Pharmacological Tools in Endocannabinoid Neurobiology

Marco Mor and Alessio Lodola

Contents

Abstract Pharmacological and biochemical investigations on the endocannabinoid system are facilitated by the availability of compounds which interact with its constituents in specific and understandable ways. This chapter describes the main representatives of several classes of chemicals employed as pharmacological tools in this field, focusing on small organic compounds having, where possible, a drug-like structure. Many compounds having different intrinsic activity and selectivity towards the G-protein coupled receptors (GPCR) CB_1 and CB_2 are now available and are currently employed in research protocols. Recently, allosteric ligands for

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CB_1 receptor and selective ligands for GPR55, a newly characterised GPCR, have also been described in the literature. As for compounds affecting endocannabinoid levels in living tissues, many classes of selective and, in some cases, drug-like inhibitors of FAAH are available, while only compounds with poor selectivity or in vivo activity are known to inhibit other enzymes involved in endocannabinoid catabolism, such as NAAA or MGL, and in endocannabinoid biosynthesis.

Keywords CB1 ligands • CB2 ligands • Anandamide Transport • FAAH inhibitors • MGL inhibitors • NAAA inhibitors

Abbreviations

2-AG	2-Arachidonoylglycerol
AEA	Anandamide
CCP	*N*-(cyclohexylcarbonyl)pentadecylamine
DAG	Diacylglycerol
ECB	Endocannabinoid
FAAH	Fatty acid amide hydrolase
GPCR	G protein-coupled receptor
MGL	Monoacylglycerol lipase
NAAA	*N*-Acylethanolamine acid amidase
NAE	*N*-Acylethanolamine
NAPE-PLD	*N*-Acylphosphatidylethanolamine phospholipase D
OEA	*N*-Oleoylethanolamine
PA	Phosphatidic acid
PEA	*N*-Palmitoylethanolamine
PLC	Phospholipase C
TRPV1	Transient receptor potential vanilloid type 1
Δ^9-THC	Δ^9-Tetrahydrocannabinol

1 Cannabinoid Receptor Ligands

The identification of cannabinoid receptors and the characterization of their endogenous ligands has triggered an exponential growth of studies exploring the endocannabinoid (ECB) system and its regulatory functions in physiological and pathological processes. Such studies have been significantly facilitated, for both in vitro experiments and animal models, by the introduction of selective cannabinoid receptor ligands and inhibitors of ECB metabolism and transport. In the last decade, the ECB system was found to be involved in several physiological functions,

both in the central nervous system (CNS) and in the peripheral one as well as in other organs. Modulation of the activity of ECBs revealed therapeutic promises in a wide range of diseases and pathological conditions (Elsohly 2002), possibly overcoming the traditional limit to the development of cannabinoid-based medicines, i. e. the socially intolerable psychoactive properties of plant-derived or synthetic agonists, mainly mediated by CB_1 receptor activation. However, this problem does not emerge when the therapeutic scope is accomplished by treatment with CB_1 receptor antagonists, as in obesity, or with agents enhancing the action of ECBs through the blockade of their metabolism or transport. Moreover, the use of selective CB_2 receptor agonists, lacking psychoactive properties, may represent another promising opportunity for certain pathological conditions. Full exploitation of the therapeutic promises for drugs acting on the ECB system, however, still needs experimental work with in vitro and in vivo models, which could elucidate the benefits and the risks associated with selective perturbations in the activity of cannabinoid receptors and of ECB distribution and metabolism.

In this chapter, compounds are described which are commonly employed as pharmacological tools in the study of the ECB system, or that may have potential usefulness in this sense. In particular, attention is focused on drug-like chemicals, also providing brief outlines of the possible therapeutic benefits of some compounds. The chapter is organised in two main sections, the former dedicated to cannabinoid receptor ligands, the latter describing the compounds affecting ECB metabolism.

2 Natural Products and Non-Selective Ligands

Among the more than 60 cannabinoids contained in extracts from the plant *Cannabis sativa*, the best known is an isomer of tetrahydrocannabinol, Δ^9-THC (Fig. 1) (Gaoni and Mechoulam 1964), which is also its main psychoactive component. It is an agonist at both CB_1 and CB_2 receptors and it partly mimics the actions of the endogenous cannabinoids (Howlett et al. 2002). The therapeutical potential of several different components still remains to be fully explored (Mechoulam 2005; Thomas et al. 2005). Among these, cannabidiol has raised remarkable interest in spite of its lack of affinity for CB_1 or CB_2 receptors, mainly for its anti-proliferative activity and its potentiation of cannabinoid actions (Mechoulam et al. 2007). Furthermore, the finding that species different from *C. sativa* have components able to bind to CB receptors opens new and interesting possibilities for pharmacological research (Gertsch et al. 2006).

The identification of Δ^9-THC as the main active component of *C. sativa* stimulated the preparation of a range of synthetic compounds having similar biological activity and different grades of chemical diversity. These efforts led to the identification of novel chemical classes able to bind CB_1 and CB_2 receptors with different profiles of selectivity and intrinsic activity, as well as facilitating the identification of the natural modulators of CB receptors, the so-called ECBs (Lambert and Fowler 2005). Cannabinoid receptor ligands can be grouped in four classes – classical,

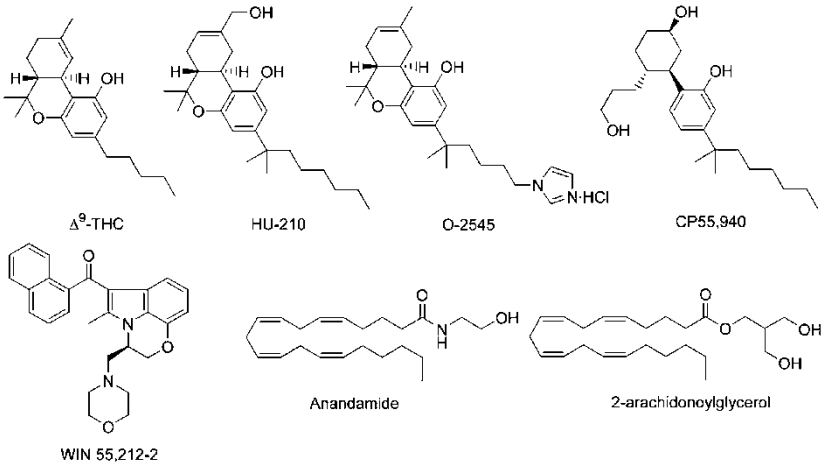

Fig. 1 Some plant-derived, synthetic or endogenous cannabinoids

non-classical, aminoalkylindole and eicosanoids – which are briefly summarised here (Pacher et al. 2006).

