCBs like AEA, 2-AG, 2-arachidonoylglycerol (noladin) ether, and the "inverted anandamide" virodhamine, reported to have various biologic activities (Maccarrone, 2017; Baggelaar et al., 2018; Cristino et al., 2020), and (2) 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 (Rodríguez de Fonseca et al., 2001; Schwartz et al., 2008; Misto et al., 2019), pain (Calignano et al., 1998; Fotio et al., 2021b), 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 biologic activities and hold therapeutic potential against vasodilation and osteoporosis (Table 3).

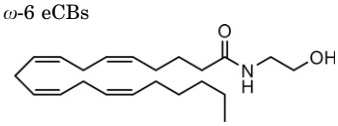
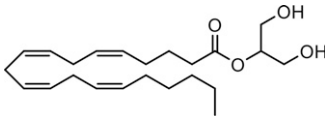
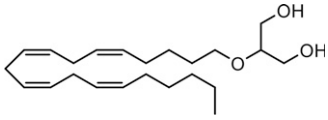
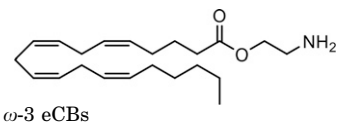
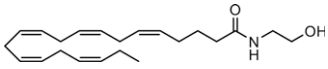
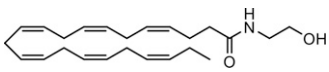
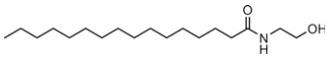
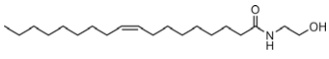
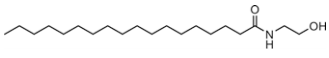
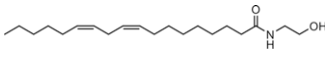
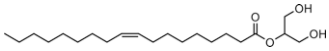
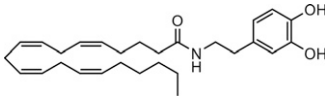
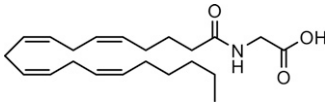
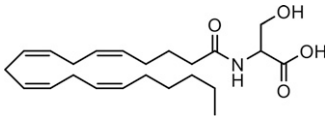
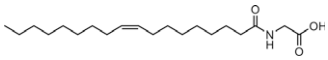
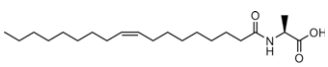
Athough THC and AEA have completely different structures, with THC being a terpene-resorcinol derivative (Table 1) and AEA being an AA amide linkage with ethanolamine (Table 3), their biologic activities were found to be closely related (Fride and Mechoulam, 1993; Vogel et al., 1993). Also of note is 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 tetraymena (Anagnostopoulos et al., 2010) where eCBs can be detected.

C. Diverse Phytocannabinoids and Endocannabinoid 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 (1) seven-transmembrane GPCRs CB_1 and CB_2 (Howlett et al., 2002), as well the recently deorphanized 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.,

TABLE 3
Major endocannabinoids and congeners

Name (abbreviation)	Chemical Structure
Major ω-6 eCBs	
N-Arachidonoylethanolamine (Anandamide, AEA)	
2-Arachidonoylglycerol (2-AG)	
2-Arachidonoylglycerol (Noladin) Ether (2-AGE)	
Virodhamine (*O*-Arachidonoylethanolamine, *O*-AEA)	
Major ω-3 eCBs	
N-Eicosapentaenoylethanolamine (EPEA)	
N-Docosahexaenoylethanolamine (DHEA)	
Major eCB-like Compounds	
N-Palmitoylethanolamine (PEA)	
N-Oleoylethanolamine (OEA)	
N-Stearoylethanolamine (SEA)	
N-Linoleoylethanolamine (LEA)	
2-Oleoylglycerol (2-OG)	
Major eCB-Amino Acids	
N-Arachidonoyl dopamine (NADA)	
N-Arachidonoyl glycine (NAGly)	
N-Arachidonoyl serine (ARA-S)	
N-Oleoyl glycine (OlGly)	
N-Oleoyl alanine (OlAla)	

2020; Im, 2021); (2) 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, which are all six-transmembrane spanning receptors; and (3) nuclear PPARs α, γ, and δ, which are transcription factors able to regulate gene expression (Maccarrone, 2020; Gomez-Cañas et al., 2023). Of note, CB_1R has been shown to move in and out of distinct microdomains of the plasma membrane known as lipid rafts, which might contribute to the control of their G protein-dependent signaling (Oddi et al., 2017; Saumell-Esnaola et al., 2021). In addition, CB_1R appears to localize also in the outer membrane of mitochondria, where it modulates energy metabolism of neuronal and nonneuronal 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 CB_2R (Soethoudt et al., 2017), also in living cells (Sarott et al., 2020), and molecular mechanism of allosteric modulation of CB_1R (Yang et al., 2022) suggest that biased signaling driven by eCBs might be better appreciated in the near 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-1990s, 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 biologic 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, which 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 N-Arachidonyl Ethanolamine. 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 classic route for the release of AEA from phosphatidylethanolamine and phosphatidylcholine precursors. In addition,

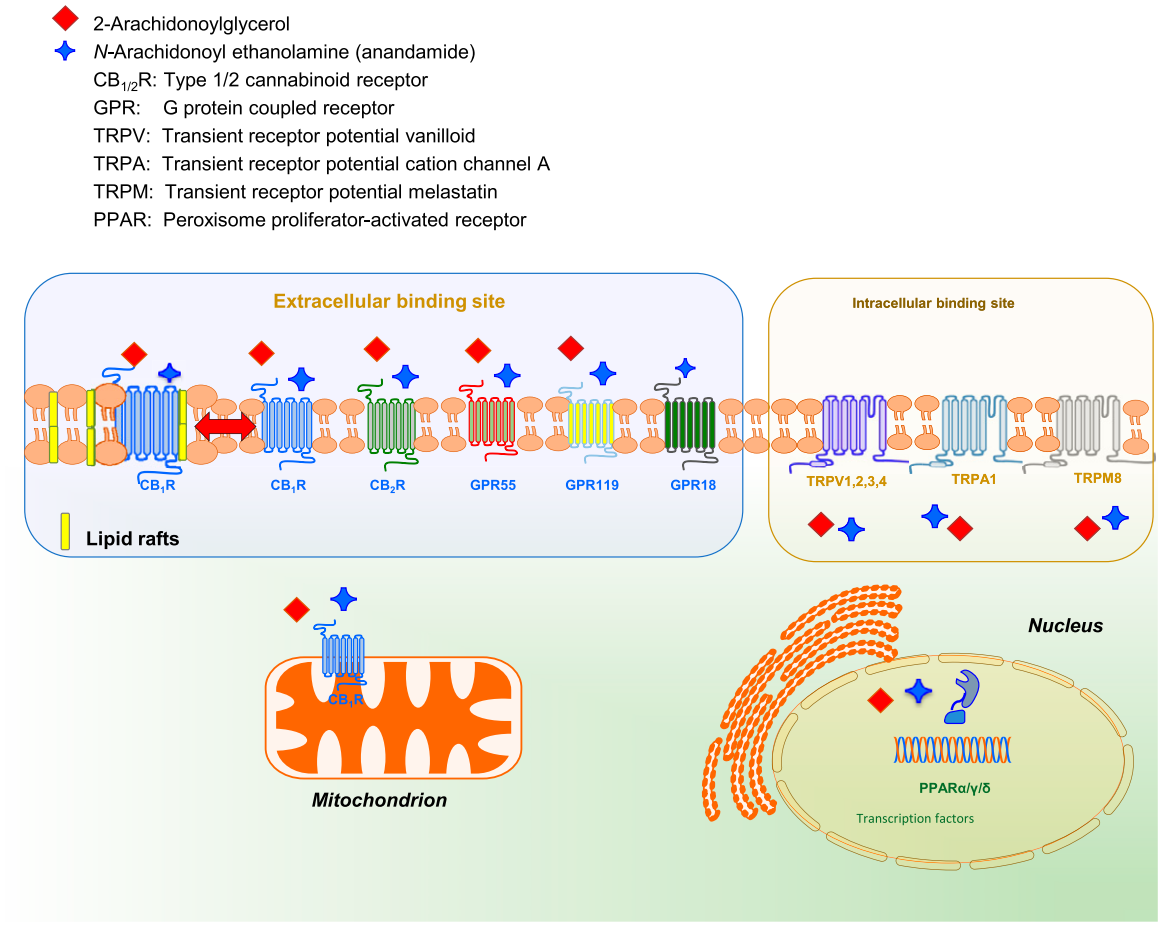


Fig. 1. Endocannabinoid binding receptors. The two major endocannabinoids anandamide and 2-arachidonoylglycerol bind to and activate metabotropic and ionotropic membrane receptors (with either an intracellular or an extracellular binding site) and nuclear receptors.

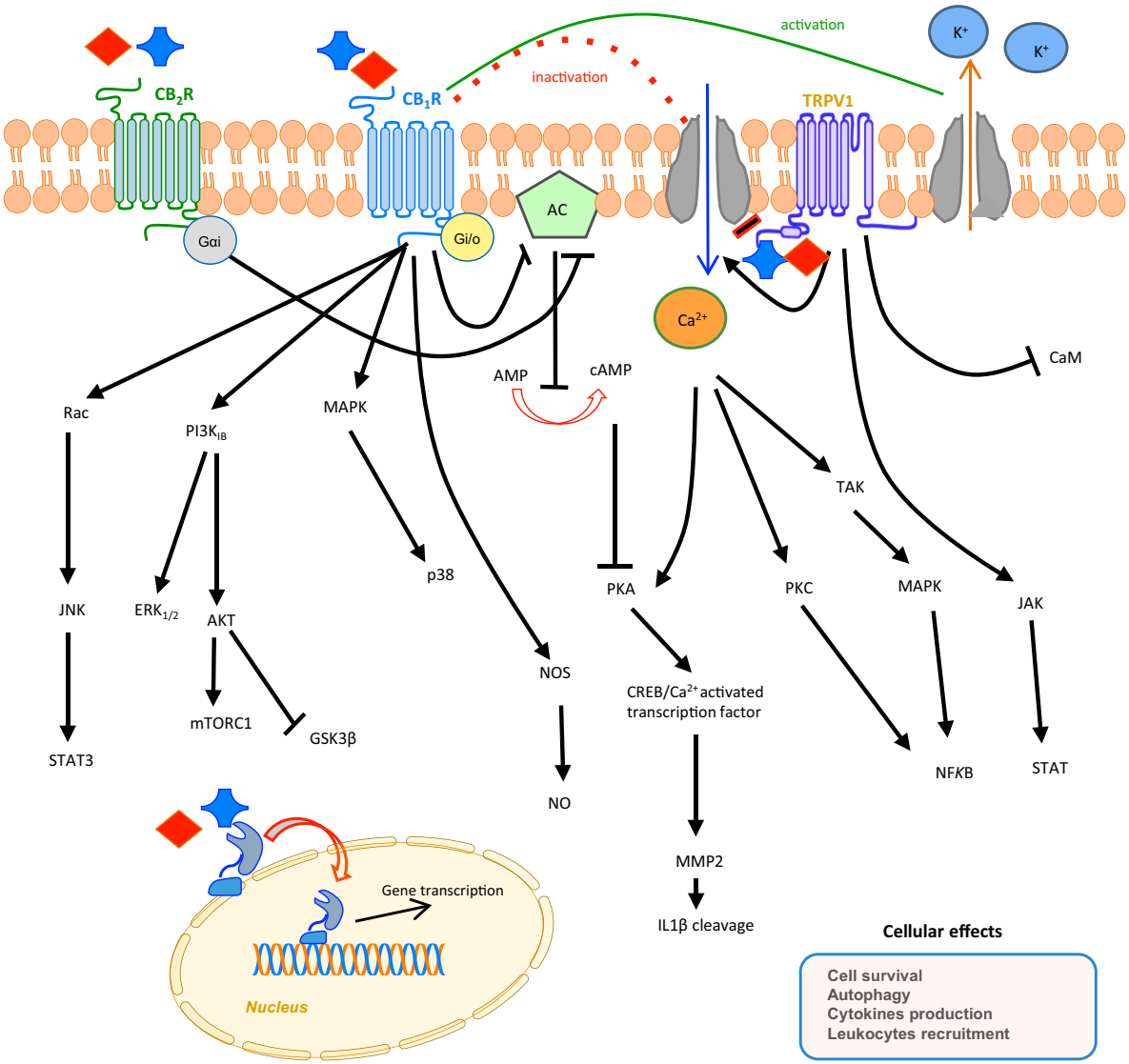
soluble phospholipase A_2, α/β hydrolase domain protein 4, phospholipase C, *lyso*-phospholipase D, protein tyrosine phosphatase non-receptor type 22, SH2 domain-containing polyinositol-5-phosphatase 1, and various glycerophosphodiesterase family members catalyze parallel routes for the biosynthesis of AEA.

Multiple pathways also exist for the degradation of AEA, which can be cleaved into ethanolamine and AA, thus terminating its biologic 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.

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 biologic 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-Arachidonoylglycerol. 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 classic biosynthetic route for 2-AG (Bisogno et al., 2003), and glutathione seems to be a key regulator in the brain (Maccarrone et al., 2008).

Alternative pathways have been discovered for the degradation of 2-AG, which is primarily cleaved to glycerol and AA by 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



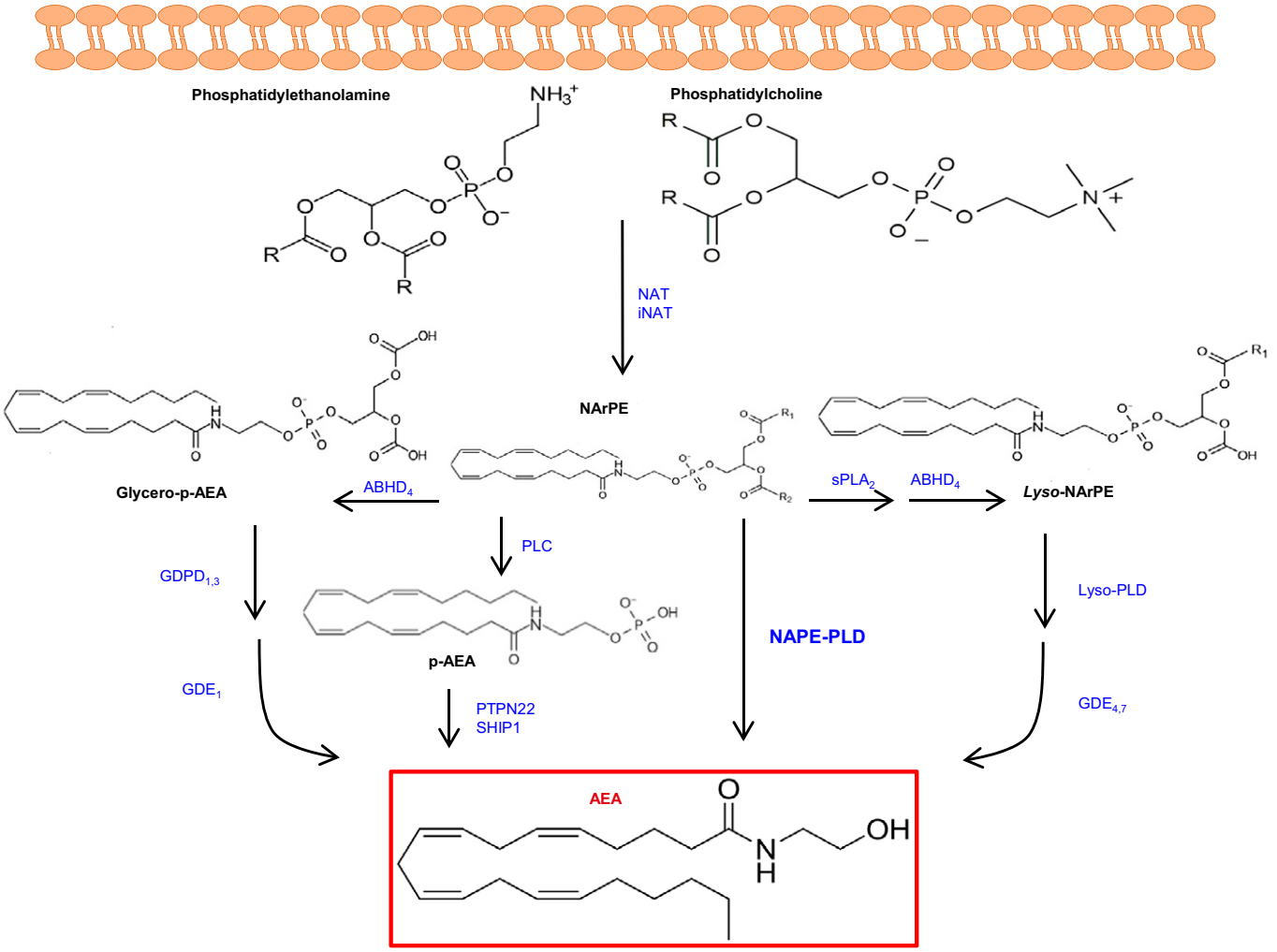
AKT: α-Serine/threonine-protein kinase
AMP: Adenosine monophosphate
cAMP: Cyclic adenosine monophosphate
CREB: cAMP response element-binding protein
ERK: Extracellular signal-regulated kinase
GSK3β: Glycogen synthase kinase-3 β
IL: Interleukin
JNK: c-Jun N-terminal kinase
MAPK : Mitogen-activated protein kinase
MMP: Matrix metallopeptidase
mTOR: Mammalian target of rapamycin
NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NOS: Nitric oxide synthase
PKA: Protein kinase A
STAT: Signal transducer and activator of transcription

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

et al., 2022), leading to oxidative derivatives like prostaglandin- or thromboxane-glyceryl esters with their own biologic activities (Baggelaar et al., 2018; Simard et al., 2022).

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 extracellulary. 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, 2020). 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

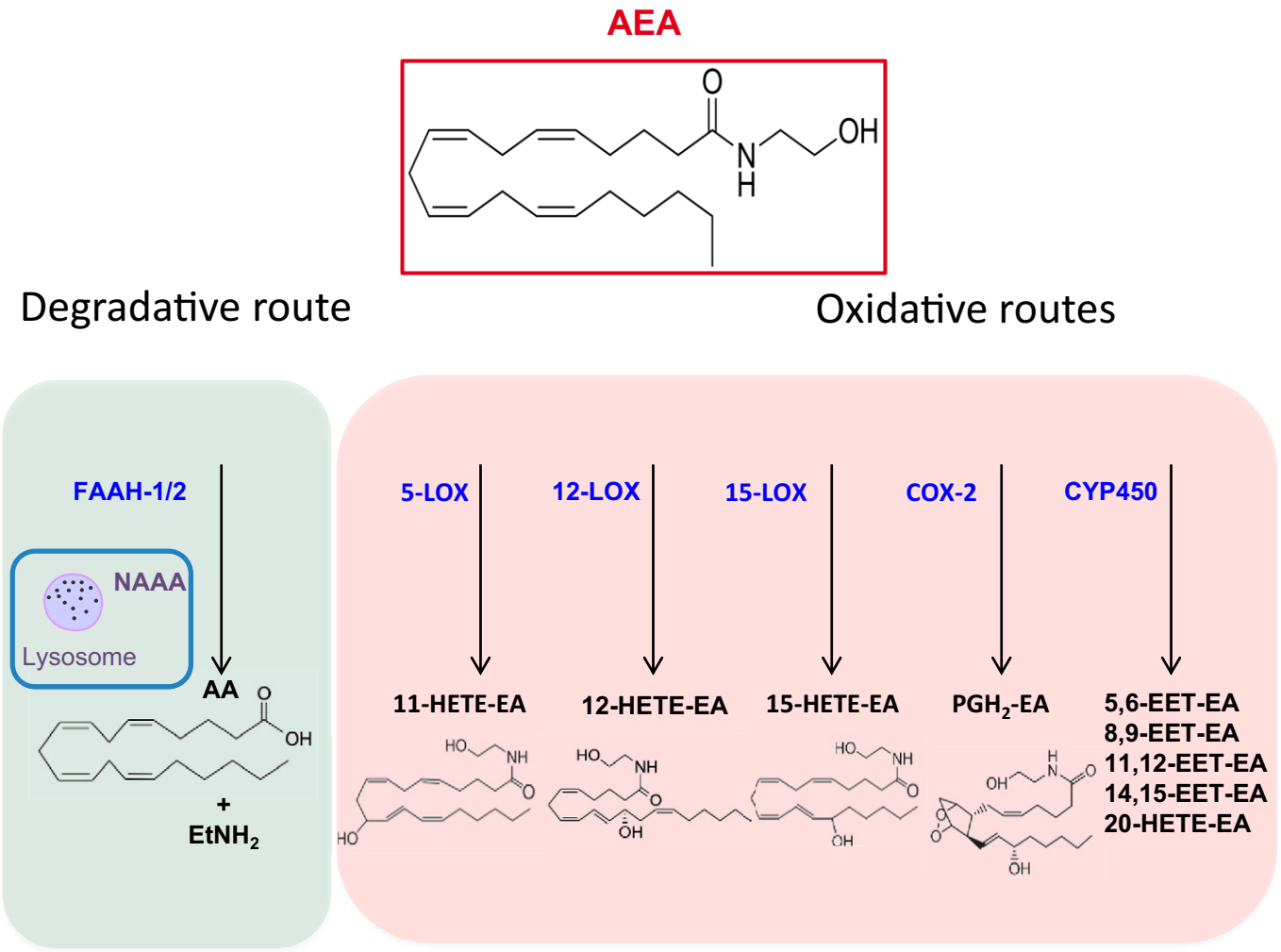


AEA: *N*-Arachidonoyl ethanolamine
ABHD4: α/β-Hydrolase domain protein 4
GDE1,4,7: Glycerophosphodiesterase isoforms 1, 4 and 7
GDPD1,3: Lysophospholipase D isoforms 1 and 3
NAPE-PLD: *N*-acyl phosphatidylethanolames-specific phospholipase D
NArPE: *N*-arachidonoyl phosphatidylethanolamine
(i)NAT: (Calcium independent) N-acyltransferase
p-AEA: Phospho-AEA
$sPLA_2$: Soluble phospholipase A_2
PLC: Phospholipase C
PLD: Phospholipase D
PTPN22: Protein tyrosine phosphatase non-receptor type 22
SHIP1: SH2 domain-containing polyinositol-5-phosphatase 1

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

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.

