THE ENDOCANNABINOID SYSTEM AND ITS THERAPEUTIC EXPLOITATION

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Abstract | The term 'endocannabinoid' — originally coined in the mid-1990s after the discovery of membrane receptors for the psychoactive principle in *Cannabis*, Δ^9-tetrahydrocannabinol and their endogenous ligands — now indicates a whole signalling system that comprises cannabinoid receptors, endogenous ligands and enzymes for ligand biosynthesis and inactivation. This system seems to be involved in an ever-increasing number of pathological conditions. With novel products already being aimed at the pharmaceutical market little more than a decade since the discovery of cannabinoid receptors, the endocannabinoid system seems to hold even more promise for the future development of therapeutic drugs. We explore the conditions under which the potential of targeting the endocannabinoid system might be realized in the years to come.

Δ9-TETRAHYDROCANNABINOL (THC). The major psychotropic component of *Cannabis sativa*, and one of about 66 'cannabinoids' found in the flowers of this plant.

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The recreational use of *Cannabis sativa* preparations is known to most people, largely as a result of the explosion in its use in the late 1960s; indeed, marijuana is still one of the most widespread illicit drugs of abuse in the world[1]. However, the medicinal use of *Cannabis* also has a millenarian history[2], although this history has been re-examined only very recently[3]. As early as 2600 BC, the Chinese emperor Huang Ti advised taking *Cannabis* for the relief of cramps, and rheumatic and menstrual pain; however, the great therapeutic potential of *Cannabis* was not scientifically assessed and publicized in the Western world until the British physician O'Shaugnessy wrote on the topic in the nineteenth century[2].

This long history of *Cannabis* use has resulted in the development of pharmaceutical drugs, such as dronabinol (Marinol; Unimed). This drug is based on (–)-Δ9-TETRAHYDROCANNABINOL (THC; FIG. 1), which, in 1964 — and after decades of attempts to isolate and determine its chemical structure — was identified as the major psychoactive component of *Cannabis*[4]. This preparation — together with Cesamet, which is based on the synthetic THC analogue nabilone (FIG. 1) — was being prescribed in the United States as an anti-emetic and appetite-stimulant to patients with cancer and AIDS[5] even before its molecular mode of action was revealed. It took the design of more potent, and enantiomerically pure, THC analogues, such as HU-210 (FIG. 1), to reveal that THC acts via specific sites of action to produce its typical psychotropic effects. Labelling of HU-210 to generate [^{3}H]HU-245, and the development of the non-classical (that is, bi-cyclic) cannabinoid CP-55,245 by Pfizer (FIG. 1), led to the identification of plasma membrane CANNABINOID RECEPTORS in 1988 (REF. 6). The serendipitous cloning in 1990 of the first of such proteins, the CB_1 receptor, came from the screening of an 'orphan' G-protein-coupled receptor (GPCR) with several possible ligands[7]. Meanwhile, several other plant cannabinoids that have little or no psychoactive action had been identified; their biosynthetic relationships have been established, and the possible contribution that they make to some of the purported therapeutic actions of *Cannabis* has been suggested. In particular, cannabidiol[8] and the cannabinoic acids (FIG. 1) seemed to be promising therapeutic tools, even though their sites of action are still not well understood. Another receptor for THC, the CB_2 receptor (located on blood cells and immune tissues), was cloned in 1993 (REF. 9), and the first endogenous ligands for cannabinoid CB_1 and CB_2 receptors — the ENDOCANNABINOIDS, as they were termed in 1995 (REF. 10) — were isolated in the early