2.1 Classical Cannabinoids

These include plant-derived compounds or synthetic analogues having a dibenzopyran scaffold, such as Δ^9-THC and HU210 (Howlett et al. 2002). Δ^9-THC (Fig. 1) binds with similar affinity to CB_1 and CB_2 receptors and behaves as a partial agonist at both of these receptor subtypes (Felder et al. 1995), with lower efficacy at CB_2 than at CB_1 receptors (Bayewitch et al. 1996). HU210 shows affinity values for CB_1 and CB_2 receptors exceeding those of many other cannabinoids. It is a potent cannabinoid receptor agonist and its pharmacological effects in vivo are exceptionally long-lasting. The enhanced affinity and efficacy shown by HU210 at cannabinoid receptors can be largely attributed to the replacement of the pentyl side chain of Δ^8-THC (an isomer of Δ^9-THC resembling it both in its affinities for CB_1 and CB_2 receptors and in CB_1 receptor efficacy) with the dimethylheptyl group. The poor water solubility of classical cannabinoids requires the use of solubilizing agents for many pharmacological uses. Addition of an imidazole at the side chain of Δ^8- THC allows the use of a water-soluble hydrochloride salt (O-2545, Fig. 1), maintaining high affinity and efficacy at CB_1 and CB_2 receptors (Martin et al. 2006).

2.2 Non-Classical Cannabinoids

These present limited modifications in their scaffold, generally being bicyclic analogues of Δ^9-THC without the central pyran ring. The most commonly used

non-classical cannabinoid is CP55940 (Fig. 1), which has CB_1 and CB_2 affinities in the low nanomolar range and exhibits relatively high efficacy at both receptor subtypes. In its [^{3}H]-labelled form, CP55940 was employed to demonstrate definitively the existence of high-affinity, saturable, stereospecific binding sites for synthetic cannabinoid agonists in rat brain and it is currently employed in competitive binding experiments (Devane et al. 1988).

2.3 *Aminoalkylindoles*

The prototype of this group is WIN55212-2 (Fig. 1), which does not show clear structural similarity with classical, non-classical or eicosanoid cannabinoids. It has been reported that WIN55212-2 binds to the CB_1 receptor in a different way from-classical and non-classical cannabinoids (Song and Bonner 1996), even if mutual displacement is observed between WIN55212-2 and non-aminoalkylindole cannabinoids at CB_1 binding site (Kuster et al. 1993). Like CP55940, WIN55212-2 exhibits relatively high efficacy at CB_1 and CB_2 receptors and possesses CB_1 and CB_2 affinities in the low nanomolar range. However, it has slightly greater affinity for CB_2 than for CB_1 receptors.

2.4 *Eicosanoids*

The prototypes and most investigated members of this group are the ECBs *N*-arachidonoylethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG), whose chemical structures are reported in Fig. 1 (Piomelli 2003). AEA acts as an endogenous ligand for CB_1 and CB_2 receptors, and for other targets, such as the transient receptor potential vanilloid type 1 (TRPV1) channel. AEA affinity for the CB_1 receptor is higher than for the CB_2 receptor. When protected from enzymatic hydrolysis, it shows a CB_1 affinity comparable to that of Δ^9-THC. Depending on the tissue and biological response being measured, it can behave as a partial or full agonist at CB_1 receptors. It has low efficacy at CB_2 receptors, where it may even act as an antagonist, depending on the interacting G proteins (Gonsorek et al. 2000). 2-AG is a full agonist at both CB_1 and CB_2 receptors. Some authors consider 2-AG the true ligand for CB_2 receptors, as AEA binds poorly to these receptors. The basal levels of 2-AG in the brain are much higher (about two orders of magnitude) than those of AEA, suggesting that only a fraction of the total is involved in ECB signalling (Sugiura et al. 2006).

Other putative ECBs are noladin ether (2-arachidonylglyceryl ether), *N*-arachidonoyldopamine and virodhamine (*O*-arachidonoylethanolamine); their biological significance and biochemical characteristics are still to be fully investigated (Piomelli 2003).

3 CB_1-Selective Ligands

Δ^9-THC and most of the cannabinoid ligands described so far show similar affinities for CB_1 and CB_2 receptors. Only recently, synthetic ligands able to discriminate between the two receptor isoforms emerged, including agonists and antagonists. The development of potent and highly selective CB_1 and CB_2 receptor antagonists is particularly noteworthy as it provided critically important tools to explore the physiological functions of these receptor subtypes (Rinaldi-Carmona et al. 1994; Rinaldi-Carmona et al. 1998).

3.1 Agonists

AEA shows some CB_1 selectivity and represents a template for CB_1-selective agonists, but its use for in vivo experiments is limited by reduced metabolic stability. The replacement of a hydrogen atom with a methyl group at the 1' position of AEA led to (*R*)-(+)-methanandamide, a rather CB_1-selective agonist endowed with improved metabolic stability. Other modifications of the ethanolamine moiety led to the potent CB_1-selective agonists arachidonoyl-2'-chloroethylamide (ACEA) and arachidonoylcyclopropylamide (ACPA), both exhibiting good CB_1 efficacy (Fig. 2) (Hillard et al. 1999).

Pharmacological investigations showed that ACEA and ACPA produce hypothermia in mice, an effect that can be inhibited by co-administration of the selective CB_1 receptor antagonist rimonabant (Fig. 3). However, unlike methanandamide, neither ACEA nor ACPA show resistance to enzymatic hydrolysis (Di Marzo et al. 2001), even if it has been shown that the addition of a methyl group to the 1' carbon of ACEA markedly decreases the susceptibility of this molecule to FAAH-mediated hydrolysis (Jarrahian et al. 2000). ACPA and ACEA also showed poor affinity for CB_2 receptors, confirming that selectivity for CB_1 receptor over CB_2 receptors can be achieved by designing structural analogues of AEA (Di Marzo et al. 2001).