The eCBs are lipids, and as such they cannot travel the aqueous cytosol without a suitable carrier (Maccarrone

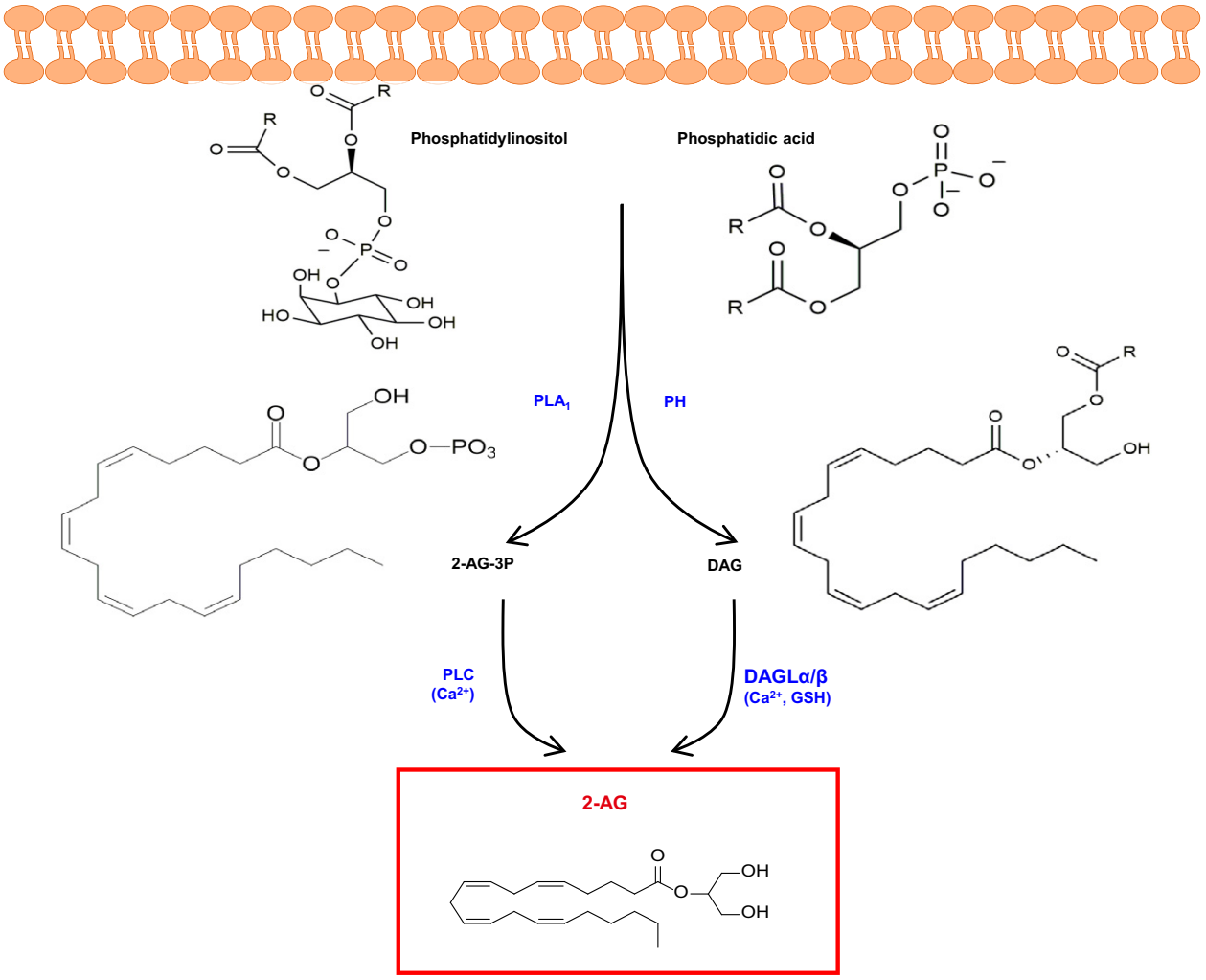


AA: Arachidonic acid; AEA: Arachidonoyl ethanolamide;
CYP450: Cytochrome P450;
EET-EA: Eicosatetraenoyl ethanolamine;
$EtNH_2$: Ethanolamide;
FAAH: Fatty acid amide hydrolase;
HAEA: 12-hydroxy-N-arachidoylethanolamide;
LOX: Lipoxygenase;
NAAA: N-Acylethanolamine acid amide hydrolase;

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.

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 with drugs that target receptors or metabolic enzymes of eCBs (Ciaramellano et al., 2023).



2-AG-3P: 2-Arachidonoylglycerol-3-phosphate
2-AG: 2-Arachidonoylglycerol
DAG: Diacylglycerol
DAGL: Diacylglycerol lipase
PH: Phosphohydrolase
PLA_1: Phospholipase A_1
PLC: Phospholipase C

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.

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, 2020). 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 CB_1 R and CB_2R, 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 CB_1 R and CB_2R partial agonist exhibiting multiple therapeutically interesting physiologic properties involving both receptor types, which include anti-inflammatory,

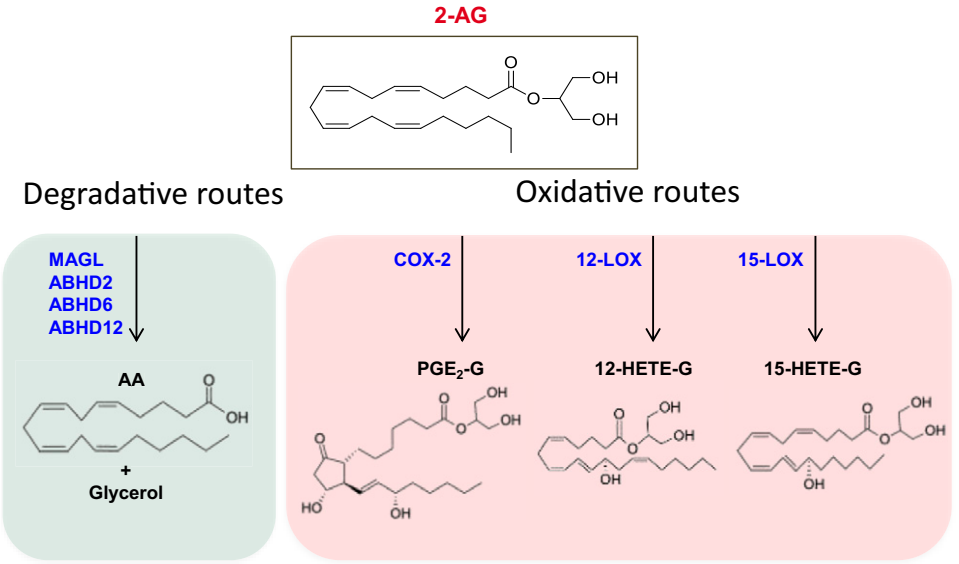


Fig. 6. Catabolic pathways of 2-arachidonoylglycerol. 2-AG can be cleaved into arachidonic acid and glycerol by different hydrolytic routes. MAGL is considered the most relevant among these catabolic pathways. Alternatively to hydrolytic routes, 2-AG can be oxidized by LOXs or COX-2 to generate various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

immunosuppressive, and analgesic effects. THC was the first cannabinoid agonist approved as a medication by the FDA under the generic name dronabinol (Marinol), although its use was restricted due to CNS-mediated psychotropic side effects.

Many additional nonselective cannabinoid agonists have provided insights into pharmacotherapuetic potential (reviewed by Robson, 2001; Pertwee 2008b, 2012). With a goal to develop cannabinoid, nonopioid 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, 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 CB_1R/CB_2R nonselective full agonist CP55940, outperforming THC with regard to CB_1R/CB_2R 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 CB_1R (Matsuda et al., 1990) and CB_2R (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 nonsteroidal 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 CB_1R/CB_2R nonselective 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 CB_1R/CB_2R 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 CB_1R or CB_2R by THC or the other nonselective agonists seems to be controlled by differential expression (induction or desensitization/downregulation) 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 methodology has characterized the sites of action at CB_1R and CB_2R (Janero et al., 2017). However, a more detailed CB_1R structure was reported in 2016 in its inactive conformation by using the long-acting CB_1 R 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 CB_1R antagonist analogs and the study of their interactions with the receptor. This work was followed by studies on the structures of the agonist-bound CB_1 R (Hua et al., 2017; Krishna Kumar et al., 2019; Hua et al., 2020), which demonstrated that activation of CB_1 R induces dramatic conformational changes of both extracellular and intracellular domains of the receptor,

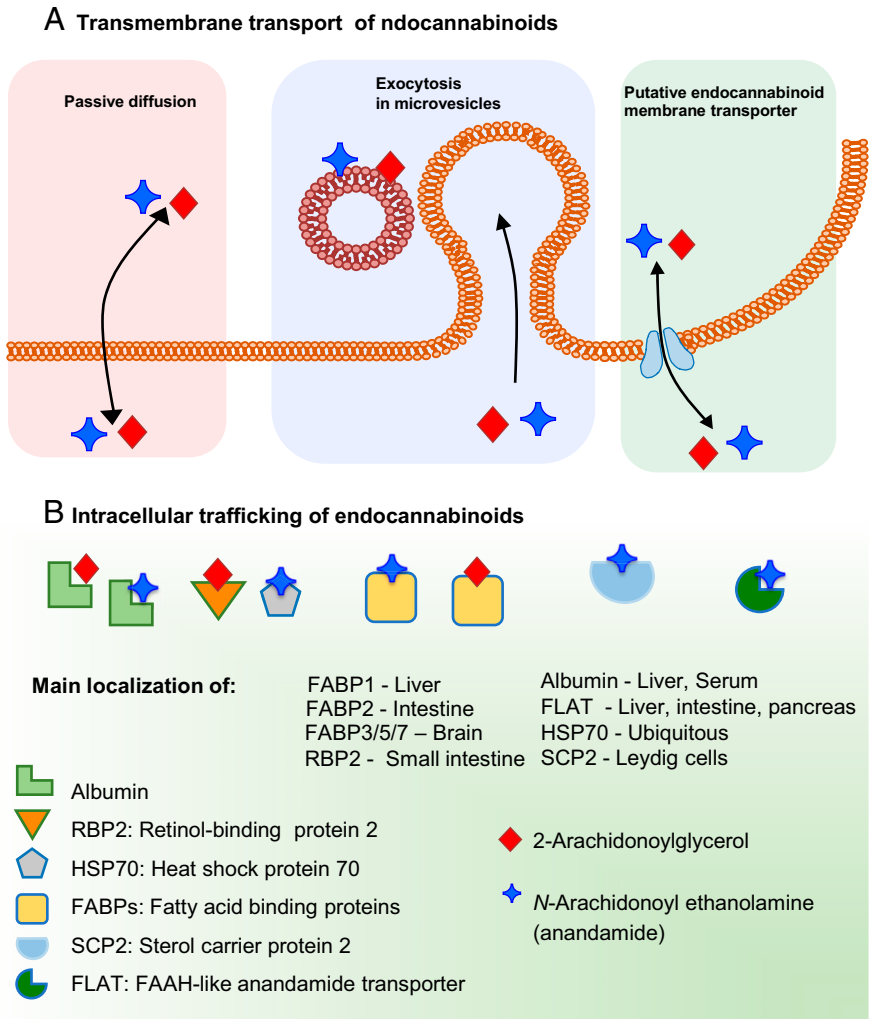


Fig. 7. Transport of endocannabinoids. (A) Anandamide and 2-arachidonoylglycerol can cross the plasma membrane via different mechanisms, which include passive diffusion, exocytosis of microvesicles and a putative membrane transporter. (B) Intracellular trafficking of anandamide and 2-arachidonoylglycerol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

accompanied by a serious contraction of the binding pocket. This more expansive conformation of CB_1R in its inactive state explains how several antagonists can be accommodated in the receptor structure.

The high-resolution crystal structure of antagonist-bound CB_2R was determined in 2019, which first 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 CB_2R (Fig. 11A). However, the antagonist AM10257 adopts a constrained binding pose in CB_2R, which is quite different from the extended binding conformation of antagonists in CB_1R (Hua et al., 2016). Of note, the adamantyl moiety of AM10257, adapting a vertical conformation, would clash with the residue Phe102N-term of CB_1R when two structures are superimposed (Fig. 11, B–D). That is the reason why the N-terminus of CB_2R forms a short helix over the orthosteric pocket, instead of the V-shaped loop that directly interacts with the antagonist in CB_1R (Hua et al., 2016; Shao et al., 2016). In addition, the extracellular part of transmembrane helices 1 and 2 in CB_2R is more compact compared with the conformations of the same helices in CB_1R, resulting in a much smaller antagonist-binding pocket than that of CB_1R (Hua et al., 2016; Shao et al., 2016). The structural analysis provides the basis for the high degree of antagonist selectivity between CB_1R and CB_2R.

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 CB_2R 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

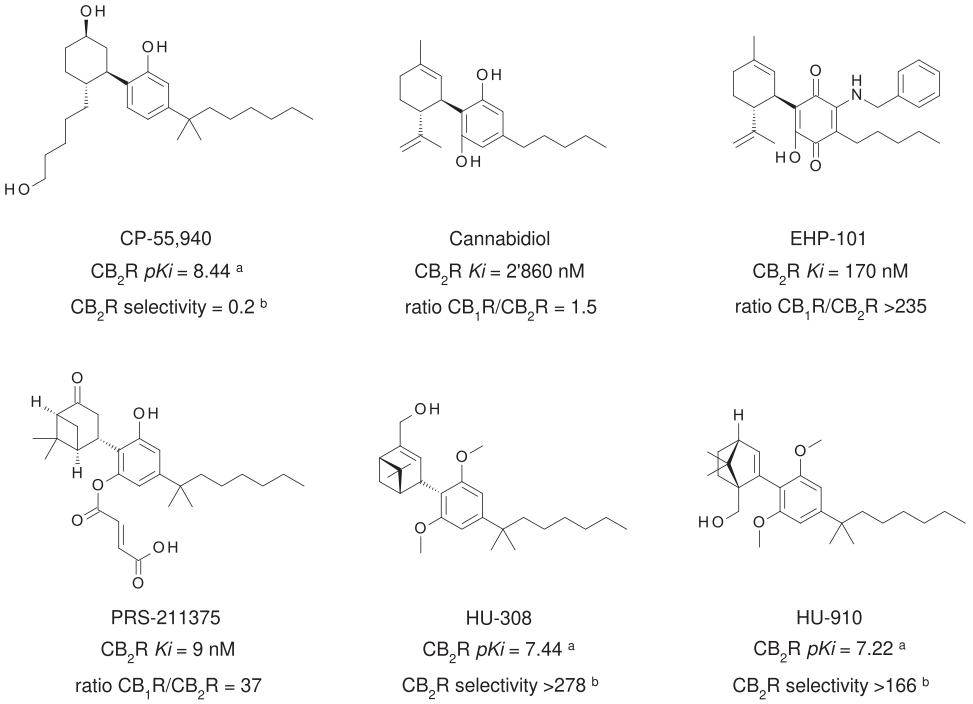


Fig. 8. Chemical structure, CB_2R binding affinity and selectivity of relevant nonclassic cannabinoids. [a]Consensus human CB_2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). [b]CB_2R selectivity (10^(*pKi* CB_2R-*pKi* CB_1R).

interactions with CB_2R, including residues from transmembrane helices 2–3 and 5–7 and the extracellular loop 2 with similar binding mode in the orthosteric ligand-binding pocket (Fig. 11, E–F). Although the core of WIN55212-2 forms π-π interactions with F1173.36 and W2586.48 of CB_2R, the rotamers of F1173.36 and W2586.48 are very similar in these two structures (Fig. 11G). The superposition of agonist-bound CB_1R and CB_2R structures shows that the agonist binding pocket volume, as well as the key residues that form interactions with ligands, are almost identical (Fig. 11, H–M).

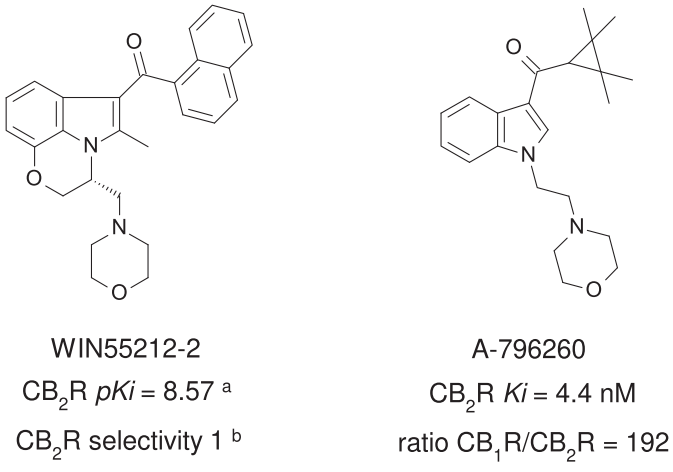


Fig. 9. Chemical structure, CB_2R binding affinity and selectivity of representative aminoalkylindole CB_2R ligands. [a]Consensus human CB_2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). [b]CB_2R selectivity (10^(*pKi* CB_2R-*pKi* CB_1R).

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_2R activation mechanism was revealed by the comparison of active and inactive structures. Though the antagonists and agonists of CB_2R share similar binding pockets, including the key interaction residues with the receptors, the interaction of CB_2R 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 classic rearrangements of N7.49 P7.50 $\times$ x Y7.53 and D3.49 R3.50 Y3.51 motifs were observed that contribute to the conformational change of the intracellular part of CB_2R, eventually forming the G-protein binding cavity (Fig. 12, B–C). However, in contrast to agonist-bound CB_1R, only the intracellular part of CB_2R exhibits obvious conformational changes while the extracellular part including the N-terminus of CB_2R undergoes minor changes during its activation (Fig. 12, D–F). The balloon-like plasticity of CB_1R during its activation indicates its higher ability to respond to a diverse array of ligands than CB_2R, which may explain the low selectivity compared with CB_1R for most classic THC-like agonists of CB_2R.