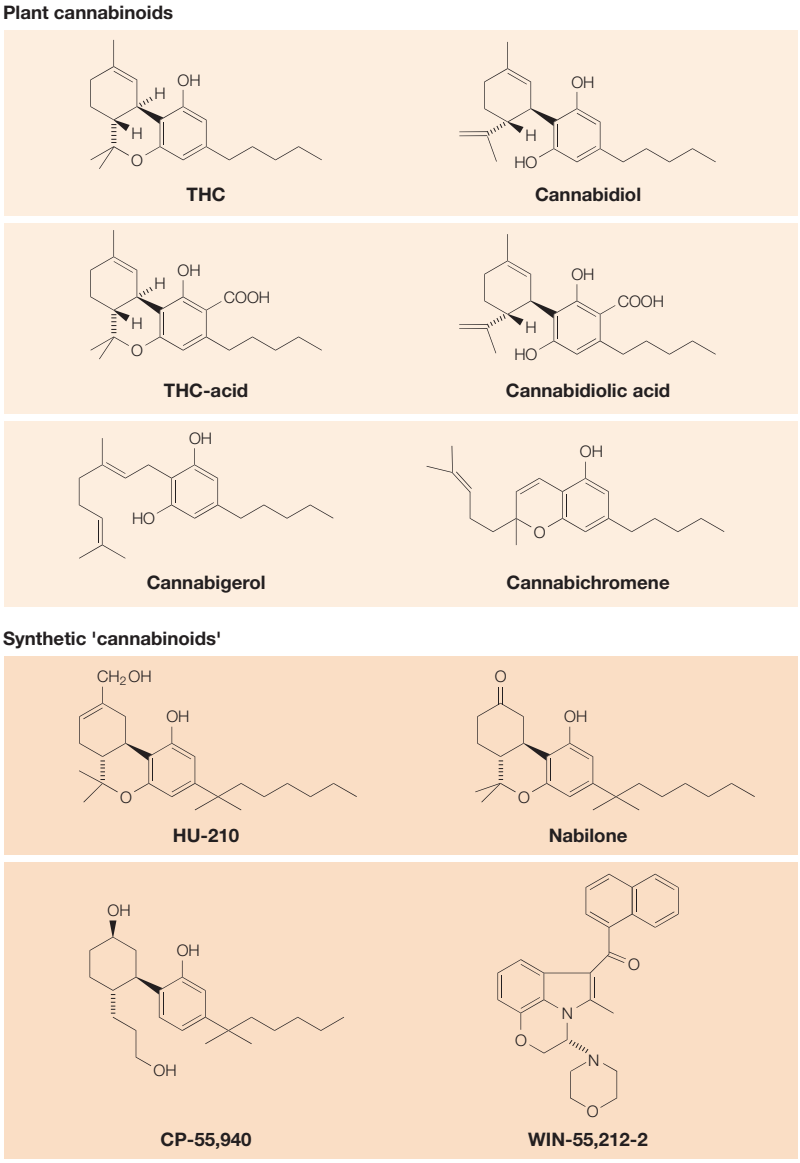

Figure 1 | **Chemical structures of some plant and synthetic cannabinoids.** Of the plant CANNABINOIDS shown, only Δ^9-tetrahydrocannabinol (THC)[4] binds to cannabinoid receptors with high affinity. Of the synthetic ones, none is selective for one type of cannabinoid receptor over the other[18].

CANNABINOIDS
Natural lipophilic products from the flower of *Cannabis sativa*, most of which have a typical bi-cyclic or tri-cyclic structure and a common biogenetic origin from olivetol.

CANNABINOID RECEPTORS
G-protein-coupled receptors for THC, so far identified in most vertebrate phyla. Two subtypes are known: CB_1 and CB_2.

ENDOCANNABINOIDS
Endogenous agonists of cannabinoid receptors in animal organisms.

1990s (FIG. 2)[11–13]. All endocannabinoids identified so far are derivatives (amides, esters and even ethers) of long-chain polyunsaturated fatty acids, specifically arachidonic acid, and exhibit varying selectivity for the two cannabinoid receptors[14] as well as for other molecular targets (FIG. 2)[15]. The two best-studied endocannabinoids are anandamide (*N*-arachidonoylethanolamine) and 2-arachidonoylglycerol (2-AG)[11–13].