3.2 Antagonists and Inverse Agonists

The gold standard among CB_1-selective ligands that counteract the effects of cannabinoid agonists is the diarylpyrazole rimonabant (SR141716A, Fig. 3)

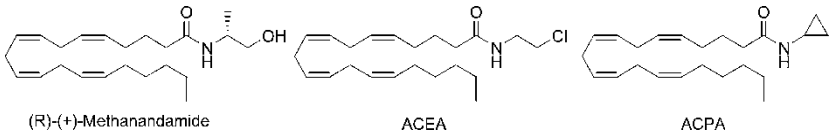

Fig. 2 CB_1-selective agonists based on the anandamide template

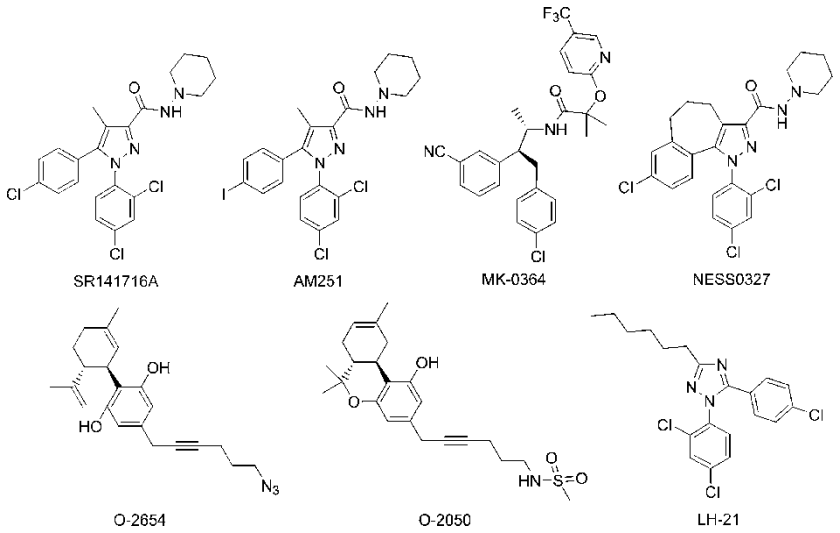

Fig. 3 CB_1-selective antagonists/inverse agonists

(Rinaldi-Carmona et al. 1994), which was also the first compound of this class to reach the market as an anti-obesity drug. It is a highly potent and selective CB_1 receptor ligand that readily prevents or reverses CB_1-mediated effects both in vitro and in vivo (Pertwee 2005a) and is now regarded as the prototypical CB_1 antagonist/inverse agonist. Other notable CB_1-selective antagonists/inverse agonists are AM251 and AM281, both belonging to the same diarylpyrazole class as rimonabant, but differing in the presence of an iodine, instead of a chlorine, on the 5-phenyl ring (AM251, Fig. 3) or in the presence of a morpholine ring instead of a piperidine one (AM281). Several pharmacological studies employed AM251, which has similar affinity and intrinsic activity at CB_1 receptors to rimonabant, while it has been claimed to be more selective, as rimonabant retains some effects in CB_1 knockout mice (Muccioli 2007). Taken together, the two pyrazole derivatives rimonabant and AM251 allowed for a better understanding of the ECB system, and represented optimal tools for exploration of the therapeutic potential of CB_1 antagonists/inverse agonists, eventually leading to the introduction of rimonabant in the clinical market. Despite their initial classification as CB_1 antagonists, there is significant evidence that rimonabant, AM251 and AM281 are inverse agonists (Pertwee 2005b). In fact, these CB_1 receptor ligands can not only attenuate the effects of CB_1 receptor agonists, but also elicit responses by themselves in some CB_1 receptor-containing tissues that are opposite in direction from those elicited by CB_1 receptor agonists, reducing the constitutive activity of CB_1 receptors.

Rimonabant was used in numerous animal models to elucidate both the role of the ECB system and the therapeutic promise of CB_1 receptor “antagonists”. Much of the pioneering research in this field was performed using this compound, taking advantage of its selectivity and oral bioavailability. Registered as Acomplia, it has

been approved for use in several countries for the treatment of obesity, though the Food and Drug Administration has expressed concern about the potential for adverse neurological and psychiatric effects, considering the widespread distribution of CB_1 receptors in the brain (Woods 2007). Efforts to generate new CB_1 antagonists/inverse agonists led to several interesting compounds, such as the highly potent and selective ligand MK-0364 (taranabant, Fig. 3) (Fong et al. 2007), a new clinical candidate for the treatment of obesity whose therapeutical potential is under investigation.

The clinical relevance of inverse agonism exhibited by rimonabant and its congeners is a matter of debate, and the availability of neutral antagonists is an urgent need in cannabinoid research, to differentiate the effects on ECB tone from those on receptor constitutive activity in animal tissues. Even if compounds with a purely antagonist profile and an established use as pharmacological tools are still lacking, neutral antagonism in biochemical assays and interesting in vivo effects have been reported for some compounds (Fig. 3), such as O-2654 (Thomas et al. 2004), O-2050 (Gardner and Mallet 2006), LH-21 (Pavon et al. 2006), and AM4113 (Sink et al. 2008).

Recently, some series of conformationally constrained derivatives have also been synthesised to achieve potency improvements by blocking their structure in the putative active conformation (Thomas et al. 2006). This strategy led to NESS0327 (Fig. 3), which has been reported to be a neutral antagonist with exceptional affinity for the CB_1 receptor in the femtomolar range. Furthermore, CB_1/CB_2 affinity ratio showed that NESS0327 is more than 60,000 times more selective for the CB_1 receptor, whereas rimonabant only showed a 285-fold selectivity (Ruiu et al. 2003). More extensive studies are needed to determine whether these new compounds will prove to be superior to rimonabant when used in vivo.

Wider dissertations regarding CB_1 receptor blockage and its therapeutical potential are available throughout this book and in dedicated reviews (Muccioli 2007; Jagerovic et al. 2008).