A. Therapeutic Potential of Cannabinoid Receptor 1

The epigenetic regulation of CB_1R expression and signal transduction pathways following Gi/o or β-arrestin

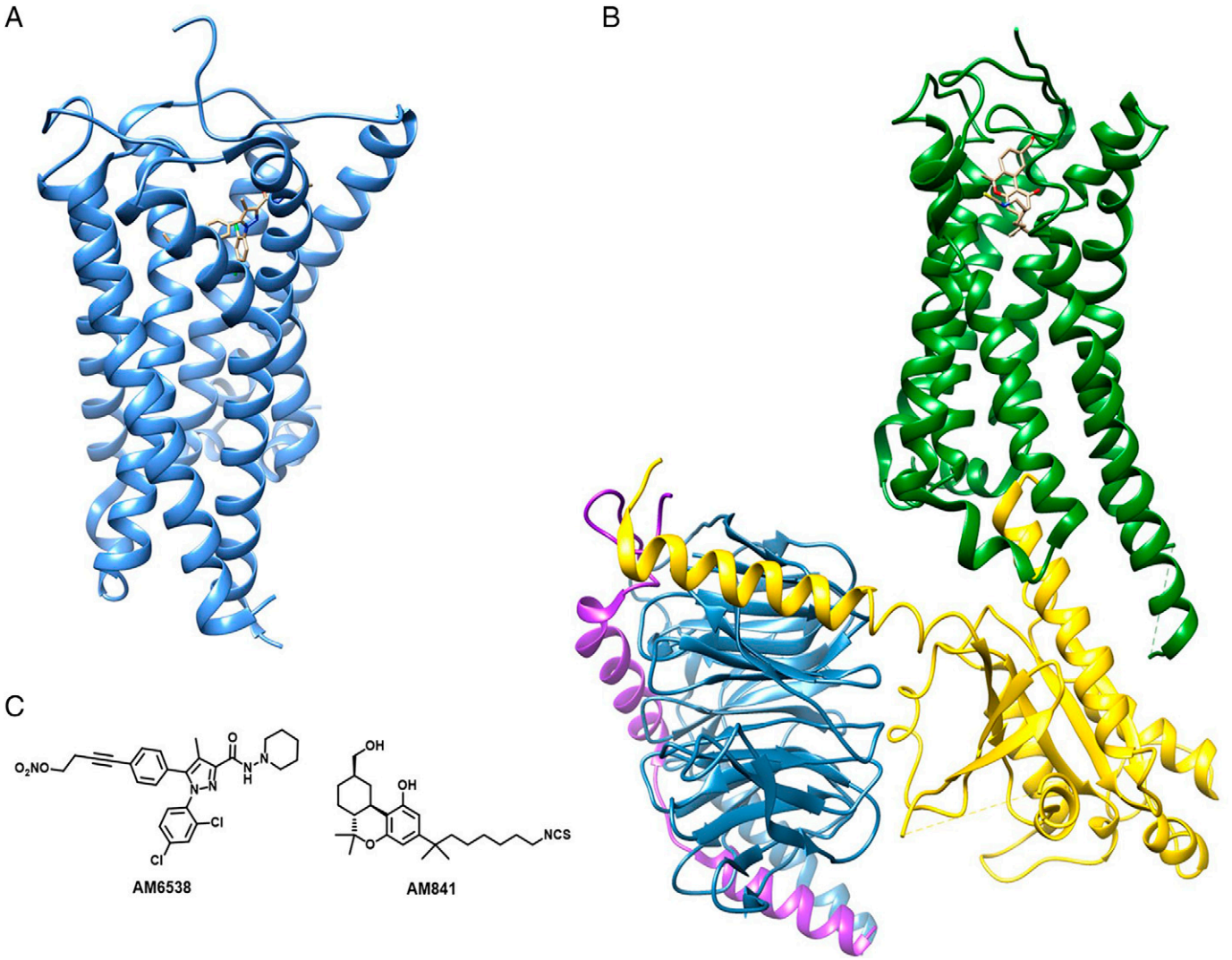


Fig. 10. (A) X-ray structure of CB_1R (blue) bound to the antagonist AM6538. (B) Cryo-EM structure of CB_1R (green) in complex with G proteins (α subunit in yellow, β subunit in blue, γ subunit in purple) and the classic cannabinoid agonist AM841. (C) Chemical structures of AM6538 and AM841.

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_1R 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_1R has been recognized as a target for pharmacotherapeutic development based on 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_1R agonists and antagonists to market has been fraught with the challenges of selectivity resulting from the abundance of CB_1R throughout all areas of the brain, including expression by neuronal and nonneuronal cells. This broad distribution increases the probability of unwanted side effects accompanying the therapeutic benefits.

1. CB_1R Agonists and Positive Allosteric Modulators. The only FDA-approved CB_1R 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, 2022d; https://adisinsight.springer.com/drugs/800025856). 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 CB_1R and CB_2 R, though therapeutic responses and untoward side effects can be attributed to one or both CB receptors, as determined by pharmacological characterization in in vivo or in vitro models. Nevertheless, targeting CB_1R for unmet

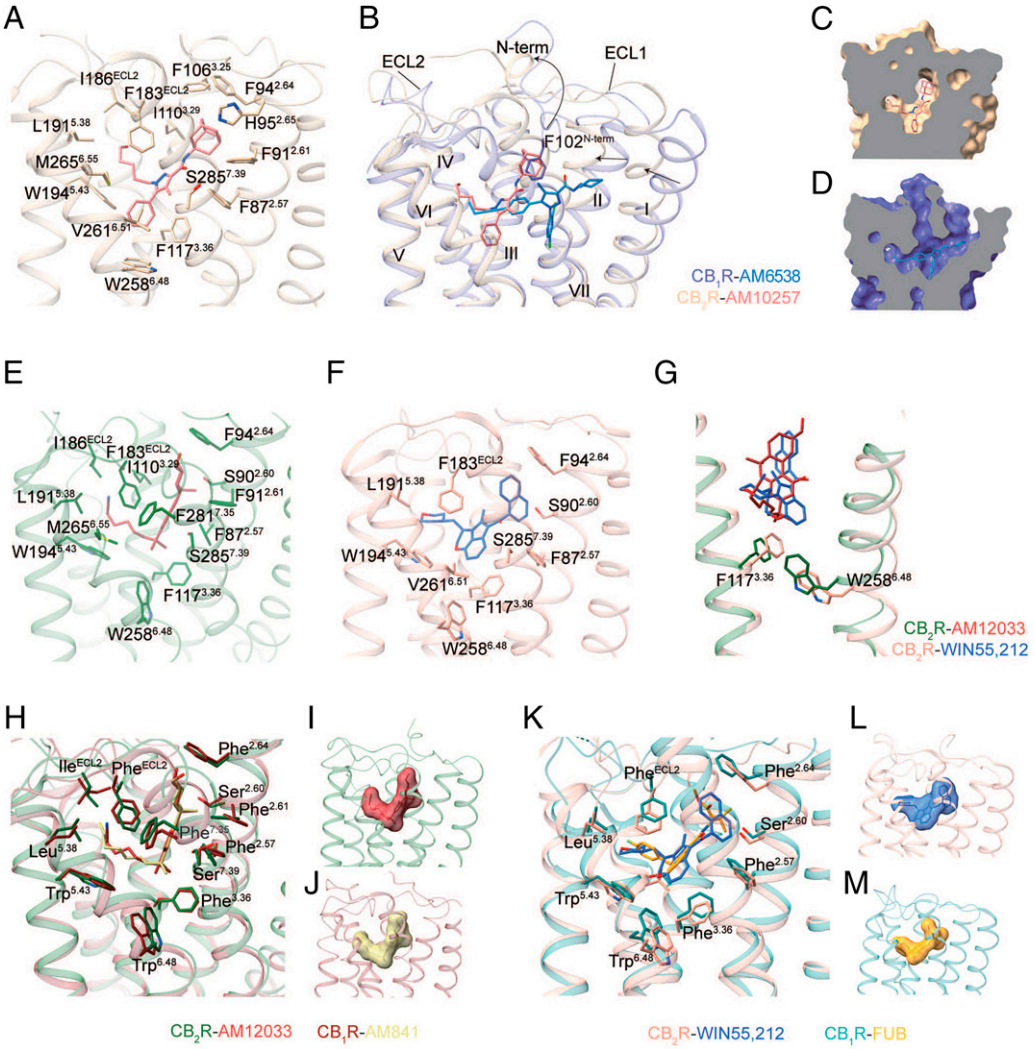


Fig. 11. Comparison of ligand binding modes in CB_1R and CB_2R. (A) The binding pocket of AM10257 in CB_2R crystal structure (PDB code 5ZTY). AM10257 and the key residues are shown in sticks as the following color code: CB_2R, brown; AM10257, light coral. (B–D) Binding pose comparison of AM6538 in CB_1R (PDB code 5TGZ), and AM10257 in CB_2R, using color code as follows: CB_1R, slate blue; AM6538, dodger blue; CB_2R, brown; AM10257, light coral. (E–F) The binding pocket of AM12033 in CB_2R (PDB code 6KPF) and WIN55,212-2 in CB_2R (PDB code 6TP0). Ligands and the key residues are shown in sticks as the following color code: AM12033, brown; CB_2R (6KPF), dark green; WIN55,212-2, royal blue; CB_2R (6TP0), dark salmon. (G) The conformational comparison of "toggle switch" residues $Trp258^{6.48}$ between AM12033- and WIN55,212-2-bound CB_2R. (H–J) Binding pose comparison of THC-like agonist in CB_1R (PDB code 6KPG) and CB_2R (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: CB_2R, dark green; AM12033, brown; CB_1R, maroon; AM841, dark khaki. (K-M) Binding pose comparison of agonist FUB in CB_1R (PDB code 6N4B) and agonist WIN55,212-2 in CB_2R (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: CB_2R, dark salmon; WIN55,212-2, royal blue; CB_1R, dark cyan; FUB, orange.

therapeutic needs has evolved based on preclinical investigations, and these opportunities will be considered in this section.

Dronabinol was developed to counteract 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 (https://adisinsight.springer.com/drugs/800007811). 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 (https://www.pdr.net/drug-summary/Marinol-dronabinol-2726). 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 (https://www.pdr.net/drug-summary/Marinol-dronabinol-2726).

Nabilone is synthesized as a 9-ketocannabinoid with a dimethylheptyl side chain (Fig. 13) and is enzymatically

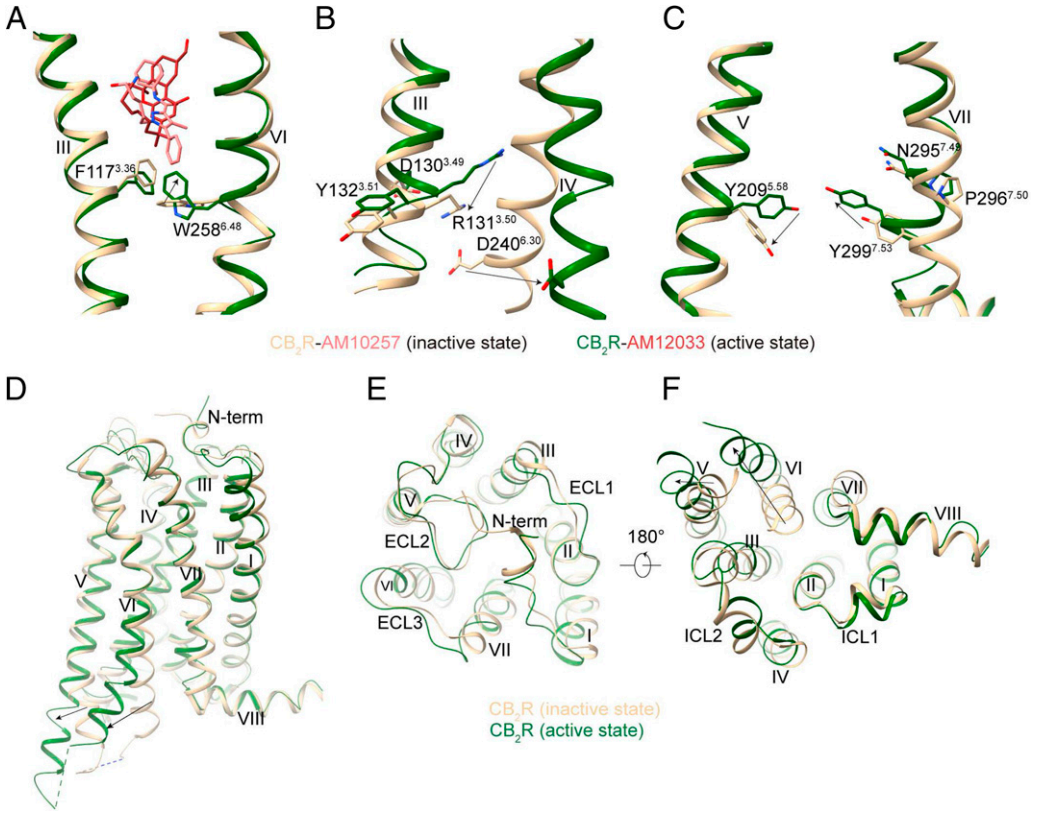


Fig. 12. Conformational changes during CB_2R activation. (A–C) The conformational change of key residues between inactive- and active-CB_2R. "Toggle switch residue" (A), $D^{3.49}R^{3.50}Y^{3.51}$ motif (B), and $N^{7.49}P^{7.50}xxY^{7.53}$ motif (C). (D–F) The overall structure (D), the extracellular region (E), and intracellular region (F) comparison of inactive- (brown) and active-state (dark green) CB_2R structures.

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 (https://www.pdr.net/drug-summary/Cesamet-nabilone-692). 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.

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 compendium for information: https://www.medicines.org.uk/emc/product/602/smpc#gref). The spray is intended to be applied at the onset of muscle contractions to reduce spasticity and pain in MS patients. Each application provides some fraction of the 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 the treatment of glioma patients while clinical trials were being conducted; however, this status was later withdrawn (see EMA notices: EMA, 2022). The EMA reports pharmacokinetic data and recommends dosing schedules for use in MS patients (https://www.medicines.org.uk/emc/product/602/smpc#gref). 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, 1991). CBD entered the market for the treatment of Dravet syndrome, infantile severe myoclonic epilepsy, Lennox-Gastaut syndrome, and tuberous sclerosis (Clarivate, 2022b). In addition, CBD and THC combinations have been approved for MS-associated spasticity and pain management (Clarivate, 2022g; https://citeline.informa.com/drugs/details/175074). CBD in the nabiximols formulation may or may not exert its

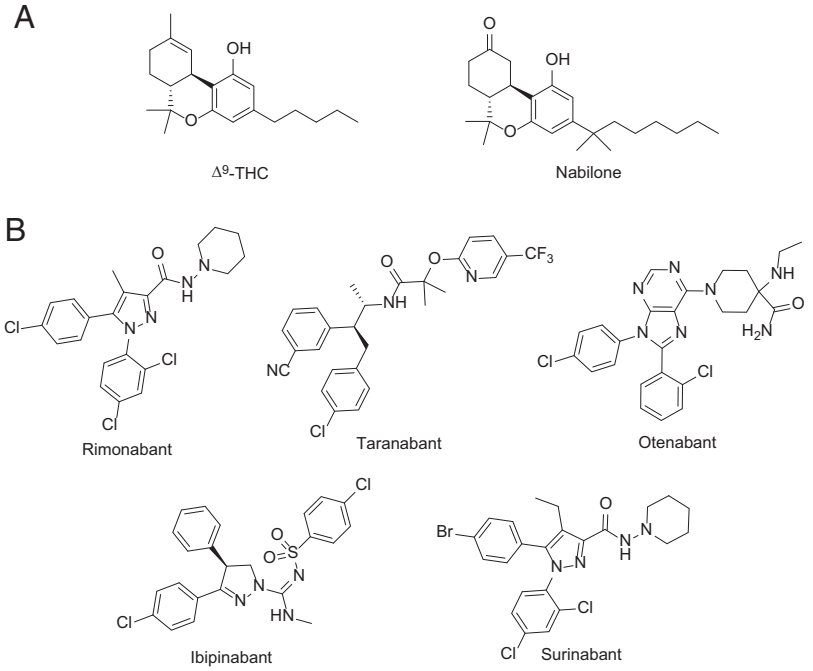


Fig. 13. Structures of the clinically tested cannabinoid agonists (A) and the selective CB_1R antagonists (B).

cellular actions via processes involving CB_1R. CBD exerts both negative and positive interactions with THC over a range of biologic and behavioral responses in animal models and humans (Pertwee, 2008a; McPartland et al., 2015). In a cloned neuronal cell model, CBD competed with the CB_1R agonist [^{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 CB_1R-coupled G_i protein as does THC (Howlett, 1984; Mukhopadhyay et al., 2002). Similar findings of low potency binding to CB_1R and inability to stimulate CB_1R 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 CB_1R pharmacology are most compelling: (1) CBD could exert a noncompetitive antagonism at CB_1 receptor (Petitet et al., 1998; Thomas et al., 2007; Laprairie et al., 2015) and (2) CBD could indirectly modulate CB_1R 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-CB_1R mechanisms proposed for CBD's neurologic actions minimally include the facilitation of serotonin signaling, activation of TRPV1 or PPARγ receptors, neuroprotection via antioxidant activity, and attenuation of proinflammatory processes (Campos et al., 2012; Ibeas Bih et al., 2015; Campos et al., 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.

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 that 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 CB_1R agonists, although the exact neurologic 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 downregulate CB_1R 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 CB_1R agonists as "orphan" drugs for the treatment of cachexia in cancer (Plasse et al., 1991). However, it is the appetite suppression by CB_1R antagonism that prompted clinical trials for weight loss in morbidly obese individuals and resulted in an explosion of basic science research linking the CB_1R to metabolic processes associated with energy storage (Piazza et al., 2017; DiPatrizio, 2021; Miralpeix et al., 2021). Studies of CB_1R-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 CB_1R stimulation on pancreatic β-cell function, diabetic insulin resistance, and hepatic steatosis (Gruden et al., 2016; Nagappan et al., 2019), as well as on female (Cecconi et al., 2020) and male (Maccarrone et al., 2021) reproductive functions, must be considered in the safety profile for CB_1R agonist medicines.

Control of chronic and episodic pain continues to be an unmet therapeutic need. The development of CB_1R agonists as antinociceptive agents by Pfizer Central Research (Johnson et al., 1981; Howlett et al., 1990; Melvin et al., 1993, 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).

CB_1R 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 CB_1R and CB_2R 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,b).

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 neurologic 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, Tourettes' 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 CB_1R, modifications have been made to pCB, aminoalkylindole, and eCB ligands. For example, AEA analogs arachidonylcyclopropylamide (ACPA) and arachidonyl-2-chloroethylamide (ACEA) exhibit 1-2 nM affinity for the CB_1R but 1-3 μM affinity for the CB_2R, and both inhibit cAMP CB_1R selectivity of the ACPA (Hillard et al., 1999). This selectivity led to the CB_1R selective (CB_1R/CB_2R Ki ratio = 0.1) dual CB_1R/CB_2R agonist CMX-020, which is currently being explored in phase 2 clinical trials for the treatment of osteoarthritis (https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=371547&isReview=true), pain including sciatica, and diabetic neuropathy (Clarivate, 2022c).