Functions of the endocannabinoid system

The components (FIGS 3,4) and possible physiological functions of the endocannabinoid system have been extensively reviewed in recent articles[16,17] and will be outlined here only briefly (although it should be noted that the accounts given of the endocannabinoid system need continuous updating). Both cannabinoid receptors cloned to date are mostly coupled to $G_{i/o}$ proteins, through which they modulate the activity of adenylate cyclases (which they mostly inhibit), mitogen-activated protein kinases (which they stimulate), and, in the case of CB_1 receptors, voltage-activated Ca^{2+} channels (which they inhibit) and inwardly rectifying K^+ channels (which they stimulate), to transduce the binding of agonists into biological responses (FIG. 3)[14]. The chemical prerequisites for the activation by synthetic and endogenous agonists of one intracellular signalling pathway rather than another are being revealed, and might open the way to new signalling-specific drugs.

The tissue distribution of CB_1 and CB_2 receptors accounts for the well-known psychotropic and peripheral effects of THC. CB_1 is one of the most abundant GPCRs found so far in the central nervous system (CNS), and reaches highest density in the basal ganglia, cerebellum, hippocampus and cortex, but is also present in the peripheral nervous system (PNS) and several peripheral organs. CB_2 receptors, by contrast, are mostly restricted to immune tissues and cells[18]. The previous knowledge of THC pharmacology[19] — and, most importantly, recent studies carried out by using pharmacological, biochemical, analytical and genetic (for example, the use of 'knock-out' mice) approaches[16] — are revealing several possible functions of endocannabinoid signalling under both physiological and pathological conditions. In the CNS and PNS, the preferential (although not exclusive) distribution of CB_1 receptors at presynaptic neurons, their coupling to the inhibition of voltage-activated Ca^{2+} channels, and the stimulation of endocannabinoid formation by increased intracellular Ca^{2+} and activation of other GPCRs makes the endocannabinoid system an ideal natural tool for modulating neurotransmitter release[20,21]. In particular, endocannabinoids in the CNS intervene in both short-term and long-term forms of synaptic plasticity, including depolarization-induced suppression of both excitatory and inhibitory neurotransmission, long-term potentiation and depression, and long-term depression of inhibition[22,23]. The implications of these actions in the regulation of cognitive functions and emotions in neuronal circuits of the cortex, hippocampus and amygdala, and in the reinforcement of substances of abuse in the mesolimbic system[24,25], have been discussed elsewhere. The abundance of both CB_1 receptors and endocannabinoids in the basal ganglia and cerebellum makes targeting this signalling system an ideal way to modulate movement and posture.

The NEUROMODULATORY actions of endocannabinoids in the sensory and autonomic nervous systems also result, mostly through CB_1 receptors, in the regulation of pain perception[26] and of cardiovascular[27], gastrointestinal[28] and respiratory[29] functions; their effects on the release of hypothalamic hormones and peptides, and the regulation of their levels by steroid hormones, lead to modulation of food intake and of the pituitary–hypothalamus–adrenal axis[30], as well as of both female and male reproduction[31]. The physiological importance of CB_2 receptors in cellular and, particularly, humoral immune responses is only now starting to be revealed[32],