3.3 *Allosteric Modulators*

Besides CB_1 antagonists and inverse agonists, there are other compounds that can modulate the effects of CB_1 receptor agonists through different mechanisms. Experiments with the synthetic indole derivative Org 27569 (Fig. 4) and its close

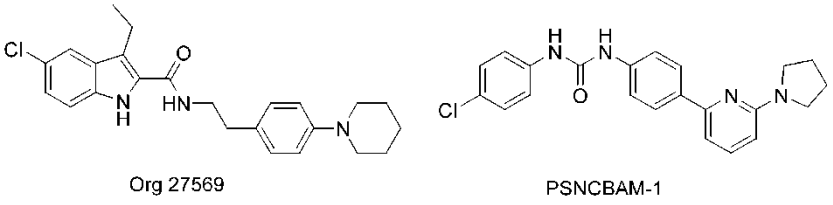

Fig. 4 Allosteric modulators of CB_1 receptors

analogues Org 29647 and Org 27759 revealed that these ligands bind allosterically to the CB_1 receptor and elicit a conformational change that increases agonist affinity for the orthosteric binding site (Price et al. 2006). These findings indicated the presence of a new way of modulating CB_1 receptor activation by endogenously released ECBs. Indeed, a new allosteric modulator of the CB_1 receptor, PSNCBAM-1 (Fig. 4), is able to increase CB_1 affinity of orthosteric agonists, antagonising their functional activity. Its effects on food intake and body weight in rats provided a first report of in vivo activity for an allosteric CB_1 receptor antagonist (Horswill et al. 2007). Future research will certainly address the question of whether allosteric modulators of the CB_1 receptor are advantageous in some pathological scenarios by comparison with the currently available orthosteric ligands (Ross 2007).

4 CB_2-Selective Ligands

4.1 Agonists

The well-known differences in receptor distribution and signal transduction mechanisms between CB_1 and CB_2 receptors are likely to account for the relative absence of CNS side effects induced by CB_2 agonists. These considerations suggest that novel therapies targeting CB_2 receptors may achieve their therapeutic potential without the risks related to the socially intolerable psychoactive properties of CB_1 agonists. Significant drug discovery efforts have thus been directed towards the development and characterization of CB_2-selective agonists, both in vitro and in vivo, mainly with the aim of evaluating and validating the CB_2 receptor as a target for the treatment of inflammatory and neuropathic pain (Guindon and Hohmann 2008). HU308 (Fig. 5) was one of the first CB_2-selective agonists employed, exhibiting low affinity for CB_1 receptors (Hanus et al. 1999). HU308 exhibits anti-inflammatory and peripheral anti-hyperalgesic properties, which are reversed by the CB_2 antagonist SR144528 but not by the CB_1 antagonist rimonabant. HU308 does not show CNS activity in the classical tetrad of behavioural tests

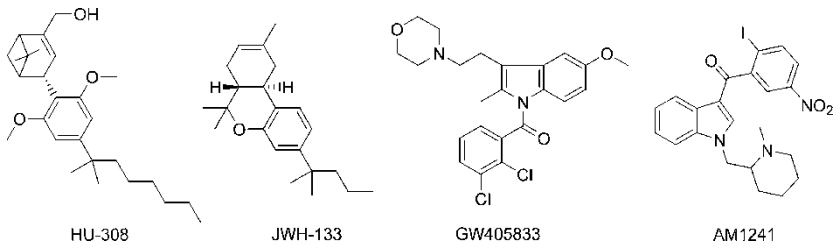

Fig. 5 CB_2-selective agonists

assessing signs of CB_1 receptor activation associated with Δ^9-THC (Gaoni and Mechoulam 1964).

Other CB_2-selective agonists (Fig. 5) widely used as pharmacological tools are the classical cannabinoid, JWH133, and the less selective aminoalkylindole, JWH015 (Howlett et al. 2002; Pertwee 2005b). Both behave as potent CB_2 agonists in functional assays, inhibiting both inflammatory and neuropathic hyperalgesia through a CB_2-dependent mechanism (Huffman et al. 1999; Jonsson et al. 2006). The indole derivative GW405833 (also indicated as L768242) behaves as a potent partial agonist at the CB_2 receptor and produces anti-hyperalgesic effects in several rodent models of pain (Valenzano et al. 2005). Another indole derivative, AM1241, is reported to give CB_2-mediated anti-hyperalgesic effects in multiple models of persistent nociception, including those induced by tissue and nerve injury, while avoiding the classical signs of CB_1 activation. While the racemate of this compound behaves either as an agonist or an inverse agonist, depending on receptor species, its (*S*) enantiomer showed slightly lower receptor affinity, but full agonist behaviour on cAMP accumulation in cell lines expressing recombinant human, rat, and mouse CB_2 receptors (Bingham et al. 2007).

The structural requirements for ligands binding at CB_2 receptors and the therapeutic potential of CB_2 receptor agonists have been thoroughly discussed in the recent literature (Whiteside et al. 2007; Poso and Huffman 2008), and several novel structures of potent and selective compounds which may be employed as pharmacological tools are being reported with an increasing rate (Giblin et al. 2007; Manera et al. 2006; Murineddu et al. 2006; Khanolkar et al. 2007; Gonsiorek et al. 2007; Ermann et al. 2008; Ohta et al. 2008; Yao et al. 2008; Kikuchi et al. 2008).

4.2 Antagonists

Fewer classes of selective CB_2 receptor antagonists have been reported so far (Muccioli 2007). Two prototypical selective antagonists are SR144528, developed from a pyrazole scaffold (Rinaldi-Carmona et al. 1998), and AM630, having an indole nucleus (Fig. 6). Both are extensively used as pharmacological tools to block CB_2 activation, but they are not pure antagonists, showing inverse intrinsic activity in some cannabinoid receptor-containing bioassay systems (Howlett et al. 2002). Potent CB_2 receptor ligands based on a quinoline carboxamide moiety have also been reported, such as JTE907 (Fig. 6) which is endowed with high selectivity for the CB_2 receptor and inverse-agonistic properties. JTE907 has been employed for in vivo investigations, showing anti-pruritic activity in a model of dermatitis when orally administered to mice (Iwamura et al. 2001; Maekawa et al. 2006). More recently, a new class of selective CB_2 receptor ligands was developed, based on a triaryl bis-sulfone backbone exemplified by Sch.336 (Fig. 6). These compounds exhibit inverse-agonistic properties at CB_2 receptors, with Sch.336 inhibiting leukocyte trafficking in rodents and blocking lung eosinophilia in mice, a model for allergic asthma (Lunn et al. 2006). A novel radioligand with the same structure,