TABLE 4
Diseases/symptoms for treatment with CB_1R agonists and antagonists registered with ClinicalTrials.gov

Generic Name Brand Name Class/Efficacy	Completed Clinical Trials	Ongoing Clinical Trials
Dronabinol	Chronic pain (with opioid treatment)	Osteoarthritis pain
Marinol	Fibromyalgia, back pain	Diabetic neuropathy
Phytocannabinoid	Migraine pain	Knee arthroplasty
Synthetically produced	Neuropathic, low back pain	Arthroscopic surgery
Δ^9-tetrahydrocannabinol (THC)	Cervical dystonia	Sleep and pain in MS
CB_1R/CB_2R partial agonist	Chest pain	Postsurgical pain-lumbar fusion
	Neuropathic pain in MS	Postsurgical pain-knee replacement
	Cramps in ALS	Pain in opioid-maintained pts
	Irritable bowel syndrome	Alzheimer's agitation
	Complex regional pain syndromes	Bipolar disorder
	Cannabis dependence	Sleep
	Cannabis use disorder	Post-traumatic stress disorder
	Marijuana withdrawal	Trauma intrusive memories
	Trichotillomania related behaviors	Glaucoma hemodynamics
	Post-traumatic stress disorder	
	Obstructive sleep apnea	
	PostSurgical N/V	
	Anti-retroviral therapy N/V	
	Brain neoplasms N/V	
	Schizophrenia	
Dronabinol derivatives		
BX-1 oral solution	Spasticity	Chemo N/V, pain in pancreatic CA
Syndros (dronabinol)	Bone pain metastatic breast CA	
Namisol THC	Postsurgical abdominal pain	
Namisol THC	Pancreatitis abdominal pain	
Namisol THC	Dementia–Alzheimer's	
Namisol THC	Dementia w/ neuropsych symptoms	
THC olive oil	Post-traumatic stress disorder	
THC olive oil	Fibromyalgia–pain	
SCI-110 THC + PEA	Tourette syndrome	Tourette syndrome
THX-110 THC + PEA	Tourette syndrome	
dronabinol + naltrexone	Opioid dependence	
Nabilone		
Cesamet	Phantom limb pain	Spinal neuropathic pain
Synthetic THC analog	Fibromyalgia	Pain and insomnia
CB_1R/CB_2R agonist	Failed back surgery pain	End-stage renal disease
	Inflammatory bowel pain	Obesity
	Diabetic neuropathies	Developmental cognitive disability
	Spinal injury muscle	Obsessive-compulsive disorder
	Spinal cord injury	Alzheimer's disease agitation
	Postsurgical N/V	
	Cancer anorexia/cachexia	
	Parkinson's disease	
	Parkinson's nonmotor symptoms	
	Alzheimer's disease	
PP-01 Nabilone+Gabapentin		Cannabis withdrawal
Nabiximols		
Sativex	Chemotherapy neuropathic pain	Diabetic neuropathy
Phytocannabinoid	Advanced malignancy pain	MS spasticity and pain
Purified Plant Extract	Pain	
THC:CBD (1:1)	Tourette syndrome	
THC: CB_1R/CB_2R agonist	Attention deficit hyperactivity disorder	
CBD: CB_1 NAM	Cannabis dependence	
(Negative Allosteric Modulator)		
Mixed THC:CBD		
THC:CBD 1:1	Endometriosis pain	
THC:CBD 1:1, 1:2	Chronic pain	
THC:CBD1:10	Crohn's disease	
THC:CBD 1: 50	Childhood epilepsy	
NanaBis Oro-MucSpray		Cancer pain
NanaBis Oro-MucSpray		Chronic widespread pain
THC or THC:CBD 1:10		Chronic spine back and neck pain
LGP1-20 THC:CBD (1:20)		Adolescent migraines
FibroCann Solution		Fibromyalgia
Pure Green SL Tablets		Osteoarthritis pain
MPL-001 THC:CBD 1:25		Postsurgical osteoarthritis pain
TN-TC11G THC:CBD1:1		Glioblastoma (w/standard of care)
TIL-T150 THC:CBD 1:5;1:25		Depression, insomnia

(*continued*)

TABLE 4—*Continued*

Generic Name Brand Name Class/Efficacy	Completed Clinical Trials	Ongoing Clinical Trials
Pure Femme SLTab 1:30 + PEA + terpenes	Menstrual symptoms	
THC or CBD		HIV cognition
THC + CBD + CBG		Chronic migraine
Pro-drug paracetamol (or acetaminophen) Biometabolite is AM404 CB_1R/CB_2R agonist	Pruritis Presurgical analgesia Pain in tonsillectomies	
SR141716 Rimonabant Acomplia, Zimulti CB_1R antagonist/inverse agonist	• Carotid atherosclerosis Cannabis dependence Diabetes w/ metformin Obesity, weight loss • Metabolic syndrome Reduce alcohol consumption Fatty liver-NASH in T2D Smoking cessation	Recovery spinal cord injury
MK-0364 Taranabant CB_1R antagonist/inverse agonist	Obesity Smoking cessation Fatty liver-NASH in T2D	
CP-945598 Otenabant CB_{1R} antagonist/inverse agonist	Nonalcoholic steato-hepatitis Obesity	
SLV319 Ibipinabant CB_1R antagonist/inverse agonist	Obesity	
SR147778 Surinabant CB_1R antagonit/inverse agonist	Obesity Smoking cessation	
ANEB-001 CB_1R antagonist/inverse agonist		Acute cannabis intoxication
GFB-024 Peripherally acting CB_1R inverse agonist monoclonal Ab	Diabetic nephropathies	
Nimacimab Peripherally acting CB_1R antagonist/inverse agonist monoclonal Ab	Diabetic gastroparesis	

Another mechanism for achieving selectivity is found in the recent development of allosteric modulators to modify the CB_1R 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, for example, via a local eCB increase as a 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 with cholesterol was also observed with CB_1R, 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 CB_1R dimerization and binding activity.

Positive allosteric modulation of CB_1R 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_1 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., 2018, 2020). Preclinical studies of CB_1R 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_1R 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_1R-biased agonists have been reviewed recently (Laprairie et al., 2016; Ibsen et al., 2017; Leo and Abood, 2021; Manning et al., 2021).

2. Cannabinoid Receptor 1 Antagonists. Sanofi discovered the first CB_1R 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_1R 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_1R antagonist to enter clinical trials for several of these indications was rimonabant (Sanofi), followed by taranabant (Merck), otenabant (Pfizer), ibipinabant (Solvay), and surinabant (Sanofi) as shown in Fig. 13. Additional indications that have been explored clinically include hepatic fibrosis and nonalcoholic fatty liver disease, renal diseases, as well as alcohol dependence and smoking cessation (Cinar et al., 2020). Recently, a CB_1R 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_1R 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 CB_1R antagonists was discontinued by other pharmaceutical companies. Notwithstanding the failure of rimonabant, its availability allowed research toward understanding the mechanism of action of CB_1R 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 CB_1R 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 CB_1R blockade led to much of the research in developing selective CB_1R 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 CB_1R blockade for several conditions with unmet medical needs, additional approaches have been explored to retain efficacy and circumvent the unwanted neuropsychiatric side effects. Among these, CB_1R antagonist/inverse agonists that cannot enter the CNS and CB_1R neutral antagonists have shown promising results in preclinical models.

The discovery of functional CB_1Rs in the periphery and the realization that they mediate many processes of the 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 CB_1R antagonist/inverse agonists may retain the therapeutic effects of CB_1R blockade without the unwanted CNS effects. Small-molecule CB_1R antagonist/inverse agonists with minimal brain exposure have shown efficacy in animal models of obesity and metabolic syndrome, alcoholic and nonalcoholic 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 CB_1R positron emission tomography tracers are used (Tam et al., 2012; Chang et al., 2019), as well as antagonism of the tetrad effects induced by CB_1R 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 CB_1R 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 with the brain-permeant CB_1R antagonist/inverse agonists (Kale et al., 2019).

In a different approach to achieving peripheral restriction, monoclonal antibodies that act as CB_1R 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.

CB_1R 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, CB_1R neutral antagonists can compete with the endogenous cannabinoid ligands without affecting the basal activity of the receptor. For this reason, it was hypothesized that CB_1R neutral antagonists could produce the therapeutic phenotypes of CB_1R antagonism without the unwanted CNS and gastrointestinal side effects. In this regard, the most extensively studied CB_1R 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 inhibiting the self-administration of heroin (Gueye et al., 2016; Schindler et al., 2016b; 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 CB_1R antagonist/inverse agonist AM251 (Sink et al., 2010; Gueye et al., 2016; He et al., 2019). Additionally, in contrast to CB_1R 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 CB_1R 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 CB_2R agonist AM1241 (Barutta et al., 2017; 2018).

On a final note, a novel and attractive dual-targeting approach is represented by the combination of CB_1R antagonists and CB_2R agonists, as evidenced by the synergy shown by coadministration of AM6545 and AM1241 for treating diabetic nephropathy (Barutta et al., 2017). Indeed, there is early evidence that CB_1R and CB_2R 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 CB_1R antagonists/CB_2R agonists.

B. Therapeutic Potential of Cannabinoid Receptor 2

The CB_2R is a class A (rhodopsin-like) GPCR (Fig. 14). It is an essential element of the ECS, and indeed CB_2R-mediated signaling plays an important role in many human health and disease conditions (Pacher and Mechoulam, 2011; Gasperi et al., 2023). Therefore, CB_2R holds tremendous therapeutic potential for treating major pathologies affecting humans.

A plethora of preclinical evidence demonstrating the anti-inflammatory and tissue-protective effects of CB_2R activation has been generated, triggering the design, synthesis, and evaluation of multiple CB_2R 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 CB_2R activators, multiple antagonists/inverse agonists and a few allosteric ligands have also been discovered. Of these, more than 20 CB_2R-selective agonists have been advanced to clinical trials. Recently, several 3D structures of CB_2R in complex with ligands have been reported (Li et al., 2019; Hua et al., 2020; Xing et al., 2020). Furthermore, a wide variety of labeled 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 has contributed the understanding of the structural basis of selective CB_2R 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 CB_2R research since the discovery of the receptor. Due to space limitations, the content of this section highlights only foundational studies and key aspects.

The CB_2R 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; http://www.immgen.org/), bone (Ofek et al., 2006), and the gastrointestinal tract (Atwood et al., 2012). CB_2R 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 CB_2R is a $G_{i/o}$ coupled GPCR, and its activation leads to an inhibition of cAMP production. In addition, the CB_2R 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

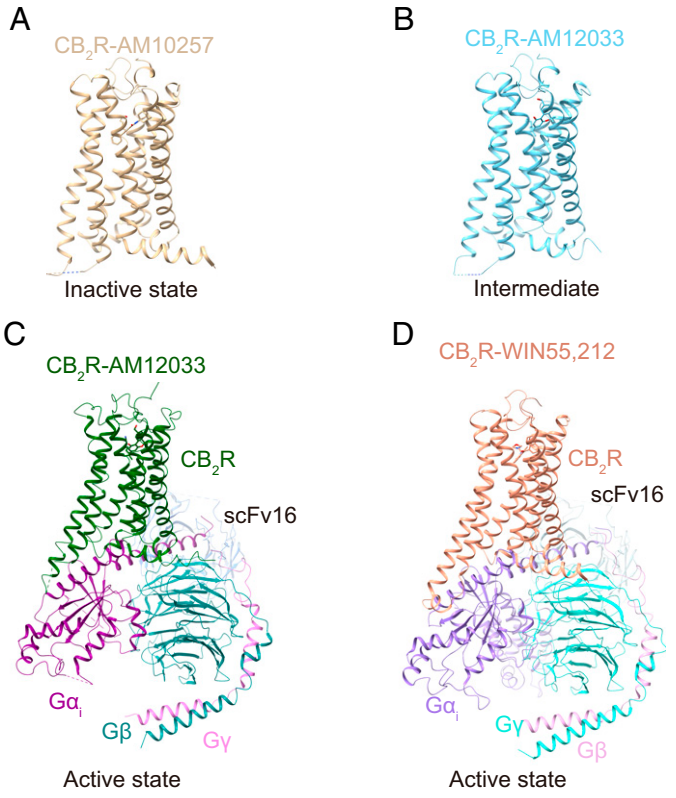


Fig. 14. Structures of the CB_2R in different states. (A) Crystal structure of antagonist AM10257-bound CB_2R (PDB code 5ZTY). (B) Crystal structure of agonist AM12033-bound CB_2R (PDB code 6KPC). (C) Cryo-EM structure of AM12033-bound CB_2R-G_i complex (PDB code 6KPF). (D) Cryo-EM structure of WIN55,212-2-bound CB_2R-G_i complex (PDB code 6TP0), using color code as follows: CB_2R-AM10257, brown; CB_2R-AM12033 (PDB code 6KPC), sky blue; CB_2R-AM12033 (PDB code 6KPF), green; CB_2R-WIN55,212-2, dark salmon; $G\alpha_i$ in CB_2R-AM12033, purple; $G\beta$ in CB_2R-AM12033, teal; $G\gamma$ in CB_2R-AM12033, orchid; scFv16 in CB_2R-AM12033, cornflower blue; $G\alpha_i$ in CB_2R-WIN55,212-2, medium purple; $G\beta$ in CB_2R-WIN55,212-2, turquoise; $G\gamma$ in CB_2R-WIN55,212-2, plum; scFv16 in CB_2R-WIN55,212-2, light blue.

et al., 2008; Atwood and Mackie, 2010). Surface and intracellular CB_2R 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 CB_2R 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). CB_2R interactions at the molecular level and its resulting downstream effects translate toward modulation of disease pathogenesis. CB_2R 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,b, 2016; Zoja 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 CB_2R-mediated immunosuppressive and anti-inflammatory effects leading to a dampening of tissue injury. In hypoactivated immune states, CB_2R activation might, however, enhance tissue damage (Pacher and Mechoulam, 2011). Under these pathologic conditions, CB_2R inverse agonists/antagonists might provide therapeutic options.

1. Cannabinoid Receptor 2 Agonists. Due to the huge therapeutic potential of CB_2R, multiple ligands have been developed. In 1996, a first patent for a CB_2R-selective antagonist was filed (Rinaldi et al., 1996). Since then, more than 1150 CB_2R patent applications have been registered. CB_2R 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 classic small molecules, including many labeled 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, 2014; Morales et al., 2016; Aghazadeh Tabrizi 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 "CB_2R ligand space" with a strong emphasis on those that made it into clinical development, all of them being activators of CB_2R. CB_2 R agonists that are launched or under active development and registered with ClinicalTrials.Gov are listed in Table 5.

a. Endocannabinoids 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 $CB_{1/2}R$ activation (De Petrocellis et al., 2004; Lambert and Fowler, 2005; Di Marzo, 2018; Cristino et al., 2020). Besides $CB_{1/2}R$ (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).

b. Plant-derived cannabinoids. THC and its thermodynamically more stable and similarly potent regioisomer Δ^8-THC served as a starting point for generating further classic cannabinoids (Razdan, 1986; Mechoulam et al., 1998). Dual CB_1R/CB_2R 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 (https://adisinsight.springer.com/drugs/800007180). Although Lenabasum activates CB_1R in addition to CB_2R, it is not psychoactive (Zurier et al., 1998). Presumably, this is the consequence of its low brain penetrance.

Preferential CB_2R 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 CB_2R-selective agonists (10^(pKi CB_2R-pKi CB_1R) > 153), and thus it has been exploited to interrogate CB_2R pharmacology (Pertwee, 1999; Soethoudt et al., 2017).The reference compound CP55940 is a potent dual CB_1R/CB_2R agonist outperforming THC with regard to $CB_{1/2}R$ binding affinity and analgesic activity (Showalter et al., 1996). Its tritiated congener has been broadly applied for the discovery and profiling of many $CB_{1/2}R$ ligands (Devane et al., 1988).

In contrast, nonpsychotropic (-)CBD exhibits moderate affinity for CB_2R (Showalter et al., 1996). CBD has been suggested to function as an inverse agonist of CB_2R (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 CB_2R and PPARγ agonist and activator of protein phosphatase 2A, which is currently investigated in phase 2a clinical trials (Del Río et al., 2016; EMA, 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 CB_2R agonist (CB_2R EC_{50} cAMP = 17.4 nM; 98% efficacy) (Gratzke et al., 2010). It showed efficacy in various rodent in vivo disease models including pain readouts in a chronic constriction injury model (Clarivate, 2022j). 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, 2022j). Interestingly, no effect was observed at higher doses. This bell-shaped curve behavior is characteristic of the pharmacodynamics studies with other cannabinoid-derived $CB_{1/2}R$ 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 CB_2R as exemplified for HU-308. This potent, selective, and bioavailable CB_2R 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 CB_2R over CB_1R (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 CB_2R agonist for studying the role of the receptor in biologic and disease processes (Soethoudt et al., 2017). HU-910 in vivo efficacy studies opened the door for exploring the potential of CB_2R 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 CB_2R signal transduction pathways in human and mouse. In contrast to being an unbiased agonist for the human CB_2R, HU-910 exhibited a preference toward G-protein activation as compared with 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 CB_2R ligands.

Aminoalkylindoles were among the earliest discovered CB_2R scaffolds. In particular, dual CB_1R and CB_2R 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).

TABLE 5
Diseases/symptoms for treatment with CB_2R agonists and antagonists registered with ClinicalTrials.gov[a]

Generic Name Brand Name Class/Efficacy	Completed Clinical Trials	Ongoing Clinical Trials
Dronabinol[b]		
Dronabinol derivatives[b]		
Nabilone[b]		
Nabiximols[b]		
Mixed THC:*CBD*[b]		
Cannabidiol		
Epidiolex	Sturge-Weber syndrome	Obsessive-compulsive disorder
CB_1R/CB_2R ligand others	Opioid-use disorder	Tuberous sclerosis complex
	Prostate cancer	Typical absence seizures
	Cannabis use disorder	Autism
	Opioid withdrawal	Fibromyalgia
	Musculoskeletal pain	Aromatase inhibitor-associated arthralgias
	Alcohol use disorder	Back pain
	• Post-traumatic stress disorder	Depressive symptoms
	• Inflammatory bowel disease	Electrical status epilepticus of slow-wave sleep
	• Knee osteoarthritis	Dental pain
	Parkinson's disease	Behavioral problems in children and adolescents with intellectual disability
	Opiate addiction	Knee arthritis
	Epilepsy	Chemotherapy-induced peripheral neuropathy
	Seizures	Bipolar disorder
	COVID-19	Hypertension
	Burn-out	Anxiety and fear
	Chronic periodontitis	Chronic pain
	Urinary stone	Early psychosis
	Schizophrenia	Post-traumatic stress disorder
	Blepharospasm	Anorexia nervosa
	Cocaine craving/dependence	Gastroparesis and functional dyspepsia
	Generalized anxiety disorder	Anxiety in advanced breast cancer
	Lennox-Gastaut syndrome	Traumatic brain injury
	Dravet syndrome	Tobacco cessation
	Psychotic disorders	Social anxiety disorder
	Infantile spasms	Rheumatoid arthritis
	Fragile X syndrome	Diabetes
	Tuberous sclerosis complex	Chronic pain
	Psoriatic arthritis	Endometriosis pain
	Hand osteoarthritis	Social anxiety disorder
	Cancer	• Radiculopathy
	Diabetic neuropathies	Sleep disturbance
	Ulcerative colitis	Insomnia
	Fatty liver	Prevention aGVHD
		Prader-Willi syndrome
		Musculoskeletal pain
Lenabasum		
CB_2R/CB_1R agonist	Cystic fibrosis	
	Dermatomyositis	
	Systemic lupus erythematosus	
Olorinab		
CB_2R agonist	Crohn's disease	
	Abdominal pain	
RG7774		
CB_2R agonist		Diabetic retinopathy
CNTX-6016		
CB_2R agonist	Chronic pain	Painful diabetic neuropathy
	Nociceptive pain	
	Pain	
EHP-101		
CB_2R agonist PPARγ agonist		Diffuse cutaneous systemic sclerosis

[a]Studies with the status "not yet recruiting, recruiting," "enrolling by invitation," "active, not recruiting," and "completed" were included in this table.
[b]See Table 4 for respective CB_1R data.

Initial aminoalkylindoles were structurally simplified. Furthermore, CB_2R selectivity was improved to lead to CB_2R agonists such as A-796260 (Fig. 9) (Frost et al., 2008), which achieved efficacy in various rodent pain models upon intraperitoneal-injection administration (Yao et al., 2008). Importantly, these antihyperalgesic effects could be blocked by pretreatment with a CB_2R antagonist.

Bicyclic (het)aryl scaffolds were investigated for CB_2R selectivity or minimal CB_1R efficacy. Several high throughput screening campaigns were conducted in the search for potent, selective, and orally bioavailable CB_2R 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

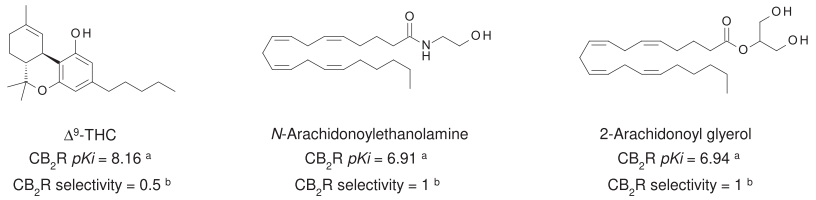


Fig. 15. Chemical structure and CB_2R binding affinity of THC, *N*-arachidonoylethanolamine, and 2-arachidonoyl glycerol. [a]Consensus human CB_2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). [b]CB_2R selectivity ($10^{\wedge}(pKi\ CB_2R\text{-}pKi\ CB_1R)$).

dual CB_1R/CB_2R 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, 2022a; https://artelobio.com/pipeline/). However, due to CNS-related side effects, phase 2 studies for the treatment of nociceptive and neuropathic pain were terminated (Kalliomäki et al., 2013; https://www.astrazeneca clinicaltrials.com/study/D3120C00006/).

Phase 2 clinical trials for the oral treatment of osteoarthritic knee pain were conducted with CB_2R agonist LY-2828360 (Fig. 17), but they were terminated despite an acceptable side-effect profile (Hollinshead et al., 2013; https://clinicaltrials.gov/ct2/show/NCT01319929; Clarivate, 2022f). The imidazopyrimidine is brain penetrant and exhibits an excellent selectivity over CB_1R (ratio CB_1R/CB_2R EC_{50} for GTPγS binding was $> 5'000$). Recently reported triazolopyrimidine-derived CB_2R agonist (at 1 nM) RG7774 (Fig. 17) is under active development in phase 2 as an innovative oral treatment of diabetic retinopathy exhibiting very high selectivity over CB_1R (ratio CB_1R/CB_2R EC_{50} 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-0355009 6 (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 CB_2R agonists made it into clinical trials. Tedalinab was investigated for the oral treatment of neuropathic pain and osteoarthritis (Clarivate, 2022n) (Fig. 19). The CB_2R-selective pyrazole carboxamide exhibits similar binding affinities for human and rat CB_2R (human CB_2R Ki = rat CB_2R Ki $\approx$ 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.