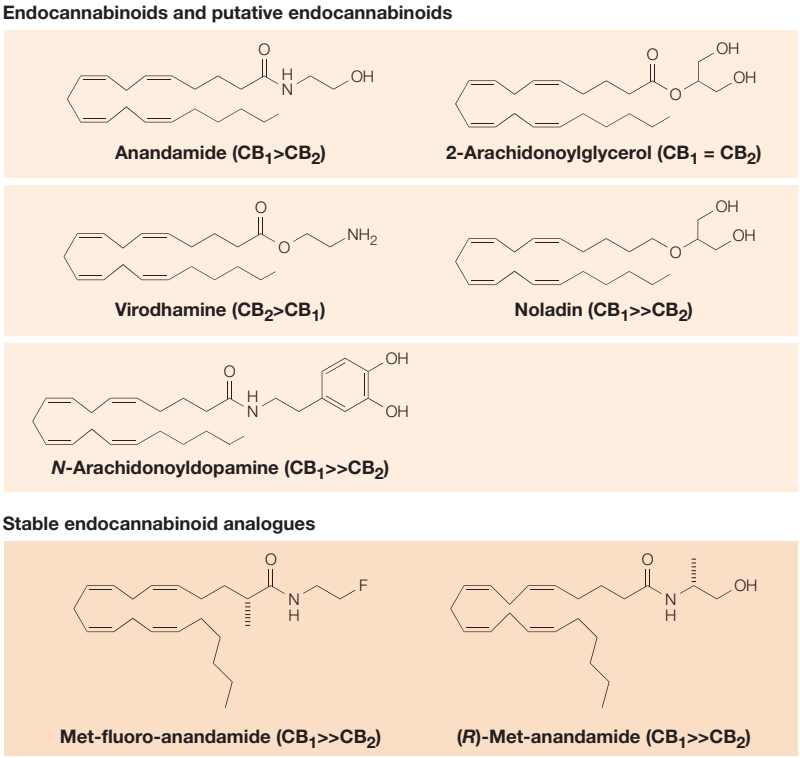

Figure 2 | **Chemical structures of endocannabinoids.** Chemical structures of the two best-studied endocannabinoids, ANANDAMIDE and 2-arachidonoylglycerol[11–13]; of three recently proposed endogenous ligands of cannabinoid receptors[159–161]; and of more metabolically stable synthetic endocannabinoid analogues[162]. The rank of affinity of each compound for cannabinoid receptor subtypes 1 or 2 is shown.

and has possible implications for inflammation and chronic pain. In general — and in view of their chemical nature as lipophilic compounds and their peculiar biosynthetic mechanisms — endocannabinoids seem to act as local mediators in an autocrine and paracrine manner, and recent evidence also points to their involvement in the control of cell metabolism, differentiation, proliferation and death[33]. As cannabinoid receptors are more ubiquitous in mammalian tissues than originally thought, the function of endocannabinoid signalling is likely to extend beyond what could be initially inferred from the knowledge of THC pharmacology.

Pathologically altered endocannabinoid signalling

The development of sensitive and specific techniques for the quantification of endocannabinoid and cannabinoid receptor levels in tissues and biological fluids[34] has enabled us to answer the following questions: is endocannabinoid signalling impaired or overactive during certain disorders, and can this explain the symptoms or the onset and progress of these disorders? Could it be that alleviation of these symptoms with *Cannabis* results from rectifying impaired levels of endocannabinoids in some disorders? Although we now know that the effects of endogenous cannabinoids and exogenously administered THC can differ both qualitatively and quantitatively, this second rather simplistic hypothesis was not too far from the truth, and provided impetus for the first studies on possible pathological alterations in endocannabinoid signalling. There is now increasing evidence that endocannabinoid levels undergo significant changes in several animal models of both acute and chronic disorders.

Neurological, psychiatric and eating disorders. Endocannabinoids are selectively and transiently elevated in specific brain areas during several pathological conditions of the CNS. Endocannabinoid levels are elevated in the hippocampus following glutamate excitotoxicity[35], and after a number of stressful stimuli: in the hypothalamus and limbic forebrain after food deprivation[36]; in the basolateral amygdala after retrieval of an unpleasant memory[37]; and in the periaqueductal grey matter after the administration of a painful stimulus[38]. Endocannabinoid signalling is enhanced to protect neurons from damage through feedback inhibition of glutamatergic neuron activity[35], or to minimize the impact of the stressful stimulus by reinforcing appetite through inhibition of anorectic signals[39,40]; by suppressing aversive memories through inhibition of signalling by GABA (γ-aminobutyric acid)[37]; and by producing central analgesia via suppression of activity of nociceptive neurons[38].