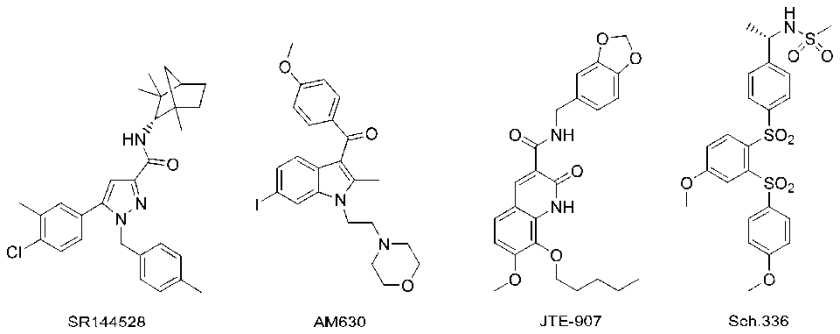

Fig. 6 CB_2-selective antagonists

named [^{35}S]-Sch225336, has also been recently proposed as a useful tool for binding assays on CB_2 receptors (Gonsiorek et al. 2006).

5 Ligands of Other ECB Receptors

Besides CB_1 and CB_2 receptors, ECBs and exogenous cannabinoids target other binding sites, which may explain why the pharmacology of cannabinoids cannot be fully explained by their activity at those two receptors only. The field is further complicated by the heterogeneity of endogenous compounds chemically related to the ECBs 2-AG and AEA, having their own spectra of targets. These compounds, thoroughly described in the previous chapters of this book, include members of the *N*-acylethanolamine (NAE) family such as *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA) shown in Fig. 7, which also activate the nuclear receptor PPARα (Fu et al. 2003; Lo Verme et al. 2005). Additional cannabinoid targets include the ion channel TRPV1 and non-CB_1/CB_2 G-protein coupled receptors, such as GPR119 and GPR55. While the pharmacology of many of these targets has been recently reviewed elsewhere (Brown 2007), in this context it is worth mentioning, as a promising pharmacological tool, the high selectivity for GPR55 shown by compound O-1602 (Fig. 7) This is an agonist with nanomolar potency at the GPR55 receptor, giving no sign of CB_1 or CB_2 activation at micromolar concentrations (Ryberg et al. 2007).

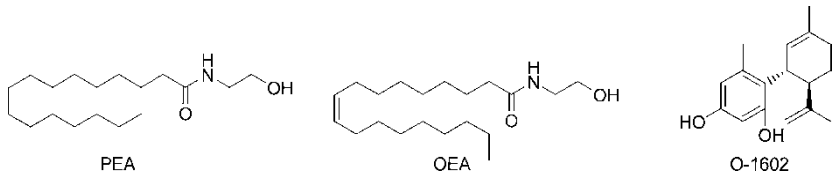

Fig. 7 PEA, OEA and the selective GPR55 agonist, O-1602

6 Modulators of ECB Metabolism

ECBs are produced on demand, as a response to elevated intracellular calcium levels. AEA and 2-AG are synthesised from membrane phospholipids through at least two distinct enzymatic reactions (Di Marzo et al. 1996, 1999). Once synthesised, AEA and 2-AG have to reach their membrane receptors to evoke cannabimimetic effects. To control ECB levels, effective mechanisms for their synthesis, trafficking and removal are present inside the cells (Piomelli 2003). Many enzymes involved in the degradation of ECBs have been identified and characterised, and their roles have been in some cases elucidated, exploiting the availability of potent and selective inhibitors. On the other hand, the details of ECB biosynthetic pathways are still more elusive, and a smaller number of well-characterised compounds able to selectively act at this level is available. A thorough description of ECB biosynthesis and inactivation is reported in Chapter 1 of this book. Here, we will focus on the pharmacological tools which may be employed to assess the role of these enzymatic components of the ECB system. The first part of this section looks at the modulators of ECB catabolism, whereas the second part reports some findings on agents blocking ECB synthesis.

6.1 ECB Catabolism

To terminate ECB signalling, these endogenous compounds must be chemically inactivated. If we focus on the two main ECBs, the amide AEA and the ester 2-AG, this is eventually accomplished by enzymatic hydrolysis. While these lipidic agents can undergo different metabolic transformations, their inactivation appears to be mainly regulated by a two-step process: ECBs must be transported into cells by a facilitated transport system (Beltramo et al. 1997) and then hydrolyzed by the action of different enzymes (Bari et al. 2006). AEA is mainly hydrolyzed, to arachidonic acid and ethanolamine, by the action of a hydrolase selective for fatty acid amides, named fatty acid amide hydrolase (FAAH) (McKinney and Cravat 2005), whereas 2-AG is degraded to arachidonic acid and glycerol, mainly by the action of a monoglyceride lipase (MGL) (Dinh et al. 2002a). Other enzymes causing the hydrolysis of AEA and 2-AG have been identified, such as FAAH-2, a homologue of FAAH expressed in periphery and only present in mammals (Wei et al. 2006), and of *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Tsuboi et al. 2005), another enzyme catalysing the hydrolysis of linear fatty acid amides in humans and rodents. While the catabolism of AEA, 2-AG and other lipid-deriving signalling molecules will disclose unexpected complexity and interconnection, the mostly established pharmacological tools are those affecting AEA transport or inhibition of FAAH, MGL and NAAA.

6.1.1 Inhibition of Anandamide Transport

AEA transport in neurons is structurally specific, displays classical saturation kinetics, and is inhibited by AEA analogues (Beltramo et al. 1997). The first inhibitor of the cellular uptake of AEA to be developed was *N*-(4-hydroxyphenyl) arachidonoylamide (AM404, Fig. 8). AM404 produces a variety of in vivo actions, including potentiation of the effects on hypothermia in FAAH knockout mice of low doses of AEA, which are blocked by CB_1 antagonists (Fowler et al. 2005). However, AM404 is not particularly selective, as it also inhibits FAAH, binds to CB_1 receptors and activates the vanilloid receptor TRPV1 at concentrations similar or below those at which it inhibits AEA uptake (Lambert and Fowler 2005). Many acyl-based compounds have been investigated for their ability to inhibit AEA uptake, leading to the identification of the arachidonoyl derivatives UCM707 and VDM11 and the oleoyl derivative OMDM-2 (Fig. 8) (Lopez-Rodrguez et al. 2003; Ortar et al. 2003).