Lead optimization toward olorinab was guided by a β-arrestin efficacy assay (Han et al., 2017). This highly potent CB_2R full agonist is a peripherally acting molecule that is devoid of psychotropic effects (https://adisinsight.springer.com/drugs/800039670; https://clinicaltrials.gov/ct2/show/NCT04043455). Olorinab (Fig. 19) was clinically assessed as an oral treatment of 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 (Pharma Intelligence, 2022). The ligand exhibits a short human half-life and was therefore administered three times a day (Clarivate, 2022i). Dual CB_1R/CB_2R agonist TAK-937 (Fig. 19) was developed as an injectable for the treatment of stroke after observing cerebroprotective effects in rat and nonhuman primate in vivo efficacy studies (Suzuki et al., 2012; Clarivate, 2022m). Yet, due to a narrow safety margin, development was halted. CB_2R-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 (Clarivate, 2022h; https://www.neurotherapia.com/research). Follow-up studies targeting MS and amyotrophic lateral sclerosis

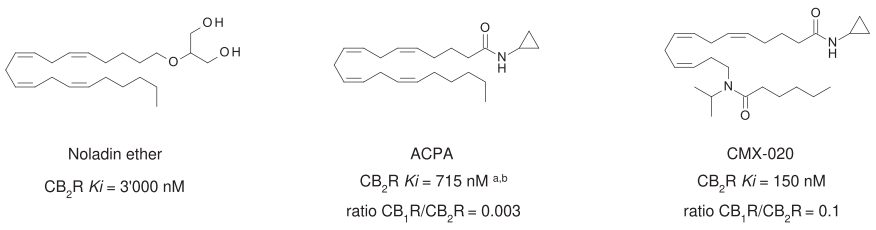
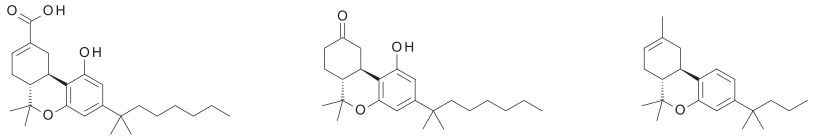


Fig. 16. Chemical structure and CB_2R binding affinity of noladin ether and synthetic eCB analogs. [a]Binding to spleen cannabinoid receptor. [b]With phenylmethanesulfonyl fluoride.



Lenabasum, Ajulemic acid
CB_2R *Ki* = 51 nM
ratio CB_1R/CB_2R = 12

Nabilone
CB_2R *Ki* = 1.84 nM
ratio CB_1R/CB_2R = 1.2

JWH133
CB_2R *pKi* = <5 [a]
CB_2R selectivity >153 [b]

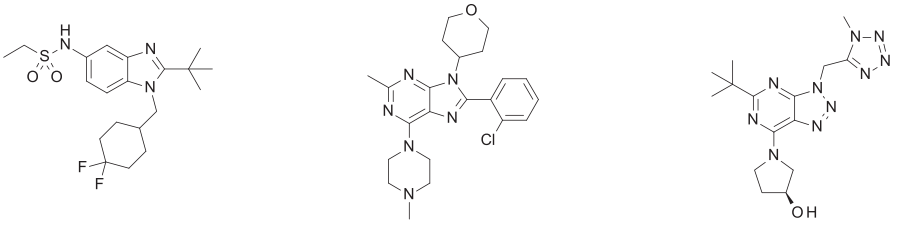
Fig. 17. Chemical structure, CB_2R binding affinity and selectivity of representative classic cannabinoids. [a]Consensus human CB_2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). [b]CB_2R selectivity (10^(*pKi* CB_2R-*pKi* CB_1R).

are foreseen. NTRX-07 preserves CB_2R 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).

CB_2R 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 CB_2R selectivity over CB_1R, and favorable pharmacokinetic properties, translating into potent analgesic effects (ED_{50} = 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 (https://clinicaltrials.gov/ct2/show/NCT00479427) and dental pain (https://clinicaltrials.gov/ct2/show/NCT00444769).

Disubstituted phenyl derivative KN 387271 (Fig. 20) is a dual CB_1R/CB_2R 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, 2022e). The selective orally bioavailable CB_2R agonist S-777469 exhibited efficacy in rodent models of scratching and skin inflammation (Odan et al., 2012; Haruna et al., 2015, 2017). However, these effects did not translate into therapeutic benefits in phase 2a trials with patients suffering from atopic dermatitis and pruritus (Clarivate, 2022l; https://www.shionogi.com/content/dam/shionogi/global/investors/pdf/e_p090803.pdf). Many additional CB_2R 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), imidazoleidine-2,4-diones (Mukhopadhyay et al., 2016), pyridines (Porter et al., 2019), and 4-oxo-1,4-dihydropyridines (El Bakali et al., 2014) 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 CB_2R agonists with favorable kinetic profiles were disclosed in a structure kinetics relationship study on a biaryl imidazoleidine-2,4-dione-based scaffold (Soethoudt et al., 2018b). An adamantyl-derived series was investigated for functional activity on the Q63R variant of CB_2R (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. Cannabinoid Receptor 2 Antagonists and Allosteric Ligands. Selective CB_2R antagonist/inverse agonist SR144528 (human CB_2R selectivity ratio = 129; mouse CB_2R selectivity ratio 10^(pKi CB_2R-pKi CB_1R) = 6'026) (Rinaldi-Carmona et al., 1998; Portier et al., 1999; Soethoudt et al., 2017) is an important pharmacological tool for antagonizing effects

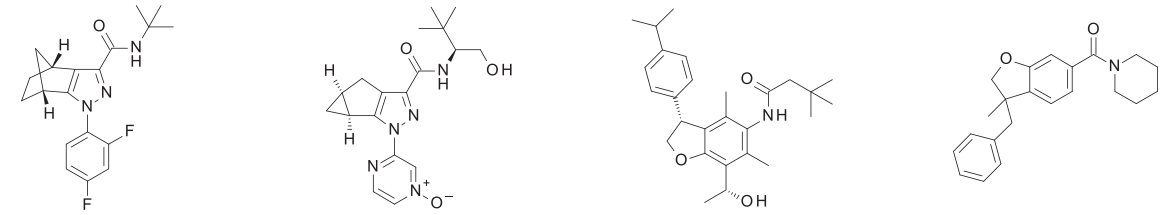


ART-27.13
CB_2R *Ki* = 0.9 nM
ratio CB_1R/CB_2R = 13

LY-2828360
CB_2R EC_{50} = 20 nM
ratio CB_1R/CB_2R >5'000

RG7774
CB_2R EC_{50} = 1 nM
ratio CB_1R/CB_2R >6'940

Fig. 18. Chemical structure, CB_2R binding affinity or functional activity, and selectivity of clinically evaluated bicyclic (het)aryl derived CB_2R ligands.



Tedalinab
CB_2R *Ki* = 12 nM
ratio CB_1R/CB_2R >80

Olorinab
CB_2R EC_{50} = 6.2 nM
ratio CB_1R/CB_2R >1'613

TAK-937
CB_2R IC_{50} = 0.74 nM
ratio CB_1R/CB_2R = 9

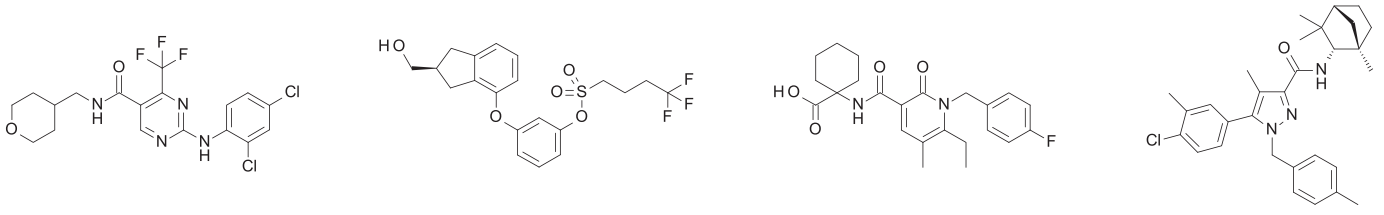
NTRX-07
CB_2R *Ki* = 422 nM
ratio CB_1R/CB_2R >27

Fig. 19. Chemical structure, CB_2R binding affinity or functional activity, and selectivity of clinically evaluated bicyclic aliphatic (het)aryl arrays.

triggered by CB_2R agonists in vitro and 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 CB_2R-mediated signal transduction pathways (Soethoudt et al., 2017).

Few ligands targeting postulated CB_2R allosteric sites (Feng et al., 2014; Pandey et al., 2020) are known. An allosteric CB_2R 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 was shown to act as a pathway-specific CB_2R 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 in silico docking studies.

Endogenously occurring RVD-hemopressin peptide pepcan-12 (Fig. 21) exhibits positive allosteric modulation of CB_2R (Petrucci et al., 2017). It was shown to increase binding of orthosteric ligands and to potentiate 2-AG- and CP55940-induced CB_2R signaling. Synthetic ligand C2 shows positive allosteric modulation of CB_2R 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 CB_2R allosteric modulator in complex with the receptor has been reported. Therefore, the design of novel ligands is mostly aided by 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 CB_2R 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 CB_2R ligands, which should be taken into account in designing selective drugs directed toward CB_2R.



GW-842166X
CB_2R EC_{50} = 63 nM
ratio CB_1R/CB_2R >476

KN 387271
CB_2R *Ki* = 1.9 nM
ratio CB_1R/CB_2R = 3

S-777469
CB_2R *Ki* = 36 nM
ratio CB_1R/CB_2R = 128

SR144528
CB_2R *pKi* = 7.88 [a]
CB_2R = selectivity 129 [b]

Fig. 20. Chemical structure, CB_2R binding affinity or functional activity, and selectivity of clinically evaluated CB_2R agonists and CB_2R inverse agonists SR144528 containing five- and six-membered central cores. [a]Consensus human CB_2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017). [b]CB_2R selectivity (10^(*pKi* CB_2R-*pKi* CB_1R).

3. Cannabinoid Receptor 2 Chemical Probes for Research and Diagnostics. A labeled 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 the target recognition element and reporter unit (Prevet and Collins, 2019). Labeled probes are of utmost importance for all research and discovery phases (Guberman et al., 2022). Due to a major debate regarding the specificity of CB_2R antibodies (Cécyre et al., 2014; Marchalant et al., 2014; Zhang et al., 2019), labeled chemical CB_2R probes are highly important tools for determining CB_2R 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 unlabeled ligands, positron emission tomography tracers focus on determining receptor expression in tissues and noninvasively measuring the distribution and receptor occupancy of drug candidates in patients (Honer et al., 2014). Nonselective [^{3}H]CP55940 and [^{3}H] WIN55212-2 are the most relevant probes for measuring equilibrium binding affinities of novel CB_2R ligands applying radioligand competition-binding assays. Selective CB_2R inverse agonist [^{35}S]SCH225336 (Lavey et al., 2005; Gonsiorek et al., 2006) was successfully applied for quantifying CB_2R expression in various cell lines and hemopoietic cells making use of the superior specific activity of its ^{35}S reporter unit, as compared with tritiated cannabinoids ($>$ 1'400 versus $\sim$20 Ci/mmol) (Fig. 22).

Tritiated pyridine [^{3}H]RO6957022 exhibits high binding selectivity targeting CB_2R (Martella et al., 2017). The CB_2R inverse agonist was used for studying drug-target binding kinetics. Its ^{11}C-labeled analog [^{11}C]RSR-056 carrying the carbon-11 reporter unit at the methoxy group is a CB_2R-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., 2015). 2-Oxoquinoline-derived [^{11}C]NE40 is the first tracer that has been used for CB_2R in vivo positron emission tomography in humans (Ahmad et al., 2013). In agreement with the known expression of CB_2R, major uptake was observed in lymphoid tissue. Despite a rapid brain uptake and washout, no CB_2R upregulation was detected in the brains of Alzheimer's disease patients (Ahmad et al., 2016). [^{18}F]RoSMA-18-d6 exhibits subnanomolar affinity for CB_2R across species and a remarkable selectivity factor of $>$ 12'000 over CB_1R (Haider et al., 2020). It showed specific and reversible target binding in vitro and in vivo and was successfully used for detecting CB_2R upregulation on postmortem human amyotrophic lateral sclerosis spinal cord tissues.

Fluorescently labeled CB_2R 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 labeled ligands that showed selectivity for CB_2R over CB_1R (Petrov et al., 2011). In flow cytometry and confocal microscopy studies, specific binding to endogenously expressed CB_2R in CD4+ T cells and B-lymphocytes was demonstrated. Cy5-labeled (Cy5-) probe is a CB_2R inverse agonist with an extended linker moiety showing low levels of nonspecific 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 CB_2R (Sarott et al., 2020; Westphal et al., 2020). Coumarin fluorophore-labeled 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 visualize in real-time CB_2R 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 CB_2R 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 biologic tissues (Hong et al., 2017). Pyrazolopyrimidine derivative NIR760-XLP6 displays high selectivity over CB_1R and improved specific binding as compared with predecessors such as NIR760-mbc94 and therefore

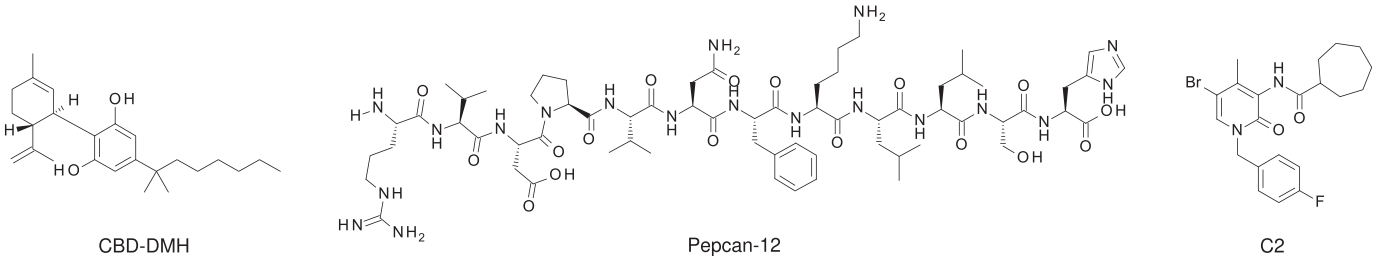


Fig. 21. Chemical structure of validated CB_2R allosteric modulators.

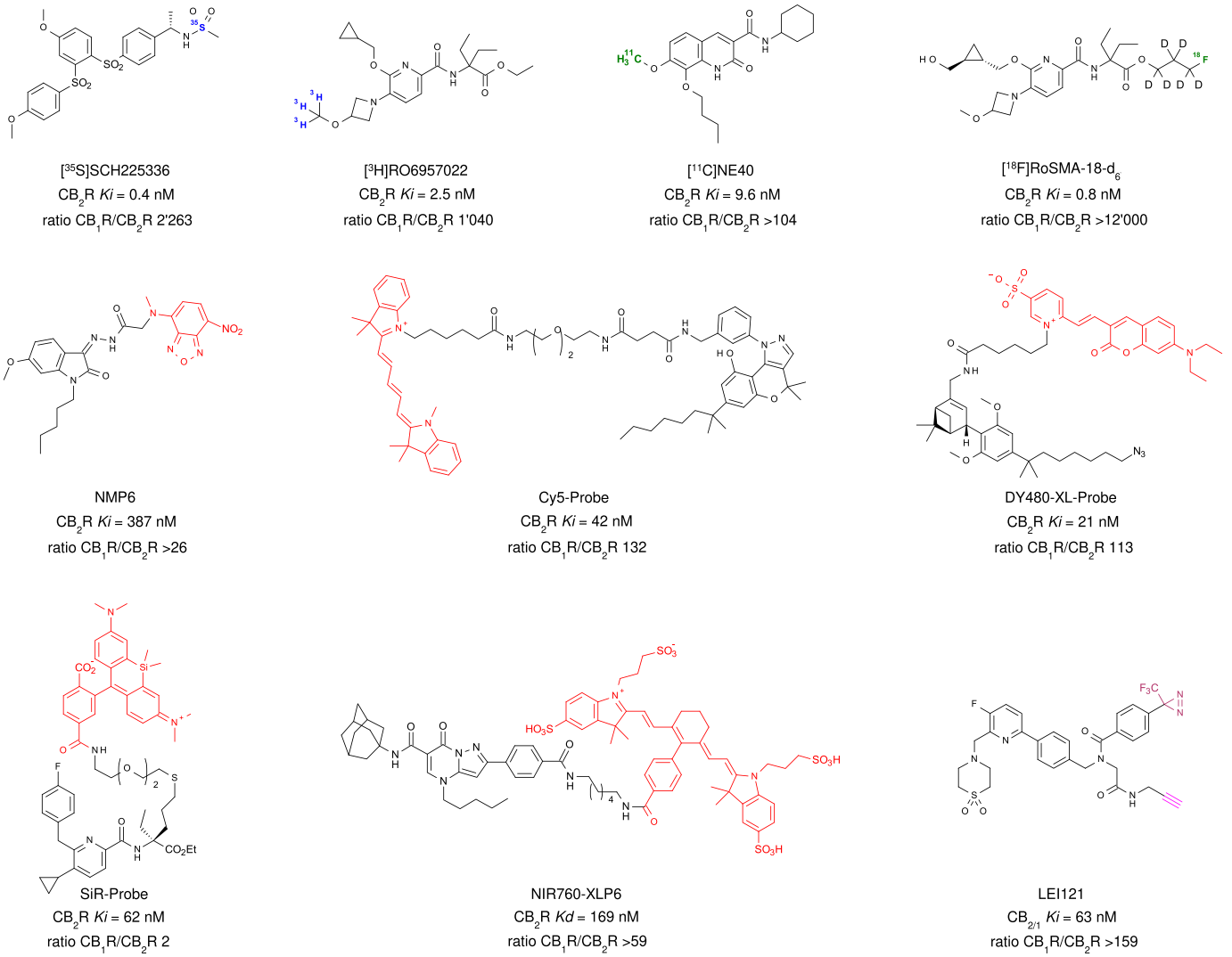


Fig. 22. Chemical structure, CB_2R binding affinity, and selectivity of CB_2R radioligands, PET tracers, fluorescent and pAfBPP probes.

holds promise for visualizing CB_2R in in vivo imaging studies (Ling et al., 2015). Alternatively to fluorescent probes, biotinylated CB_2R ligands have been applied for visualization of the receptor after conjugation with streptavidin-AlexaFluor488 (Martin-Couce et al., 2012).

While reversible noncovalent interaction with CB_2R 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 CB_2R (Szymanski et al., 2011; Mallipeddi et al., 2017). Furthermore, CB_2R-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., 2018a).

C. Summary of Clinical Status of Cannabinoid Receptor 1 and Cannabinoid Receptor 2 Agonists

In summary, three phytocannabinoid preparations (dronabinol, nabiximols, and CBD) are currently available for treatment of diseases via stimulation of CB_1R, CB_2R, both, or neither (Table 6). Although the need for selective full agonist stimulation of CB_1R is limited due to side effects, selective CB_2R agonists are in phase 2 clinical trials. We are at the stage of defining which human diseases can best be treated with these CB_2R agonists. Mixed CB_1R/CB2R-directed agonist preparations and numerous selective CB_2R ligands are either

on the market or under clinical development (reported in Table 6). Overall, more than 20 new molecular entities that activate CB_2R have been investigated in humans for a wide range of indications. Structurally, they cover a huge chemical space including fatty acid derivatives, classic and nonclassic cannabinoids, as well as multiple diverse synthetic ligands, thus resulting also in the coverage of a broad range of physicochemical properties.