Anandamide levels were also increased in a clinical case of hemispheric stroke[41], which, taken together with the findings that CB_1 receptors seem to contribute significantly to protection from stroke in animals[42] and that 2-AG is protective in a model of head trauma[43], support the notion that endocannabinoids are neuroprotective agents. Indeed, endocannabinoid signalling is also elevated in several animal models of neurodegenerative diseases: in the basal ganglia of reserpine- or 6-hydroxy-dopamine-treated rats (two models of Parkinson's disease)[44,45]; in the hippocampi of β-amyloid-treated rats (a model of Alzheimer's disease) (V.D.M., unpublished observations); and in the brains and spinal cords of mice with chronic relapsing experimental allergic encephalomyelitis (CREAE), a model of multiple sclerosis[46].

The function of this upregulated signalling, as inferred from studies with CB_1 agonists/antagonists and knockout mice, is presumably to counteract neuronal hyperactivity, local inflammation and therefore damage — or, in the case of multiple sclerosis, to inhibit tremors and spasticity[47]. However, the progressive nature of disorders such as Parkinson's and Alzheimer's diseases and multiple sclerosis could result in a permanent, as opposed to transient, hyperactivation of the endocannabinoid system. Such hyperactivation could even contribute to the development of the symptoms of Parkinson's and Alzheimer's diseases (such as inhibition of motor activity and loss of memory, respectively — two typical effects of CB_1 agonists)[44,48]. Hyperactivation also results, in some cases, in a compensatory downregulation of CB_1-receptor expression[49,50]. Interestingly, post-mortem analysis of the brains of patients with Alzheimer's disease revealed an overexpression of normally unexpressed CB_2 receptors, which indicates that endocannabinoids might confer

ANANDAMIDE
One of the most studied endocannabinoids, named from the Sanskrit word 'ananda' for 'bliss'.

NEUROMODULATORY
A physiological action consisting of the capability of modulating neurotransmitter release and/or action.