The existence of a specific transporter had been questioned, and in fact hydrolytic activity of FAAH may be responsible for the maintenance of a concentration gradient of AEA. In this regard, FAAH inhibition by compounds also acting as reuptake blockers can be functionally relevant and, to some extent, limits their usefulness as pharmacological tools (Glaser et al. 2003). However, detailed investigations demonstrated that AM1172 (Fig. 8), a hydrolysis-resistant analogue of AM404, inhibits AEA internalisation without interacting with FAAH in brain neurons and astrocytoma cells (Fegley et al. 2004), confirming that membrane transport and intracellular hydrolysis are two discrete steps in AEA deactivation. It has been claimed, however, that in different conditions AM1172 may be a weak

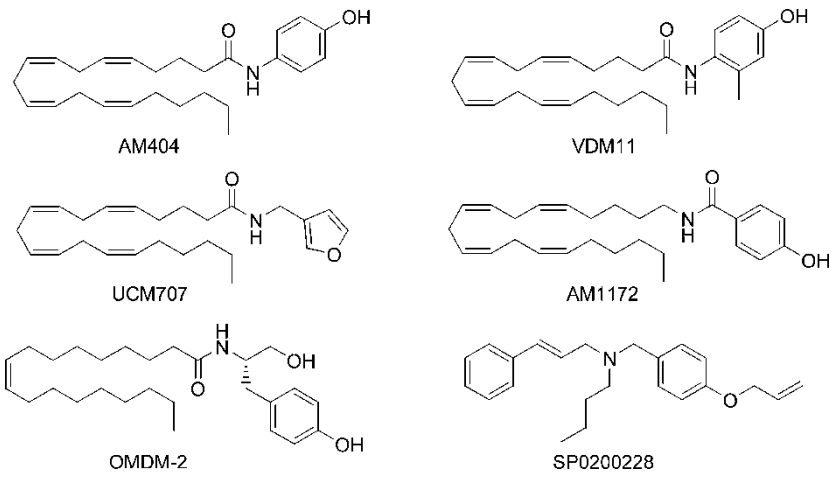

Fig. 8 Inhibitors of anandamide transport

inhibitor of FAAH (Hillard et al. 2007; Fowler et al. 2004; Vandevoorde and Fowler 2005).

A new generation of ECB transport inhibitors is strongly needed, particularly with improved drug-likeness and selectivity compared to the cited fatty acid amides. A different chemotype is represented by a class of trialkylamines (e.g. SP0200228, Fig. 8) that inhibit AEA accumulation and amplify the analgesic effects of AEA (Piomelli 2005). However, research in this area is delayed by the elusive nature of the ECB transport system, whose molecular identity is still undefined.

6.1.2 FAAH Inhibitors

The characterization of FAAH and MGL in many tissues boosted the search of drug-like selective inhibitors, to be used for a characterization of the physiological and pathological roles of ECBs and as potential new drugs. In fact, partly as a result of experiments with FAAH and MGL inhibitors, there is now evidence that ECB levels are unbalanced in certain pathological conditions, supporting the hypothesis that such inhibitors can have therapeutic applications (Piomelli 2005).

Immediately after the discovery of AEA, a widely used inhibitor of its enzymatic hydrolysis was the non-selective, irreversible serine protease inhibitor, phenylmethylsulphonyl fluoride (PMSF, Fig. 9) (Deutsch and Chin 1993), which also inhibits MGL, but only at higher concentrations (Ho and Hillard 2005). More potent covalent inhibitors of FAAH, such as methyl arachidonyl fluorophosphonate

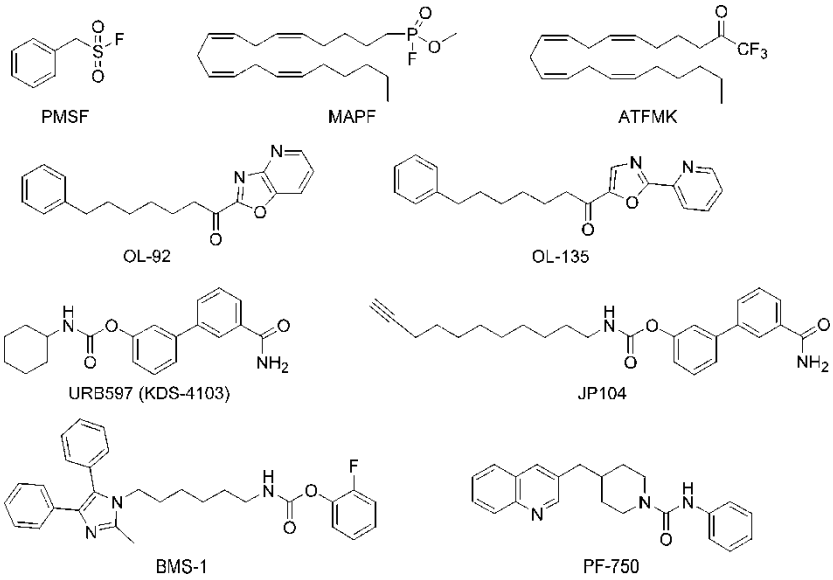

Fig. 9 Representatives of main FAAH inhibitor classes

(MAFP) (De Petrocellis et al. 1997) and palmitylsulfonyl fluoride (AM374) (Deutsch et al. 1997), were also used, but all these compounds were poorly selective. Looking for more selective inhibitors, less reactive compounds were taken into consideration. An electron-withdrawing trifluoromethyl group close to a carbonyl makes arachidonoyl trifluoromethyl ketone (ATFMK) a reversible FAAH inhibitor, considered as a transition state analogue (Koutek et al. 1994). A similar strategy, also exploiting selective recognition at the FAAH catalytic site, led to the class of α-ketoheterocycles, which includes very potent and selective inhibitors such as OL-92 and OL-135 (Boger et al. 2005) (Fig. 9). These are competitive inhibitors presumably acting via reversible hemiketal formation with the active site Ser241 (Boger et al 2000). OL-92 and OL-135 proved to be exceptionally potent and selective FAAH inhibitors in vitro, enhancing ECB signalling and producing analgesia in vivo (Lichtman et al. 2004).