Dronabinol, nabilone, and CBD, exerting their action through both CB_1R and CB_2R activation, have been introduced to the market. Oral THC is used for the treatment of anorexia, cachexia, and chemotherapy-induced emesis (Clarivate, 2022d). Buccal THC has been launched for cancer pain (https://adisinsight.springer.com/drugs/800027102). Other routes of administration, e.g., inhalable and sublingual formulations, are under exploration. Nabilone was launched for treating patients who suffer from chemotherapy-induced nausea and vomiting (https://adisinsight.springer.com/drugs/800025856). Clinical trials for the treatment of Parkinson's disease and pain are in advanced stages. CBD, a nonclassic 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, 2022b). 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, 2022g; https://citeline.informa.com/drugs/details/175074). Nonpsychoactive dual CB_1R/CB_2R agonist Lenabasum (Zurier et al., 1998) is in phase 3 trials for the treatment of systemic sclerosis and dermatomyositis (https://adisinsight.springer.com/drugs/800007180; Corbus Pharmaceuticals, 2022). Most advanced selective CB_2R agonists are the synthetic cannabinoids olorinab (https://adisinsight.springer.com/drugs/800039670) 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 to provide an oral treatment for patients suffering from diabetic retinopathy (Clarivate, 2022k). 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 (https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=371547&isReview=true). Pain, in particular neuropathic pain, is also the focus of the selective synthetic CB_2R agonists CNTX-6016, whose structure has not been yet disclosed (https://centrexion.com/science/pipeline/; https://clinicaltrials.gov/ct2/show/NCT04857957), and NTRX-07 (https://www.neurotherapia.com/research; Clarivate, 2022h). CNTX-6016 is in phase 2 and NTRX-07 in phase 1 trials. Dual CB_1R/CB_2R agonist ART-27.13 is in phase 2 trying to provide treatment options for cachexia and cancer-related anorexia (https://artelobio.com/pipeline/). CBD derivative EHP-101, which activates both PPARγ and CB_2R, is aimed at MS and scleroderma patient populations in phase 2 clinical trials (EMA, 2022). Ten additional new

TABLE 6
CB_2R agonist that are launched or under active clinical development

Drug	Chemical Class	Mode of Action	CB_2R/CB_1R in vitro Pharmacology	Indication(s)	Highest Phase of Development
Dronabinol (THC, Syndros, Marinol)	Classic cannabinoid	CB_2R/CB_1R agonist	pKi=8.16/8.48[a]	Appetite loss, CINV, anorexia, cancer pain	Launched
Nabilone (Cesamet)	Classic cannabinoid	CB_2R/CB_1R agonist	Ki=1.84/2.19 nM	CINV	Launched
Lenabasum (Ajulemic acid)	Classic cannabinoid	CB_2R/CB_1R agonist	Ki=51/628 nM	CF, SLE, RA, systemic sclerosis, dermatomyositis	Phase 3 (systemic sclerosis since 2017; dermatomyositis since 2018)
Olorinab (ADP-371)	Tricyclic 3,5,5-fused pyrazole 3-carboxamide	CB_2R agonist	EC_{50}=6.2/>10^4 nM[b]	IBS-related pain, IBS with predominant constipation or diarrhea	Phase 2b (since 2017)
CMX-020	Arachidonic acid analog	CB_2R/CB_1R agonist, TRPV1 agonist	Ki=150/21 nM	Pain, OA, DnP	Phase 2 (since 2015)
RG7774	Triazolopyrimidine	CB_2R agonist	EC_{50}=1/>10^4 nM[c]	DR	Phase 2 (since 2020)
CNTX-6016	Piperidine based ligand	CB_2R agonist	—	Pain Np, DnP	Phase 2 (since 2020)
ART-27.13 (AZD-1940)	Benzimidazole	CB_2R/CB_1R agonist	Ki=0.9/12 nM	Pain, cachexia, CINV	Phase 2 (since 2021)
EHP-101 (VCE-004.8)	Cannabidiol derivative	CB_2R agonist, PPARγ agonist	Ki=170/>4x10^4 nM	MS, ScD	Phase 2 (since 2020)
NTRX-07 (MDA-7)	2,3-Dihydro-1-benzofuran	CB_2R agonist	Ki=422/>10^4 nM	AD, Np pain, cognitive disorder	Phase 1 (since 2019)

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

[a]Consensus human CB_2R binding affinity values from a multicentric collaborative profiling effort between multiple independent academic laboratories and industry (Soethoudt et al., 2017).

[b]Functional activity in β-Arrestin-2 assay on human cannabinoid receptors (Han et al., 2017).

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

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 CB_1R/CB_2R agonist TAK-937 was terminated due to a narrow safety margin (Clarivate, 2022m), S-777469 due to the lack of a pharmacological effect (Clarivate, 2022l; https://www.shionogi.com/content/dam/shionogi/global/investors/pdf/e_p090803.pdf), while for KN 387271 (Clarivate, 2022e) and PRS-211375 13 (Clarivate, 2022j) business reasons were reported.

It is clear from this summary that there are many therapeutic opportunities for both CB_1R and CB_2R agonists (Pacher and Kunos, 2013), yet the untoward effects of the CB_1R at the CNS have limited the clinical progression of CB_1R agonists that penetrate the blood-brain barrier and preclude their use in the nonhospitalized 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 CB_1R-mediated side effects while simultaneously activating CB_2R-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 on 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 understimulating the receptors. In contrast, a negative allosteric modulator would impart noncompetitive antagonism in a situation of excessive eCB tone. Research findings not discussed in the present review have recognized the presence of CB_1R and CB_2R 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 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 $A_{2\varepsilon}$ ($cPLA_{2\varepsilon}$) (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. $cPLA_{2\varepsilon}$ belongs to the $cPLA_2$ family with a serine residue as catalytic nucleophile. Since for *N*-acylation of phosphatidylethanolamine $cPLA_{2\varepsilon}$ 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 $cPLA_{2\varepsilon}$-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 $cPLA_{2\varepsilon}$ 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 coexisting 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. N-Acyl Phosphatidylethanolamine-Specific Phospholipase D. 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 classic 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 has 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 in demonstrating 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 CB_1R 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 multistep 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 $cPLA_{2\gamma}$ (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 physiologic significance in human tissues also remains unclarified.

B. Enzymes of N-Arachidonyl Ethanolamine 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 lipoxygenases (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 physiologic significance of these AEA oxygenation pathways remains unclear.

1. Fatty Acid Amide Hydrolase. 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 CB_1R-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 allosteric kinetics (Dainese et al., 2020). Additionally, colocalization 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, 2023).

2. N-Acylethanolamine Acid Amide Hydrolase. 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 and Lübke, 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. Fatty Acid Amide Hydrolase 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 CB_1R activation by accumulated anandamide (Kathuria et al., 2003; Gobbi et al., 2005; Bortolato et al., 2007).

Importantly, unlike direct-acting CB_1R agonists such as THC, URB597 is not rewarding to nonhuman primates, suggesting a 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 CB_1R 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

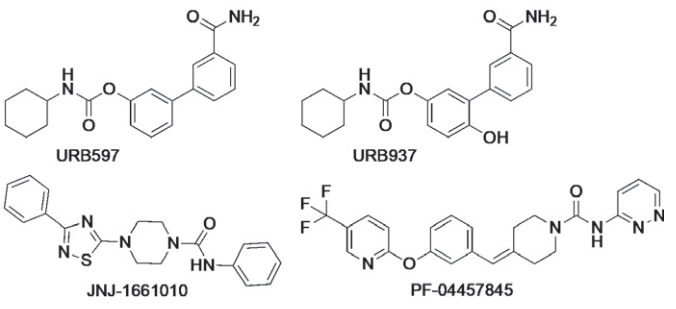


Fig. 23. Chemical structures of representative inhibitors of FAAH.

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 CB_1R blockade, leading to the suggestion that they were mediated by PPARα agonists, such as *N*-oleoylethanolamine and *N*-palmitoylethanolamine, rather than by AEA acting at CB_1R. 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).

Several compounds (URB597, PF-04457845, SSR411298, APD8477, V158866, BIA 10-2474, and JNJ-42165279) have also been tested in clinical trials (Table 8). Of note, the FAAH inhibitor BIA 10-2474 led to adverse neurologic 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 neurologic effects and BIA 10-2474 was shown to have multiple off-targets, inhibition of FAAH is considered to be safe.

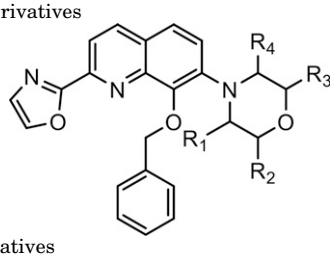
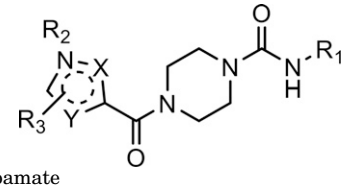
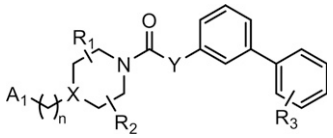
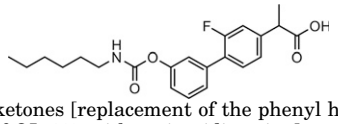
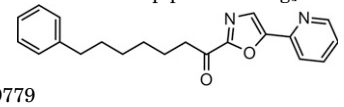
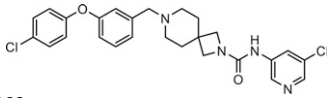
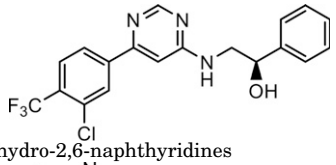
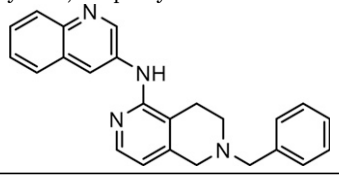
D. N-Acylethanolamine Acid Amide Hydrolase

The search for potent, selective, and systemically active NAAA inhibitors started in 2009 with the identification of the β-lactone derivative *N*-[(3*S*)-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).

Due to the 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 et al., 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 with (*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

TABLE 7
Potential therapeutic use of patented FAAH inhibitors[a]

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

[a]For further details see Fazio et al. (2020).

(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 UV 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 al., 2013, 2018). The striking effects produced by ARN077 on critical mediators of the allergic response (e.g.,

TABLE 8
Diseases/symptoms for treatment with FAAH inhibitors registered with ClinicalTrials.gov[a]

Generic Name Brand Name Class/Efficacy	Completed Clinical Trials	Ongoing Clinical Trials
FAAH inhibitors		
PF-04457845	Tourette syndrome Cannabis withdrawal Fear conditioning Acute pain • Chronic pain • Knee osteoarthritis	Cannabis use disorder
URB597		Schizophrenia
SSR411298	Major depressive disorder Cancer pain	
APD8477	Peripheral neuropathic pain	
V158866	Neuropathic pain	
JNJ-42165279	Major depressive disorder Social anxiety disorder Autism	

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

interleukin 4 and immunoglobulin E) (Sasso et al., 2018) and the efficacy demonstrated by *N*-palmitoylethanolamine as an adjuvant treatment of 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., 2019b) 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., 2021a) 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.

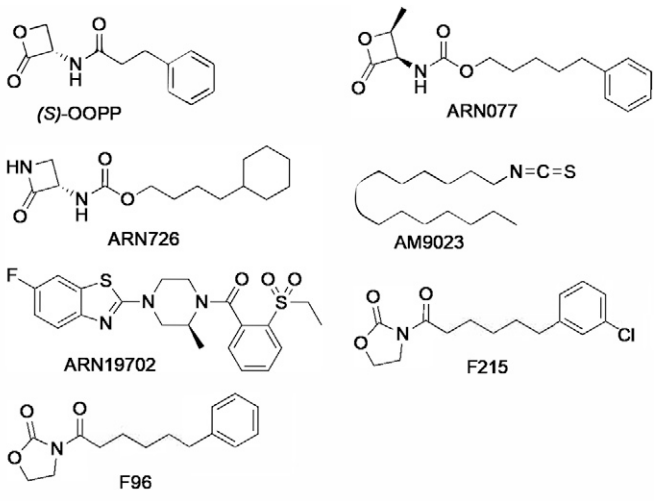


Fig. 24. Chemical structures of representative inhibitors of NAAA.

IV. Therapeutic Potential of Metabolic Enzymes of 2-AG

A. Metabolism of 2-Arachidonoylglycerol

The endocannabinoid 2-AG can be produced via two distinct biologic pathways. The metabolic pathway uses *sn*-2 arachidonoyl-containing triglycerides, which are hydrolyzed by hormone-sensitive lipase, carboxyl esterases, or other lipases toward *sn*-2 arachidonoyl DAGs (Baggelaar et al., 2018). The signaling pathway utilizes phosphatidylinositol-4,5-bisphosphate, which is converted by PLC β in the CNS or PLCγ2 in immune cells. The PLC enzymes are activated by Ca^{2+} ions and integrate G_q protein-coupled receptor activation and extracellular Ca^{2+} influx via ionotropic receptors and voltage-gated Ca^{2+}-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 *dagl*α and *dagl*β, 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 CB_1R or CB_2R occurs through hydrolysis of the ester bond, thereby generating AA and glycerol. MAGL (also termed monoglyceride lipase) is the main enzyme responsible for the inactivation of 2-AG in the brain (Dinh et al., 2002), whereas α/β-hydrolase domain protein 6 and 12 may play a role in 2-AG hydrolysis in specific cell types (Marrs et al., 2010; 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 proinflammatory 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 next sections their therapeutic potential is described. For an extensive review on chemical probes of the endocannabinoid system, see also Punt et al. (2023).

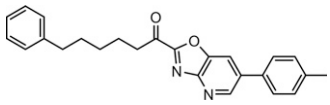
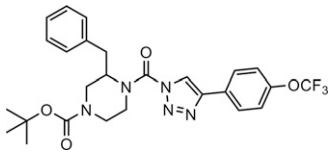
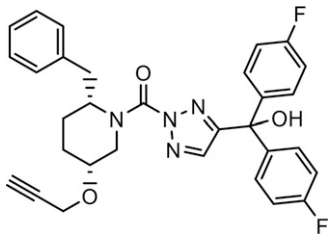
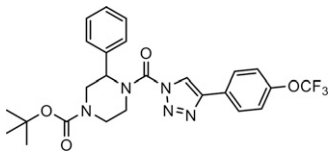
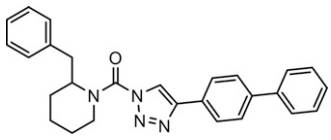
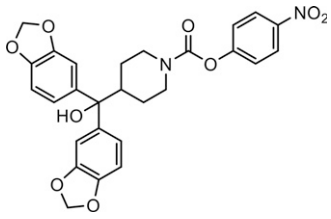
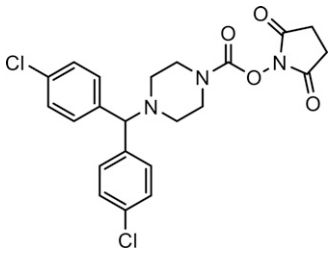
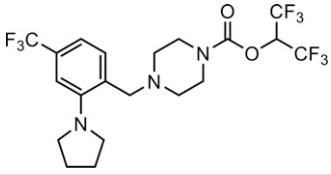
B. Therapeutic Potential of Diacylglycerol Lipase-α

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).

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 neurologic 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 (Shonesy et al., 2014; Jenniches et al., 2016), 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 physiologic 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 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, 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. 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., 2007). Interestingly, the surface localization of DAGLα was shown to be a dynamic process controlled by protein kinase C. DAGLα colocalized 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, 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 *dagl*α 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

TABLE 9
Modulators of 2-AG metabolism

Name	Target	Phase	Structure	Reference
LEI-105	DAGL	Preclinical		Baggelaar et al., 2015
DO34	DAGL	Preclinical		Ogasawara et al., 2016
DH376	DAGL	Preclinical		Ogasawara et al., 2016
DO53	Negative control compound	Preclinical		Ogasawara et al., 2016
KT109	DAGL	Preclinical		Hsu et al., 2012
JZL184	MAGL	Preclinical		Long et al., 2009
MJN110	MAGL	Preclinical		Niphakis et al., 2013
ABX-1431 (Lu-AG06466)	MAGL	Phase 2		

subcellular localization of DAGLα and its activity result in abnormal neurotransmission and neurologic 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 is the main enzyme responsible for the production of 2-AG in immune cells, including microglia that are the brain resident macrophages. DAGLβ is a 70 kDa multidomain, integral membrane serine

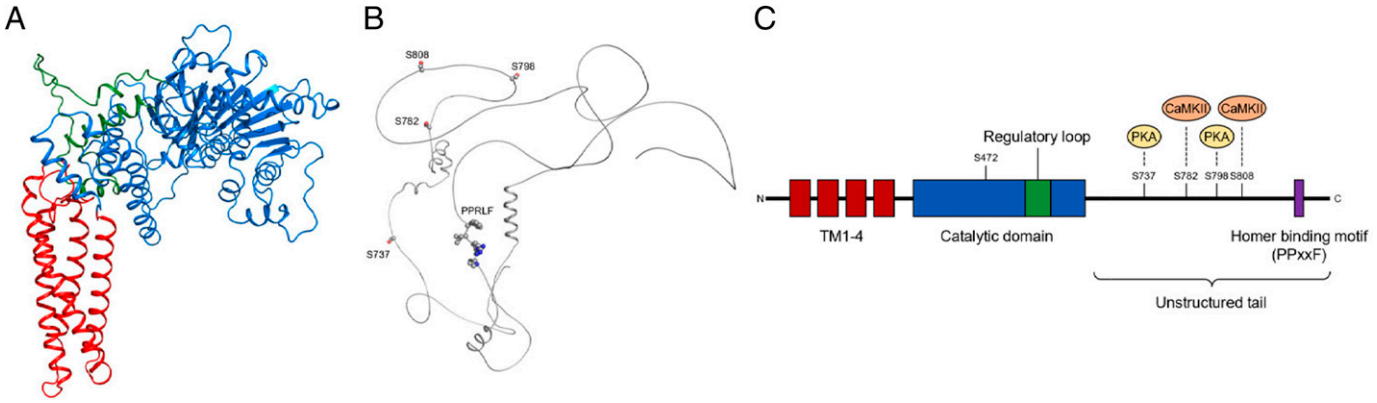


Fig. 25. (A) Structured part of the AlphaFold model for human DAGLα, residues 1-681; red: transmembrane domain, blue: catalytic domain, green: regulatory loop. (B) Unstructured tail region from the AlphaFold model, residues 682–1042 highlighting potential phosphorylation sites, as discussed in the text,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

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 depolarization-induced suppression of inhibition 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 is 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). PLC$_{\gamma 2}$, 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 PLC$_{\gamma 2}$ 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 PLC$_{\gamma 2}$.

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 (Wilkerson et al., 2016; Shin et al., 2018), 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 earlier, nonselective 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 is a membrane-associated serine hydrolase, which was cloned in 1997, and consists of two tissue-specific splice-variants with a molecular weight of 33 kDa and 36 kDa. 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 noncatalytic 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 CB_1 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 CB_1R, 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., 2013). 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 (Hernández-Torres et al., 2014) 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., 2015a). For recent reviews, see Gil-Ordóñez et al. (2018), Deng and Li (2020), and Van Egmond et al. (2021). Of note, chronic, high dosing of MAGL inhibitors caused desensitization and downregulation of CB_1R, and behavioral tolerance to CB_1R agonists. A therapeutic window for antinociceptive efficacy without CB_1R 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 (Müller-Vahl et al., 2021, 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 Clinical Trials.gov at the date of this review are shown in Table 10.

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 toward the development of ECS modulators have been primarily dedicated to eCB degradation inhibitors, in particular FAAH and MAGL blockers (Blankman and Cravatt, 2013; Fowler, 2021; van Egmond et al., 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

TABLE 10
Clinical trials for MAGL inhibitor Lu-AG06466 listed in ClinicalTrials.gov

Identifier	Status	Condition	Title
NCT04597450	Ongoing	PTSD	Lu-AG06466 in Participants With Post Traumatic Stress Disorder
NCT04990219	Ongoing	Multiple sclerosis	A Study of Lu-AG06466 for the Treatment of Spasticity in Participants With Multiple Sclerosis
NCT05028673	Completed	Healthy	A Study to Evaluate a New Tablet Formulation of Lu-AG06466 in Healthy Participants
NCT04713254	Completed	Healthy	Drug Drug Interaction Study With Lu-AG06466 in Young Healthy Men
NCT04405323	Completed	Healthy	Study That Evaluates the Effect of CYP3A4 Inhibition on Lu-AG06466 in Healthy Men and Women
NCT04419636	Completed	Healthy	Binding of Lu-AG06466 in the Brain in Healthy Men
NCT05081518	Terminated	Focal epilepsy	A Study of Lu-AG06466 in Participants With Treatment Resistant Focal Epilepsy
NCT05201092	Completed	Healthy	A Study Investigating Lu-AG06466 in Healthy Men
NCT05219838	Completed	Healthy	Binding and Effects of Lu-AG06466 in the Brain of Healthy Men
NCT04974359	Terminated	Fibromyalgia	A Study to Evaluate Lu-AG06466 in Participants With Fibromyalgia
NCT05177029	Terminated	Healthy	Safety and Tolerability Study of Lu-AG06466 in Healthy Young Japanese and Caucasian Participants

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, focusing 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 CB_1R/CB_2R-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 with FAAH and MAGL inhibitors.

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 the 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 minutes), 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 or 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 (Km = 0.3 μM), cortical neurons (Km = 1.2 μM), and human neuroblastoma CHP100 cells (Km = 0.2 μM) (Beltramo et al., 1997; Maccarrone et al., 1998), with values comparable to those obtained with the transporters of serotonin (Km = 0.3–0.5 μM), dopamine (Km = 0.9–1.2 μM), and noradrenaline (Km = 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 Km 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 with that of FAAH. In an experiment on [^{3}H]AEA uptake competition in U937 cells, different eCBs congeners (AEA, 2-AGE, *O*-AEA, and NADA, shown in Table 3) competed with [^{3}H]AEA uptake, suggesting that a common cellular membrane uptake mechanism seemingly competes for one target related to cellular eCB uptake (Chicca et al., 2012).