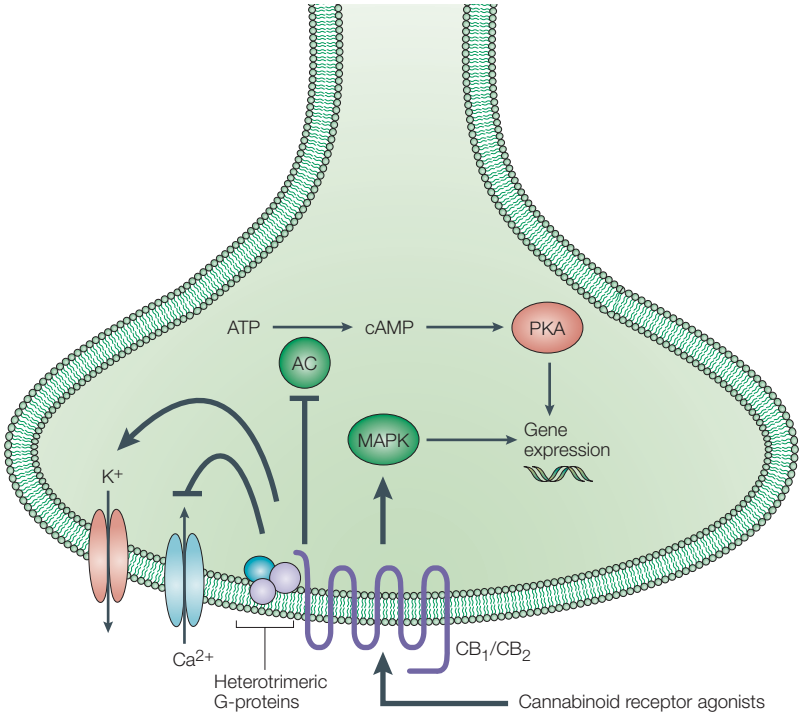

Figure 3 | **Major signalling pathways associated with cannabinoid receptor activation by agonists.** Activation of both cannabinoid CB_1 and CB_2 receptors, and the subsequent stimulation of $G_{i/o}$ heterotrimeric proteins, is well known to be coupled to inhibition of adenylate cyclase (AC) with corresponding inactivation of the protein kinase A (PKA) phosphorylation pathway, or to stimulation of mitogen-activated protein kinase (MAPK). These intracellular events lead to, among other effects, the regulation of expression of several genes. However, more complex protein phosphorylation cascades — specifically, those involving phosphoinositide-3-kinase and protein kinase B — are also proposed to be triggered by CB_1 receptors[14–18]. Furthermore, stimulation, rather than inhibition, of AC by CB_1, but not CB_2, receptors, via G_s proteins, has also been described occasionally. CB_1-, but not CB_2-, receptor stimulation of $G_{i/o}$ proteins is also directly coupled to inhibition of voltage-activated Ca^{2+} channels and stimulation of inwardly rectifying K^+ channels in neurons, with subsequent inhibition of neurotransmitter release. The choice between which of these pathways is modulated by cannabinoid receptor activation also depends on the type of agonist under study[14–18]. cAMP, cyclic AMP.

protection in this disorder by activating CB_2 receptors, and possibly by interfering with inflammatory reactions[51]. In animal models of Huntington's chorea, by contrast, CB_1-expressing fibres in the basal ganglia are progressively lost from the early stages of the disorder onwards, which results in impaired levels of both endocannabinoids and CB_1 receptors — this subsequently contributes to the hyperkinesia typical of the first phase of the disease[52,53]. Importantly, lower levels of CB_1 receptors have also been found in post-mortem brains from patients with Huntington's chorea[54].

It is interesting to note, with respect to the role of endocannabinoids in food intake and energy balance, how the endocannabinoid system seems to become overactive in both the hypothalamus and adipocytes of animal models of genetic obesity[39,55]. This observation might explain the transient inhibition of food intake and the more persistent reduction in fat mass that is observed following treatment of mice and rats with the CB_1-receptor antagonist/inverse agonist[56] SR141716 (Rimonabant; Sanofi-Synthelabo), and the lower susceptibility of CB_1 knockout mice to develop obesity following a high-fat diet[57].

Cardiovascular disorders. Elevated levels of macrophage and/or platelet endocannabinoids are found in the blood of rats during haemorrhagic and septic shock, or following liver cirrhosis and experimental myocardial infarction; these endocannabinoids produce the hypotension typical of these pathological states[58–61]. The levels of CB_1 receptors and endocannabinoids are also elevated in the liver and blood, respectively, of cirrhotic patients[60].

Gastrointestinal disorders. The concentration of anandamide, and/or the expression of cannabinoid CB_1 receptors, is elevated in three mouse models of intestinal disorders: small intestine inflammation[62]; cholera-toxin-induced intestinal hyper-secretion and diarrhoea[63]; and peritonitis-induced paralytic ileus[64]. Interestingly, although enhanced signalling at CB_1 receptors affords tonic protection against the symptoms in the first two disorders, it contributes to a reduction of intestinal motility during paralytic ileus. A recent study showed that genetically engineered mice lacking either the CB_1 receptor or the major enzyme that catalyses anandamide inactivation are more and less susceptible, respectively, to developing colonic inflammation when treated with di-nitro-benzene-sulphonic acid[65]. The importance of the role of the endocannabinoid system in the control of intestinal functions in men is indicated by the occurrence of occasional diarrhoea after the repeated administration of rimonabant (see below).

Reproductive disorders. There are a number of similarities between mice and humans in the endocannabinoid control of reproductive functions. Anandamide, by acting preferentially at cannabinoid CB_1 receptors, has a dual function in mouse embryo implantation, which it stimulates at low concentrations and inhibits at higher concentrations[66]. Accordingly, defective anandamide hydrolysis leads to high levels of this compound in the blood of pregnant women, which correlates with premature abortion or failure of implanted oocytes fertilized *in vitro*[67,68].