Amino acid conjugates of arachidonic acid also provided FAAH inhibitors, possibly acting as competing substrates (Lambert and Fowler 2005). This is the case for *N*-arachidonoylserotonin, which displays dual activity as a FAAH inhibitor and a TRPV1 antagonist, being highly effective against both acute and chronic peripheral pain (Maione et al. 2007).

Among the FAAH inhibitors developed so far, the *O*-aryl carbamates represent the most promising class of clinical candidates for the treatment of CNS and peripheral disorders. The *O*-aryl carbamate class includes potent and selective inhibitors based on the *N*-cyclohexylcarbamic acid *O*-aryl ester scaffold (Kathuria et al. 2003; Mor et al. 2004), such as URB597 (KDS-4103, Fig. 9), which have been intensively investigated to reveal a pharmacological profile characterised by a unique combination of analgesic, anxiolytic-like, and anti-depressant-like properties (Piomelli et al. 2006; Jayamanne et al. 2006; Gobbi et al. 2005; Bortolato et al. 2007). URB597 inhibits FAAH by carbamoylation of the active nucleophile Ser241, giving irreversible inactivation as revealed by mass spectrometry (Alexander and Cravatt 2005) and computational investigations (Lodola et al. 2008). Furthermore, URB597 inhibits FAAH in vitro at nanomolar concentrations, showing no displacement of radiolabelled ligands from CB_1 and CB_2 receptors. Consistently, URB597 does not evoke catalepsy or hypothermia in vivo, supporting the hypothesis that selective enhancement of ECB transmission by chemical inactivation of FAAH does not produce the typical signs of intoxication by exogenous cannabinoids (Russo et al. 2007; Kathuria et al. 2003). Several classes of carbamate derivatives and related compounds, exemplified here by JP104 and BMS-1 (Fig. 9), have been developed by academic and industrial groups. For more detailed information, the reader is referred to review articles dedicated to FAAH inhibitors (Vandevoorde 2008; Labar and Michaux 2007).

Recently, the issue of in vivo selectivity has been raised for URB597 and other FAAH inhibitors. It has been shown that these derivatives display multiple off-targets, such as rat liver carboxylesterases (Zhang et al. 2007), due to the tendency of the carbamic fragment to react with different serine hydrolases. Although the impact of these observations for the clinical development of this class of compounds has not been assessed, efforts were made to reduce the intrinsic reactivity of

FAAH-carbamoylating agents, exploiting the strong nucleophilicity of its active serine, which is part of an unusual serine–serine–lysine catalytic triad (Lodola et al. 2005). A novel class of inhibitors based on the chemically stable urea group has been recently disclosed. As an example from this class, PF-750 was found to inhibit FAAH in a time-dependent manner by covalently modifying the nucleophile Ser241, avoiding any interactions with all the other mammalian serine hydrolases tested so far (Ahn et al. 2007).

6.1.3 MGL Inhibitors

MGL is a traditional serine hydrolase endowed with a serine–histidine–aspartate catalytic triad (Karlsson et al. 1997) and it is responsible for 2-AG hydrolysis in rat cerebellar membranes (Saario et al. 2004). Several compounds able to inhibit MGL have been reported so far, but their therapeutic potential remains almost unexplored because of their poor selectivity and/or limited in vivo potency (Saario and Laitinen 2007b). The situation is also complicated by the presence of more than one molecular entity endowed with MGL activity (Muccioli et al. 2007). The first compounds reported to inhibit MGL hydrolytic activity were identified among non-specific serine hydrolase inhibitors, such as MAFP, PMSF, AM374 (Saario and Laitinen 2007a; Dinh et al. 2002b). Commonly used lipase inhibitors, such as RHC-80267 or tetrahydrolipstatin (THL or orlistat, Fig. 10), are also known to inhibit 2-AG hydrolysis in rat cerebellar membranes, but their inhibitory effect on DAGLα, an enzyme responsible for 2-AG synthesis, hampers their use for in vivo investigations (Bisogno et al. 2006).

Looking for selective inhibitors of MGL, various analogues of 2-AG have been synthesised, leading to the identification of α-methyl-1-arachidonoylglycerol (α-Me-1-AG), which inhibits both cytoplasmic and membrane MGL at micromolar concentrations, with a weak effect on FAAH activity (Ghafouri et al. 2004).

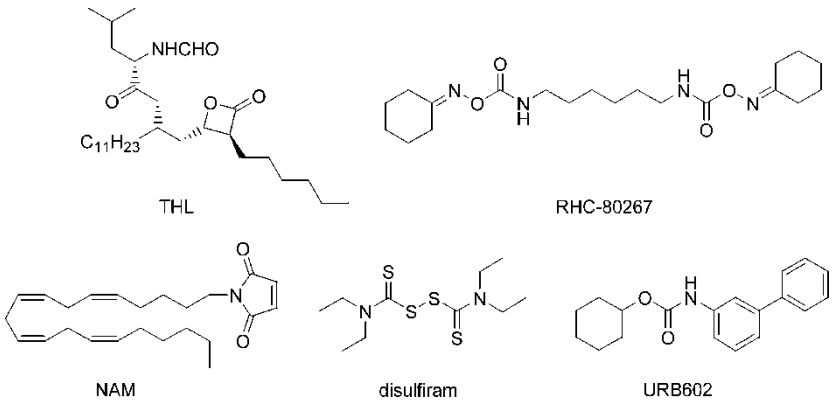

Fig. 10 MGL inhibitors

α-Me-1-AG and other 1-AG derivatives were also synthesised, but potency improvements were not associated with selectivity towards FAAH inhibition (Vandevoorde 2008).