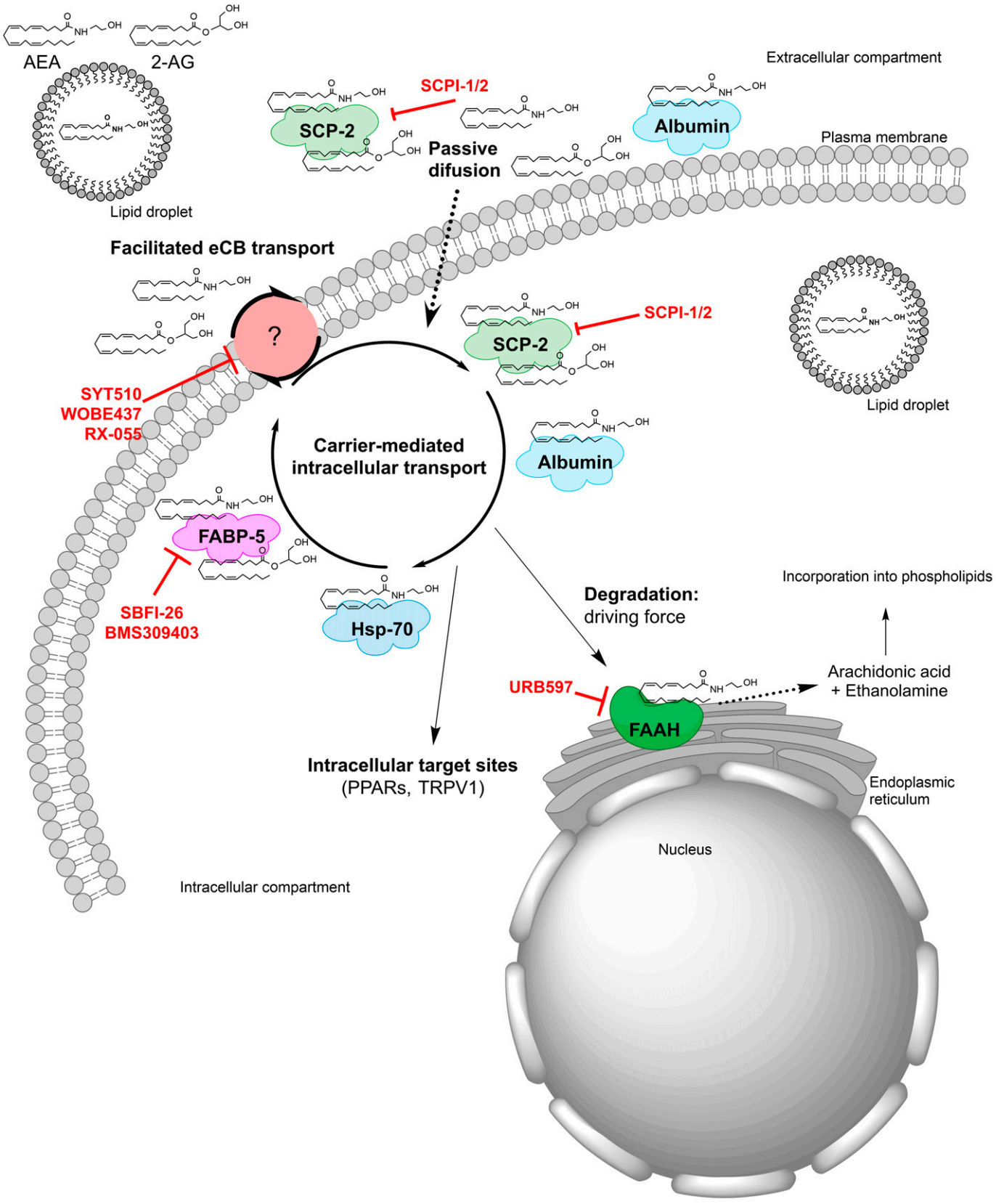


Fig. 26. Model of eCB membrane transport and trafficking showing druggable targets. Molecular pharmacology and possible implications for therapeutic intervention of using diverse eCB transport inhibitors are shown. See text for details and abbreviations.

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., 2011). 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 were 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 IC_{50} values between SCP2-expressing and wild-type 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 were 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 regarding their druggability; i.e., it remains unclear whether competing for extracellular AEA protein binding would exert robust cannabimimetic effects, as well as diverse CB_1R/CB_2R-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 CB_1R/CB_2R 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.

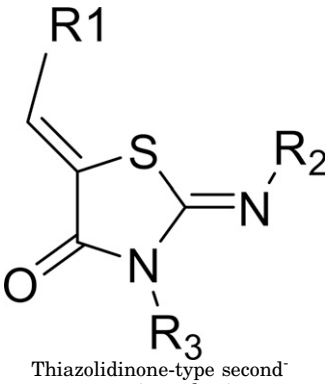
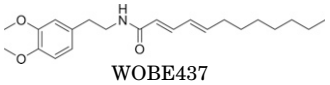
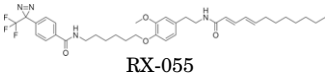
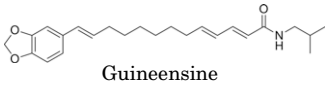
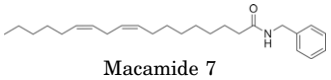
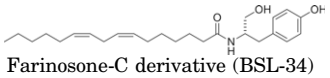
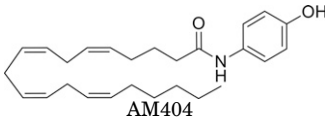
B. Evolution of Pharmacological Inhibitors of N-Arachidonyl Ethanolamine Transport

In the 1990s, the first AEA uptake inhibitors were synthesized. Based on the observed substratespecificity, initially mainly structural analogs of AEA were synthesized and tested for [^{3}H]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 IC_{50} value ~1 μM in neurons and an IC_{50} 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 toward uptake inhibition over FAAH inhibition (IC_{50} > 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 IC_{50} 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).

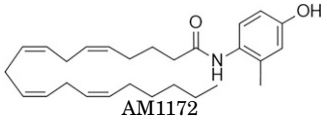
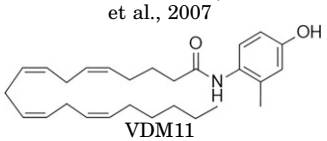
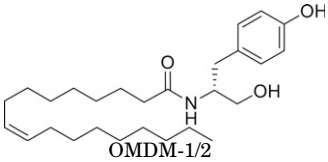
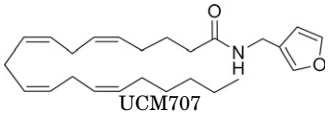
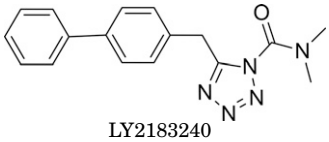
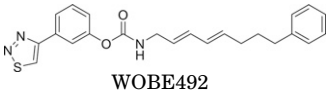
The quest for better AEA cellular uptake inhibitors continued with the aim to increase their

TABLE 11
Potent inhibitors of AEA cellular uptake relative to inhibition of AEA degradation (by FAAH) or intracellular transport (by FABP5)

Compound	AEA Cellular Uptake Inhibition			FAAH	**FABP5**
	IC_{50} (μM)	Cell Type	AEA Cellular Uptake Kinetics	IC_{50} value (μM)	K_i value (μM)
 Thiazolidinone-type second-generation selective endocannabinoid reuptake inhibitors (SERIs) Patent ES2845636T3	0.08-0.50	U937	ND	>10	>10
 WOBE437 Chicca et al., 2017	0.01 0.137 0.055 ~1 [50% inh.]	U937 HMC-[a] Neuro2A Rat cortical neurons	K_m = 0.25 μM, V_{max} = 67.4 fmol/min/cell in U937 cells	>10	>50
 RX-055 Chicca et al., 2017	0.014 ~1 [35% inh.]	U937 Neuro2A	ND	4.0	ND
 Guineensine Nicolussi et al., 2014b	1.32 0.29 0.62	U937 U937 HMC-1[a]	ND	46.8	>100
 Macamide 7 Hajdu et al., 2014	0.67	U937	ND	4.1	ND
 Farinosone-C derivative (BSL-34) Burch et al., 2013	0.23	U937	ND	>10	ND
 AM404 Beltramo et al., 1997; Maccarrone et al., 1998; Piomelli et al., 1999; De Petrocellis et al., 2000; Rakhshan et al., 2000; Jarrahian et al., 2000; Deutsch et al., 2001; Porter et al., 2002; Glaser et al., 2003; López-Rodríguez et al., 2003a; Fowler et al., 2004; Vandevoorde and Fowler, 2005; Dickason-Chesterfield et al., 2006; Hillard et al., 2007; Nicolussi et al., 2014a	1.0 5.0 2.2 10.2 10.9 8.1 10.2 4 $K_i \approx 14$ 14.9 20 1.8 4.9 >100 44.4	Rat cortical neurons Rat cortical astrocytes Astrocytoma C6 glioma RBL-2H3 RBL-2H3 RBL-2H3 RBL-2H3 RBL-2H3 RBL-2H3 RBL-2H3 U937 Cerebellar granular neurons HMC-[a] HeLa[a]	K_m = 1.2 μM, V_{max} = 90.9 pmol/min/mg in neurons K_m = 0.32 ± 0.1 μM, V_{max} = 171 pmol/min/mg in astrocytes K_m = 0.6 ± 0.1 μM, V_{max} = 14.7 ± 1.5 pmol/min/mg in astrocytoma K_m = 0.7 ± 0.1 μM, V_{max} = 0.39 fmol/min/cell in C9 glioma cells K_m = 11.4 ± 2.3 μM, V_{max} = 17.5 ± 2.1 × 10^{-17} mol/min/cell in RBL-2H3 cells K_m = 0.13 ± 0.01 μM, V_{max} = 140 ± 15 pmol/min/mg in U937 cells	5.9 3.6 0.5 0.8 >30 K_i = 0.60 6 2.1	0.39

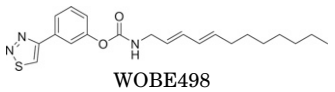
(*continued*)

TABLE 11—*Continued*

Compound	AEA Cellular Uptake Inhibition			FAAH	**FABP5**
	IC_{50} (μM)	Cell Type	AEA Cellular Uptake Kinetics	IC_{50} value (μM)	K_i value (μM)
 AM1172 Fegley et al., 2004; Dickason-Chesterfield et al., 2006; Kaczocha et al. 2006; Hillard et al., 2007	2.5 2.1 24.0 86.6 68.9	Astrocytoma Rat cortical neurons Cerebellar granular neurons RBL-2H3 HeLa [a]	ND	>5 K_i ≈3.2 >100 >50	ND
 VDM11 De Petrocellis et al., 2000; Fowler et al., 2004; Vandevoorde and Fowler, 2005; Dickason-Chesterfield et al., 2006; Kaczocha et al., 2006; Hillard et al., 2007; Kaczocha et al., 2012; Nicolussi et al., 2014a	10.2 6.1 11.2 9.9 1.1 5.5 >100 23.8	C6 glioma C6 glioma RBL-2H3 RBL-2H3 U937 Cerebellar granular neurons HMC-1[a] HeLa [a]	ND	2.0 1.2 0.4 K_i ≈ 0.44 11.1[74] 5[111] 1.6 - 2.9	1.75
 OMDM-1/2 Ortar et al., 2003; Fowler et al., 2004; Dickason-Chesterfield et al., 2006; Kaczocha et al., 2006; Hillard et al., 2007; Chicca et al., 2012; Kaczocha et al., 2012; Nicolussi et al., 2014a	K_i ≈ 3 16.6 3.2 9.1 3.93 5.2 3.2 3.1 >100 4.9	RBL-2H3 C6 glioma RBL-2H3 RBL-2H3 U937 U937[64] U937 HMC-1[a] HeLa[a] Cerebellar granular neurons	ND	>50 54 23.4 >100 K_i ≈ 9.7 >100 >50	3.85 >100
 UCM707 López-Rodríguez et al., 2003a; 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., 2014a	0.8 1.34 1.8 41 25 20.1 3.6 56.4 30.3 4 3	U937 U937 U937 C6 glioma RBL-2H3 RBL-2H3 HMC-1[a] HeLa[a] Cerebellar granular neurons Cortical neurons Cortical neurons $FAAH^{-/-}$	K_m = 1.1 μM, V_{max} = 151 pmol/min/mg in WT cortical neurons K_m = 1.3 μM, V_{max} = 157 pmol/min/mg in $FAAH^{-/-}$ cortical neurons	30 8.32 >100 K_i ≈ 0.37 20.5 50	25.8
 LY2183240 Moore et al., 2005; Alexander and Cravatt, 2006; Dickason-Chesterfield et al., 2006; Ortar et al., 2008; Nicolussi, 2014; Nicolussi et al., 2014a	0.000270 0.015 0.00095 1.93 29.7	RBL-2H3 RBL-2H3 U937 HMC-1[a] HeLa[a]	K_m = 4.69 ± 0.46 μM, V_{max} = 0.02 fmol/min/cell in RBL-2H3 cells	0.014 0.0021 0.0124 0.001	ND
 WOBE492 Nicolussi et al., 2014	0.000005 0.12	U937 HMC-1	ND	0.000014	ND

(*continued*)

TABLE 11—*Continued*

Compound	AEA Cellular Uptake Inhibition			FAAH	**FABP5**
	IC_{50} (μM)	Cell Type	AEA Cellular Uptake Kinetics	IC_{50} value (μM)	K_i value (μM)
 WOBE498 Nicolussi et al., 2014	0.00005 0.25	U937 HMC-1	ND	0.000015	ND

ND, not determined.
[a]Cells lacking FAAH-activity.

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 the inhibition of [^{3}H]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, 2003a) 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 $IC_{50} = 3 \pm 1$ 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., 2003b). 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 seconds) and inhibited FAAH (Glaser et al., 2003). Ligresti and colleagues convincingly showed saturable AEA uptake within 90 seconds 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 nonphysiologic AEA concentrations (1–100 μM) were used, which may easily mask the transport kinetics seen with concentrations of 50 to 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 $IC_{50} = 270 \pm 29$ 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 afterward, 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., 2002) 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 with 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., 2014b) show robust AEA uptake kinetics, although with a lower Vmax than in FAAH-expressing cells. Moreover, an energy-independent and saturable export of [^{3}H]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 toward 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 CB_1R (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 CB_1 R 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 CB_1R- and TRPV1-mediated mechanisms (Llorente-Berzal et al., 2015). Finally, in nonhuman primates, AM404 reinforced anandamide or cocaine self-administration behavior and induced reinstatement of drug-seeking behavior in abstinent monkeys by a CB_1R-dependent mechanism (Schindler et al., 2016a).

C. Preclinical Development of Selective Endocannabinoid Reuptake Inhibitors

The *N*-isobutylamide 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., 2014b). Guineensine did not show a relevant inhibition of FAAH activity ($IC_{50} > 50$ 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 CB_1R 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 that 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-(9*Z*,12*Z*)-octadecadieneamide (macamide 7, shown in Table 11), which exhibited a nanomolar IC_{50} 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 ($IC_{50} = 232$ nM) over FAAH inhibition ($IC_{50} > 10$ μM), with close structural similarity to OMDM-2 (Burch et al., 2013).

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., 2014a). Interestingly, some of these *N*-alkylcarbamates (e.g., WOBE492 and WOBE498) showed a FAAH-independent AEA uptake inhibition in HMC-1 cells with IC_{50} values below 300 nM (Nicolussi et al., 2014a). This study led to the identification of (2*E*,4*E*)-*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_{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_1R/CB_2R, 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_1R/CB_2R-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 neurologic disorders and inflammation at Synendos Therapeutics, though their target protein has not yet been published.

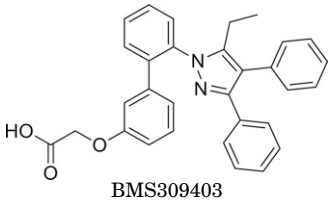
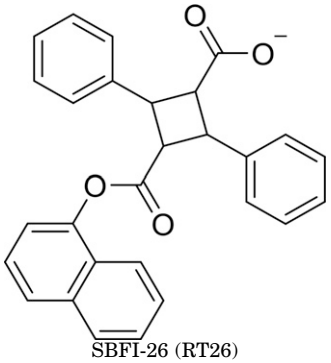
D. Preclinical Development of Fatty Acid Binding Protein Inhibitors

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., 2018). Studies using FABP1 knockout mice revealed a markedly diminished impact of a 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, a reduction of AEA uptake and hydrolysis could be monitored after preincubation 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., 2014a).

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., 2019a), 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 remodeling 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

TABLE 12
Additional and less potent inhibitors of AEA cellular uptake relative to inhibition of AEA degradation (by FAAH) or intracellular transport (by FABP5)

Compound	AEA Cellular Uptake Inhibition		FAAH	FABP5
	IC_{50} (μM)	Cell Type	IC_{50} Value (μM)	K_i Value (μM)
 BMS309403 Sulsky et al., 2007; Kaczocha et al., 2009; Berger et al., 2012; Kaczocha et al., 2012; Nicolussi et al., 2014a	~100 [57% inh.] >100 [48% inh.] 24.7 >100 [30% inh.]	N18TG2 COS7-FAAH-eGFP U937 HMC-1*	>100 >100	0.35 0.75 0.89
 SBFI-26 (RT26) Berger et al., 2012: Zhou et al., 2019a	~20 [40% inh.]	HeLa[a]	>50	0.9

[a]Cells lacking FAAH-activity.

cytosolic FABP5, which is an AEA carrier and binds numerous highly abundant fatty acids, was also shown to bind 2-AG. Using fluorescence polarization and a labeled fatty acid probe that was displaced from FABP5, a Ki = 8.7 μM was determined (Nicolussi, et al., 2014a). 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 CB_1R 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 CB_1R 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 coculture 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 CB_1R, DAGLα (the neuronal isoform) or MAGL, concluding that there are no changes in either CB_1R activation or 2-AG metabolism. Although nonsignificant 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 with previous reports (Kaczocha et al., 2009; 2014); however, there were no changes in AEA levels measured in FABP5−/− neurons compared with 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 CB_1R 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 CB_1R (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 physiologic 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 Endocannabinoid 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 a 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).

The new generation of selective inhibitors for AEA uptake (WOBE437, 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 the desensitization of CB_1R (Reynoso-Moreno et al., 2021).

VI. Therapeutic Potential of Additional Targets Within the "Endocannabinoidome"

A. Definition of the Endocannabinoidome

Two realizations during the past 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 biologic fluids, although they seldom share with eCBs the capability of modulating the activity of CB_1R and CB_2R (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 aforementioned eCB-like molecules (Di Marzo, 2018). The main components of the eCBome are summarized in Table 14.

In particular, beyond *N*-acyl-ethanolamines and 2-monoacylglycerols, whose existence was known even before the discovery of AEA and 2-AG, several other subfamilies 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; (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

TABLE 13
Drug candidates that target eCB transport in late preclinical stage

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

SERI, selective eCB reuptake inhibitor.

cascade" enzymes COX-2 and LOXs) of the di- and polyunsaturated members of the aforementioned families (namely *N*-acyl-ethanolamines and 2-monoacylglycerols), a subfamily 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 docosahexeanoic 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 bidimensional 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 subfamilies, depending on its fatty acid and amine moieties, can modulate the activity of one or more different receptors that 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 CB_1R and CB_2R (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 CB_1R and CB_2R 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 Ca^{2+} (Ca_{v3}) 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 CB_1R (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. While 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 these 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 noneuphoric cannabinoid CBD (Watanabe et al., 1996; Bisogno et al., 2001).

As mentioned, several non-THC cannabinoids, as well as AEA and 2-AG, have also been suggested to influence the activity of the aforementioned eCBome receptors, including (1) orphan GPCRs, particularly for CBD, which antagonizes GPR55 and seems to modulate some opioid and serotonin receptor subtypes (reviewed by de Almeida and Devi, 2020); (2) TRPV1-4 or TRPA1 channels, which are activated by several noneuphoric cannabinoids—with TRPV1 now being widely considered also as an alternative physiopathological target for AEA, unsaturated NAEs, and 2-AG—and TRPM8, which is antagonized 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) $Ca_{v3.3}$ 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.