Cancer. Finally, increased endocannabinoid signalling is found in some human malignancies compared with the corresponding healthy tissues[69,70], as well as in human cancer cells with a high degree of invasiveness[69,71]. These observations — together with the finding that stimulation of either CB_1 or CB_2 receptors causes blockage of the proliferation of cancer cells or induction of their apoptosis *in vitro*[69,71,72], and inhibition of cancer growth, angiogenesis and metastasis *in vivo*[73–76] — indicate that endocannabinoids might represent one of the many adaptive responses aimed at counteracting tumour-cell growth[69]. This possibility is supported by the recent finding that inhibitors of endocannabinoid inactivation can retard tumour growth both *in vitro* and *in vivo*[69,77]. By contrast, aberrant overexpression of CB_2 receptors on haematopoietic precursor cells has been suggested to be

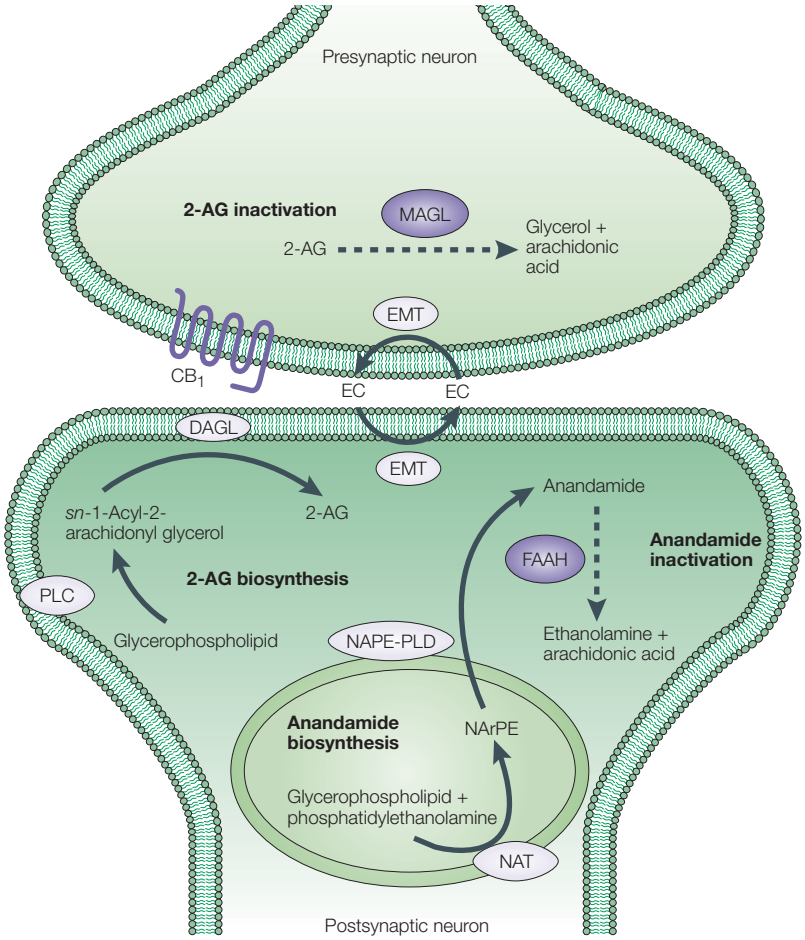

Figure 4 | **Anabolic and catabolic pathways of endocannabinoids and their most likely subcellular localization.** Hydrolytic enzymes are involved in both the biosynthesis of endocannabinoids (ECs) and in their inactivation (BOX 1). The enzymes for 2-arachidonoylglycerol (2-AG) biosynthesis, the phospholipases C (PLC)[82,83] and the *sn*-1-selective diacylglycerol lipases (DAGLs)[88] seem to be mostly localized on the plasma membrane. The DAGLs, in particular, are located on postsynaptic neurons in the adult nervous system[88], whereas the monoacylglycerol lipase (MAGL) for 2-AG inactivation is localized in presynaptic neurons[110], which supports a possible role as retrograde messenger at presynaptic CB_1 receptors for this compound[157]. The anandamide biosynthetic enzymes *N*-acyltransferase (NAT)[84] and *N*-acylphosphatidyl-ethanolamine-specific phospholipase D (NAPE-PLD)[85] and the inactivating enzyme fatty acid amide hydrolase (FAAH)[104] are all located on intracellular membranes. FAAH seems to be most abundant on neurons postsynaptic to CB_1 receptors[158], indicating that anandamide acts principally on these neurons. However, whether NAT and NAPE-PLD are pre- or postsynaptic is not known. Finally, an as yet uncharacterised ENDOCANNABINOID MEMBRANE TRANSPORTER (EMT) seems to facilitate both endocannabinoid release and re-uptake[80,94–97], and might be localized on both pre- and postsynaptic neurons. NArPE, *N*-arachidonoyl-phosphatidyl-ethanolamine.