Sulfhydryl-specific compounds, such as *p*-chloromercuribenzoic acid (*p*-CMB) or mercury chloride, inhibit MGL, indicating that cysteine residues may have a key role in regulating MGL hydrolytic activity. Thus, *N*-arachidonylmaleimide (NAM, Fig. 10) inhibits 2-AG hydrolysis in rat cerebellar membranes with nanomolar potency (Saario et al. 2005). It has been proposed that the maleimide scaffold can covalently bind a cysteine sulfhydryl group by a Michael addition mechanism, thus explaining the irreversible inhibition of the enzyme (Saario et al. 2005). These findings prompted researchers to screen disulfide-containing agents, leading to the observations that disulfiram (a well-known inhibitor of aldehyde dehydrogenase, used for decades to treat alcoholism) inhibits human purified MGL, and that its inhibition is reversed by the reducing agent dithiothreitol (DTT) (Labar et al. 2007). The efficacy of sulfhydryl-specific compounds is underlined by the erroneous identification of a putative inhibitor (URB754, structure not shown) as an MGL inhibitor, while the observed activity was actually due to a bis(dimethylthio) mercury impurity present in a commercial sample of this compound (Makara et al. 2005, 2007; Tonidandel et al. 2006).

At present, the most strongly characterised inhibitor of MGL is the carbamate derivative URB602 (Fig. 10), giving MGL inhibition at micromolar concentrations and no detectable FAAH inhibition, nor [^{3}H]-WIN55212-2 displacement from CB_1 or CB_2 receptors at 100 μM (Hohmann et al. 2005). Biochemical investigations indicated that URB602 inhibits recombinant MGL through a rapid non-competitive and reversible mechanism, excluding the formation of a stable carbamoylated adduct at the MGL active site (King et al. 2007). Despite its low potency, URB602 was utilised in vivo to provide evidence for the involvement of 2-AG in pain suppression. URB602 enhanced non-opioid stress-induced analgesia in the tail-flick test, when administered via microinjection into the periaqueductal grey matter (Hohmann et al. 2005), or intrathecally into the lumbar spinal cord (Suplita et al. 2006). Moreover, URB602 decreased pain behaviour in a formalin-induced inflammatory pain model (Guindon et al. 2007). URB602 was also recently reported to inhibit MGL activity in microglial cells with micromolar potency (Muccioli et al. 2007).

6.1.4 NAAA Inhibitors

Beside the central role played by FAAH in the degradation of AEA and other *N*-acylethanolamines such as PEA and OEA, another important enzyme shown to be involved in the degradation of NAEs in macrophages and peripheral tissues is NAAA (Tsuboi et al. 2007). Potent and specific NAAA inhibitors are needed as tools to clarify the biological role of this enzyme, but at present no drug-like potent and selective NAAA inhibitor has been reported. NAAA is less sensitive to PMSF and MAFP than FAAH or MGL (Ueda et al. 1999, 2001), but it is inhibited by the

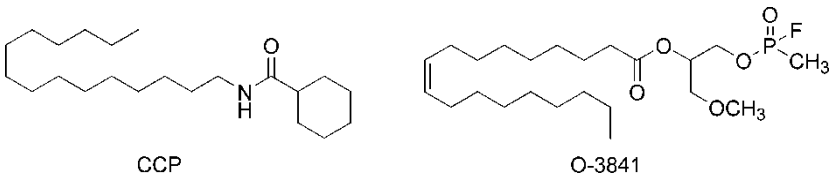

Fig. 11 NAAA and DAG lipase α inhibitors

sulfhydryl-specific agent *p*-CMB (Ueda et al. 2001), consistently with the presence of a catalytic cysteine in the active site (Tsuboi et al. 2005). Several NAE derivatives were shown to inhibit NAAA with some selectivity over FAAH. The most potent inhibitor identified within this class is *N*-(cyclohexylcarbonyl)pentadecylamine (CCP, Fig. 11), which inhibits NAAA at micromolar concentrations, and does not inhibit FAAH at concentrations up to 100 μM (Tsuboi et al. 2004). New compounds endowed with higher potency, good selectivity and a pharmacokinetic profile allowing in vivo administration are still required to assess the physiological role of this interesting acid amidase.

6.2 ECB Synthesis

Since AEA and 2-AG are synthesised on demand, and increased production and release of these ECBs is responsible for unwanted signs and symptoms of certain disorders, selective inhibitors of their biosynthesis would not only constitute important experimental tools, but may also have therapeutic perspectives.

NAEs, including AEA, are principally produced by two successive enzymatic reactions: (a) *N*-acylation of phosphatidylethanolamine to generate *N*-acylphosphatidylethanolamine (NAPE) by Ca^{2+}-dependent *N*-acyltransferase (NAT); (b) hydrolysis of NAPE to yield NAE, by a specific phospholipase D (NAPE-PLD) (Okamoto et al. 2007).

Hydrolysis, by a DAG lipase, of the ester bond at *sn*-1 position of a diacylglycerol (DAG) containing a *sn*-2 arachidonoyl chain produces 2-AG. In turn, DAG can be generated either from phosphoinositides by a specific phospholipase C (PLC) or from phosphatidic acid (PA) by PA phosphohydrolases. At present, two different isozymes of DAG lipase (α and β) have been identified (Bisogno et al. 2003).

Potent and selective inhibitors of the enzyme NAT have yet to be discovered, whereas it is known that NAPE-PLD activity can be blocked by *p*-CMB, which suggests the presence of catalytically important cysteine residues in its active site (Wang et al. 2006). Recently, it was reported that the lipase inhibitor THL shows detectable inhibition of NAPE-PLD at micromolar concentrations (Bisogno et al. 2006).

Some inhibitors are available for enzymes involved in the synthesis of 2-AG. THL and MAFP inhibit DAG lipase α with nanomolar potencies, but they are not

selective and thus of limited utility as pharmacological tools. A noteworthy compound is the phosphonate ester O-3841 (Fig. 11), which inhibits DAG lipase α in the nanomolar range, lacks any detectable inhibitory effect on NAPE-PLD, FAAH, MGL, and does not interact with CB_1 and CB_2 receptors up to the concentration of 25 μM (Bisogno et al. 2006). These findings suggest that O-3841 may be used as a pharmacological tool to investigate the role of 2-AG biosynthesis on physiological processes and pathological conditions.

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