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 biologic 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-CB_1R, non-CB_2R 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

TABLE 14
Lipid signals from the eCBomes, their targets and metabolic enzymes

	Family	Most Studied Subfamily (If Applicable)	Most Studied And/Or Most Tissue Abundant Members	Best Established Molecular Target(s)	Best Established Anabolic Enzyme(s)	Best Established Catabolic Enzyme(s)	Main Potential Therapeutic Applications (Based On The Best Established Pharmacological Actions)	References
eCBome	NAEs (AEA and its congeners)		*N*-Arachidonoyl-ethanolamine (anandamide)	CB_1R (↑), CB_2R (↑), TRPV1(↑), TRPV2(↓), TRPM8(↓), Cav3.3(↓)	NAPE-PLD, ABHD4+GDE1	FAAH-1	Chronic and inflammatory pain; anxiety; depression; (neuro)inflammatory disorders; cancer. *Obesity, NASH and type 2 diabetes*	Mechoulam, 2023
			N-Docosa-hexaenoyl-ethanolamine	GPR110(↑),CB_2 R(↑)		FAAH-1	Inflammation; neurodegenerative disorders; cancer.	Watson et al., 2019
			N-Oleoyl-ethanolamine	PPARα(↑), TRPV1(↑), GPR119(↑)		FAAH-1, FAAH-2	Obesity, type 2 diabetes, steatosis and related disorders	Bowen et al., 2017
			N-Palmitoyl-ethanolamine	PPARα(↑), GPR55(↑)		NAAA, FAAH-1	Chronic and inflammatory pain; eczema, neuroinflammatory disorders; migraine; COVID-19	Petrosino and Di Marzo, 2017
	MAGs (2-AG and its congeners)		2-Arachidonoyl-glycerol	CB_1R(↑), CB_2R (↑), TRPV1(↑), TRPM8(↓)	DAGLα, DAGL*β*	MAGL, ABHD6, ABHD12	Chronic and inflammatory pain; anxiety; depression; (neuro)inflammatory disorders, obesity and type 2 diabetes	Baggelaar et al., 2018
			2-Oleoyl- and 2-Linoleoyl-glycerol	GPR119(↑), TRPV1(↑)			Type 2 diabetes	Hansen and Vana, 2019
			1- or 2-Palmitoyl-glycerol	PPARα(↑)			Obesity, type 2 diabetes, steatosis and related disorders	Depommier et al., 2021
	Primary amides		Oleamide	TRPV2(↓), CB_1 R(?)(↑)	GLYATL3+PAM	FAAH-1	Sleep disorders	Leggett et al., 2004; Schiano Moriello et al., 2018
	N-Acyl-amino acids (lipo-aminoacids)	*N*-Acyl-glycines	*N*-Oleoyl-glycine *N*-Palmitoyl-glycine *N*-Arachidonoyl-glycine	PPARα(↑), FAAH-1(↓), Cav3.3(↓), GPR132(↑), GPR118 (?)(↑)	GLYATL3	PAM FAAH-1	Brain trauma and its consequences, neuroprotection, nicotine and opiate addiction, pain	Huang et al., 2001; Foster et al., 2019; Donvito et al., 2019; Piscitelli et al., 2020
		N-Acyl-alanines	*N*-Oleoyl-alanine	PPARα(↑), FAAH-1(↓)	?	?	Nicotine and opiate addiction	Ayoub et al., 2020
		N-Acyl-serines	*N*-Oleoyl-serine	Cav3.3(↓)		?	Osteoporosis	Milman et al., 2006
		N-Acyl-taurines	*N*-Oleoyl- and *N*-Arachidonoyl-taurine	TRPV1(↑), TRPV4(↑), Cav3.3(↓)		FAAH-1	Skin wound healing, type 2 diabetes and dyslipidemia	Grevengoed et al., 2019; Sasso et al., 2016.
	N-Acyl-neurotransmit-ters	*N*-Acyl-dopamines	*N*-Arachidonoyl-dopamine	TRPV1(↑), CB_1 R(↑), FAAH(↓), Cav3.3(↓)	?	COMT, Cyp epoxygenases	Pain, cancer, nausea, neuroinflammatory and neurodegenerative disorders	De Petrocellis and Di Marzo, 2014

(*continued*)

TABLE 14—*Continued*

	Family	Most Studied Subfamily (If Applicable)	Most Studied And/Or Most Tissue Abundant Members	Best Established Molecular Target(s)	Best Established Anabolic Enzyme(s)	Best Established Catabolic Enzyme(s)	Main Potential Therapeutic Applications (Based On The Best Established Pharmacological Actions)	References
		N-Acyl-serotonins	*N*-Arachidonoyl-serotonin	TRPV1(↓), FAAH(↓), Cav3.3(↓)		Cyp epoxygenases	Chronic and inflammatory pain, anxiety, depression, (neuro)inflammatory disorders, epilepsy, IBS	
OxyeCBome	COX-2 derivatives	Prostamides	Prostamide $F_{2\alpha}$	FP/Alt4-FP heteromer (↑)	COX-2 +PGFS	?	Glaucoma, obesity, alopecia pain	Woodward et al., 2013
		Prostaglandin-glycerol esters	Prostaglandin E_2 -glycerol ester	P2Y6(↑)	COX-2 +PGES	MAGL	Hippocampal excitotoxity	Brüser et al., 2017
	15-LOX derivatives	Linoleoylethanolamide derivatives	13-hydroxy-octadecaenoyl-ethanolamide	TRPV1(↑)	15-LOX	?	?	Simard et al., 2022
		Anandamide derivatives	15-hydroxy-eicosatetraenoyl-ethanolamide	CB_1R(↑), CB_2R (↑) TRPV1(↑)			?	
	Cyp epoxygenase derivatives	Epoxyeicosatrienoyl-ethanolamides	5,6- Epoxy-eicosatrienoyl-ethanolamide	CB_2R(↑), TRPV4(?) (↑)	Cyp epoxygenase	?	Cardiovascular disorders, hypertension, inflammation	Snider et al., 2009
		Epoxyeicosatrienoyl-neurotransmitters	Epoxyeicosatrienoyl-dopamine and -serotonin	CB_1R(↑), CB_1R(↑) TRPV1(↑)		?	Neuroinflammation	Arnold et al., 2021
μbeCBome	Microbiota-derived *N*-Acyl-amides	Glycine and alanine derivatives	Commendamide, *N*-Myristoyl-alanine	GPR132(↑)	Bacterial *N*-acyl-transferase (*choA*/*glsB*)	?	Cancer, intestinal inflammation	Cohen et al., 2017
			Diacylated glycine lipids	?	Bacterial *N*-acyl-transferase (*choA*/*glsB*) + and *O*-acyl-transferases (*choB*/*glsA*)	?	Intestinal fitness, eubiosis	Lynch et al., 2019
		Serinol derivatives	*N*-Oleoyl-serinol	GPR119(↑)	Bacterial *N*-acyl-transferases	?	Type 2 diabetes	Cohen et al., 2017
		N-Acyl-tyramines, *N*-Acyl-tryptamines, *N*-Acyl-aminoacids	*N*-Lauroyl-tryptamine	GPR183(↓)	Bacterial *N*-acyl-transferases	?	Immune disorders	Chang et al., 2021

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 CB_1R and CB_2R 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. 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.

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 CB_1R and CB_2R, 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 noncannabinoid receptors as their main targets and share with AEA or 2-AG the capability of being inactivated by FAAH (as in the case of *N*-acylethanolamines, -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 CB_1 and CB_2 receptors, but also of other eCBome mediators with targets whose functions in disease might also be 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 a potential treatment of obesity and metabolic disorders through the impairment of 2-AG biosynthesis and subsequent CB_1R activation (Bisogno et al., 2013; Janssen and van der Stelt, 2016), might reduce the levels of 2-monoacylglycerols acting at CB_2R, GPR119, and TRPV1, which, unlike CB_1R, may have beneficial effects in such pathologic conditions (Di Marzo and Silvestri, 2019). In yet other pathologic 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 multitarget drugs, 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.

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 that, like the eCBome, is also strongly influenced by diet, medications, and other environmental and 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 micronutrients, have been well characterized and include, among others, (1) short chain fatty acids (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.

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 CB_1R antagonists and TRPV1 agonists (namely capsaicin) (Cluny

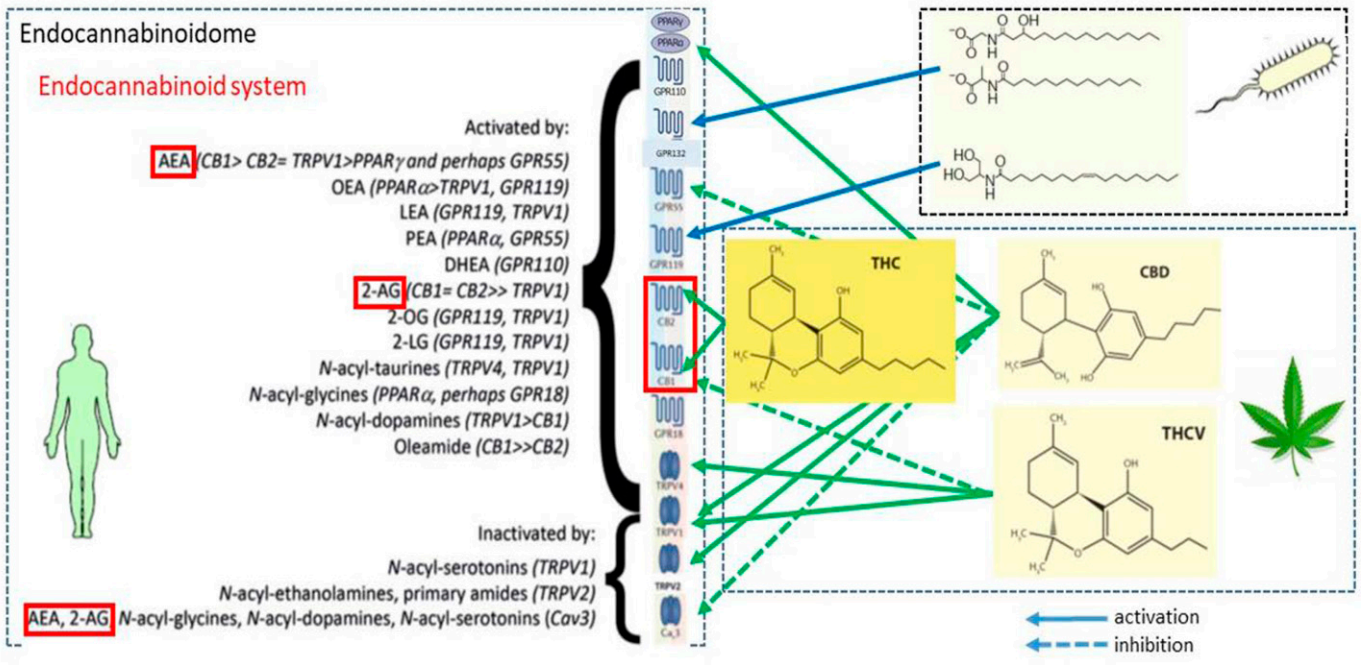


Fig. 27. The eCBome receptors as a pharmacological substrate for plant-derived cannabinoids and host or commensal bacteria-derived eCBs and eCB-like molecules. The elements of the ECS as part of the eCBome are shown squared in red. The chemical structures of commendamide, *N*-miristoyl-alanine, and *N*-oleoyl-serinol are shown from the top right and down. CBD, cannabidiol; THC, Δ^9-tetrahydrocannabinol; THCV, Δ^9-tetrahydrocannabidivarin.

et al., 2015; Shen et al., 2017; Mehrpouya-Bahrami 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, some therapeutic effects of pCBs were likewise found to be accompanied by corresponding beneficial actions on the gut microbiome. Clearly,

TABLE 15
Endocannabinoidome mediators and their synthetic analogs under clinical testing

Name	Company (and Commercial Name) when Available	Indication(s)	Main Proposed Target	Clinical Trials Gov Identifier
N-Palmitoylethanolamine (PEA)	Epitech Italy (Normast, Pelvilen, Glialia)	Neuropathic pain from spinal cord injury Covid-19 Fibromyalgia Chronic pain Chronic pelvic pain in endometriosis Frontotemporal Dementia Tourette's syndrome (in combination with dronabinol)	PPARα GPR55[a] TRPV1[b] CB_2R[b]	NCT01851499 NCT04568876 NCT04488926 NCT02699281 NCT02372903 NCT04489017 NCT03066193
N-Oleoylethanolamine (OEA)	University of California Davis Davis, California, United States NutriForward LLC (RiduZone, 90% OEA)	Post-prandial inflammation Overweight and obesity	PPARα TRPV1 GPR119	NCT05017428 NCT04614233
Bimatoprost (Prostamide $F_{2\alpha}$ analog)	Allergan (Lumigan)	Intraocular Pressure, Glaucoma, Ocular Hypertension Eyelash hypotrichosis, alopecia	FP/Alt4-FP heteromer FP[a]	Several completed studies Several completed studies

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.

[a]Still controversial.

[b]These targets are activated indirectly via elevation of endogenous ligand levels or activity.

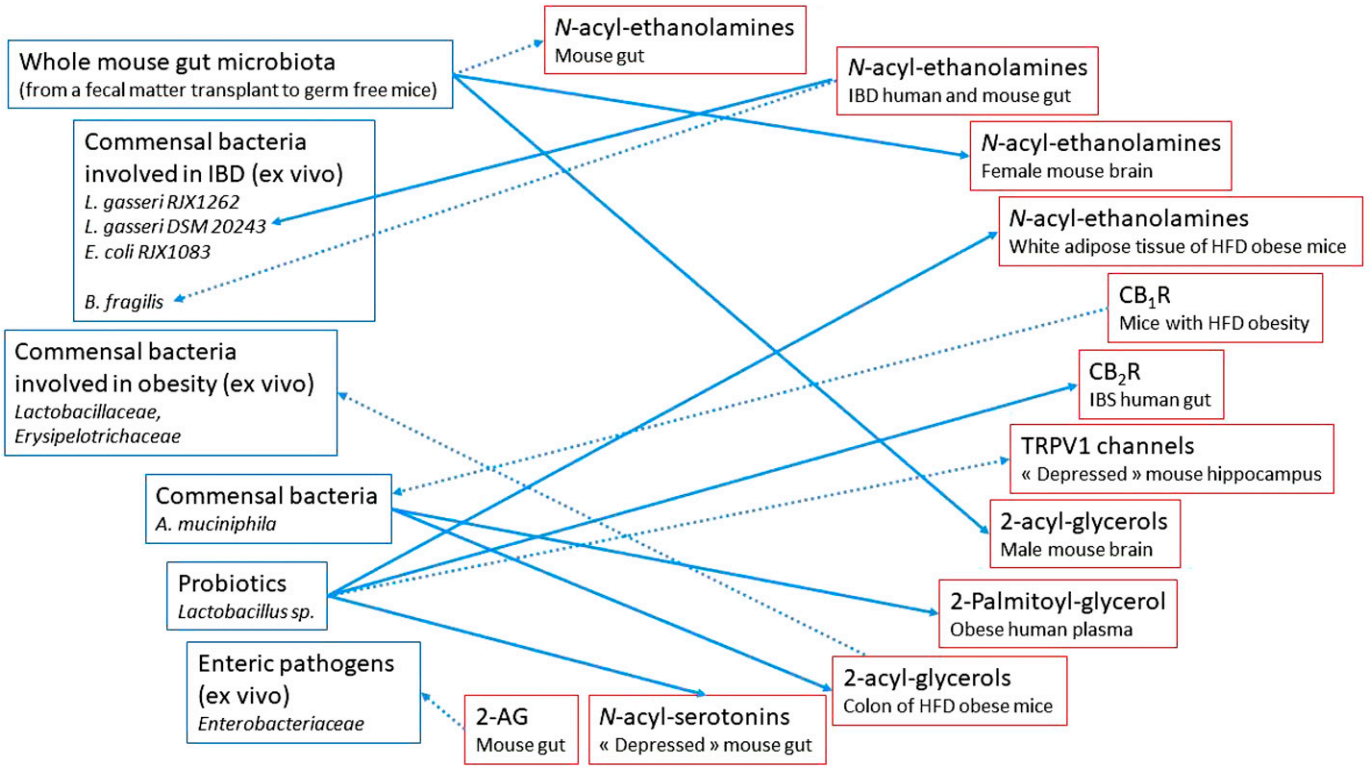


Fig. 28. Reciprocal modulation of the ECS and the gut microbiome. The emerging microbendocannabinoidome (μbeCBome), also summarized in Table 14, enlarges the span of microbe-host communication and expands it to the eCBome.

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*-acylethanolamines 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, b). 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 these effects were exerted directly on eCBome mediator biosynthesis or degradation. Clearly, in vitro studies, using, for example, cocultures of commensal bacteria or probiotic species (or their culture media) with mammalian intestinal cells or organoids, are again needed to understand whether these in vivo effects are the consequence of direct bacterial interactions with host cells.

VII. Conclusion

Plant-derived cannabinoids and endocannabinoids 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 biologic 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 us to turn pCBs and ECS-oriented drugs from threats to a treasure trove for human health.

Among the various components of the ECS, CB_1R, CB_2R, and FAAH have been the most largely exploited to develop therapeutic drugs for human diseases.

Shortly after the discovery of CB_1R, many therapeutic opportunities identified it 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 CB_1R but devoid of activity at other receptors (e.g., CB_2R, GPR55) continues to remain a challenge. Organ-system selectivity is a second goal for minimizing CNS actions of CB_1R agonists and antagonists and is being met by the development of peripherally restricted ligands that have limited access to brain CB_1R (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 CB_1R 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 $G_{12/13}$ 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 noncompetitive inhibition to those receptors receiving an endocannabinoid signal. Additionally, future goals for antagonists would be the development of biased antagonists, negative allosteric modulators, as well as neutral antagonists that do not affect the basal activity of CB_1R. A still unexplored approach with therapeutic potential that is closer to the concept of polypharmacology involves specifically inhibiting CB_1R and activating CB_2R. Thus, the future for CB_1R pharmacotherapeutics can be predicted to move from phytocannabinoid preparations to agonists and antagonists that exhibit greater selectivity through one of these strategies.

Also, CB_2R 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 pathologic 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 CB_2R ligands has been developed that can be categorized as eCBs and related fatty acid derivatives, pCBs, or synthetic CB_2R ligands. The majority of these ligands include agonists, modulators, neutral antagonists, inverse agonists, allosteric ligands, as well as labeled chemical probes. Altogether, a large, structurally diverse chemical space is covered. Generally, early CB_2R modulators are dual CB_1R/CB_2R agonists that are mostly not quite "drug-like." In contrast, recent ligands often combine high potency for CB_2R with favorable overall ADME profiles including low lipophilicity, aqueous solubility, and favorable plasma protein binding, which translate into excellent pharmacokinetic profiles and consequently improved developability. To overcome CB_1R-driven psychotropic effects, two strategies were followed: limiting exposure toward the periphery or enhancing the selectivity over CB_1R 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 CB_2R agonists are in phase 2 clinical trials. While no CB_2R-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 CB_2R modulators still poses challenges for the development of CB_2R-based therapies. The generation of translational animal models and a better understanding of CB_2R and the ECS in general will help unlock the receptor's full therapeutic potential. Recently discovered high-quality labeled chemical probes have enabled a better understanding of

CB_2R expression, mechanism of action, and translatability of results toward the human situation. The in-depth understanding of signaling bias as well as CB_2R receptor homo- and heterodimers might translate into different functional properties and ultimately tailor-made CB_2R therapeutics. Deeper insights into drug-target binding kinetics, their impact on receptor function, and, in particular, the recently reported structures of antagonist- and agonist-bound CB_2R and knowledge on allosterism will facilitate rational drug design. Together with the huge chemical space available to generate tailor-made CB_2R 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 to study enzyme activity, its regulation, and relevance in the pathophysiological process. Over the years, different approaches have been 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 also demonstrated 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 multitarget 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 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 neurologic 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 CB_1R, CB_2R, 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 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 programs 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 Cinzia Rapino (University of Teramo, Teramo, Italy) and Marina Fava (Campus Bio-Medico University of Rome, Rome, Italy) for their help in preparing Tables 1 to 3 and Figures 1 to 7, respectively. A.M. thanks Christos Iliopoulos Tsoutsouvas (Northeastern University, Boston, MA, USA) for his support in collecting information on CB_1R and preparing Table 4 and Figures 8 and 9. D.P. thanks Yannick Fotio (University of California, Irvine, CA, USA) for his support in preparing Figures 23 and 24. M.vd S. thanks Na Zhu and Anthe Janssen (Leiden University, Leiden, Netherlands) for preparing the schemes in Tables 1, 3, 7, and 9 and for Figure 25.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Maccarrone, Di Marzo, Gertsch, Grether, Howlett, Hua, Makriyannis, Piomelli, Ueda, Van der Stelt.

Note Added in Proof: Two mistakes were found in Figure 4 and Figure 6 published in the Fast Forward version published May 10, 2023. Figures 4 and 6 have now been corrected.

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