associated with, and possibly a causative factor of, human acute myeloid leukaemias[78].

In summary, it can be concluded from these ANALYTICAL STUDIES ON ENDOCANNABINOIDS and cannabinoid receptors that altered endocannabinoid signalling accompanies several disorders. Such changes in signalling sometimes represent an attempt to counteract a pathological process, and in other instances are one of the causative factors underlying the disease or its symptoms. Although it is premature to view endocannabinoids as markers of pathological states, a general conclusion from the studies carried out in the past decade is that, in most cases, endocannabinoids seem to have a protective role, which, in certain diseases, can become 'too much of a good thing'. In view of the parallels that exist between many experimental models and the corresponding conditions seen in clinical studies, this conclusion opens the way to the therapeutic use of substances that, depending on the type of disorder, either prolong the half-life of endocannabinoids or prevent their formation or action (see below).

'Endocannabinoid enzymes' as drug targets

If the endocannabinoid system is involved in pathological states, then cannabinoid CB_1 and CB_2 receptors — which, however, recent evidence suggests are not the sole molecular targets for the endocannabinoids[15] — can certainly be considered as new targets for drug development. Furthermore, in view of the findings discussed in the previous section, attempts to pharmacologically manipulate endocannabinoid levels might also result in novel pharmaceuticals. Hence, an understanding of how these molecules are made, and how cells regulate their levels under physiological and pathological conditions, has been recognized as a high priority in cannabinoid research.

Most of the pathways and enzymes for the biosynthesis and degradation of endocannabinoids have now been identified (BOX 1; TABLE 1) and have been extensively reviewed[16,17,79]. Both anandamide and 2-AG are produced by the hydrolysis of precursors that derive from phospholipid remodelling (FIG. 4)[80–83]. The hydrolysis of the phosphodiester bond of *N*-arachidonoylphosphatidylethanolamine, a minor component of animal membranes, yields anandamide in one step. The enzyme that catalyses this reaction, which was identified in the 1980s[84] but remained uncharacterized until only a few months ago, is *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD) (BOX 1)[85]. NAPE-PLD, a member of the zinc-metallo-hydrolase family of enzymes of the β-lactamase fold, is chemically and enzymatically distinct from other PLD enzymes, and is almost equally efficacious with most NAPEs as substrates. It is therefore responsible for the formation of other biologically active *N*-acylethanolamines (NAEs), such as the C16:0, C18:0 and C18:1 congeners. This basic information on the structural and enzymatic properties of NAPE-PLD should soon result in the development of selective inhibitors of the enzyme. The NAPE precursors for anandamide and NAEs are, in turn, produced from the action of an as yet uncharacterized *trans*-acylase enzyme, which catalyses the transfer of an acyl group from the *sn*-1 position of phospholipids to the nitrogen atom of phosphatidylethanolamine in a Ca^{2+}-sensitive manner[86,87].

2-AG is obtained from the hydrolysis of *sn*-1-acyl-2-arachidonoyl-glycerols (DAGs) through the action of two *sn*-1-selective-diacylglycerol lipases (DAGL-α and DAGL-β), which have been recently cloned and characterized (FIG. 4; BOX 1)[88]. These two isoforms seem to be members of the serine lipase (Ser-lipase) family, because they contain the typical lipase-3 and Ser-lipase signature sequences. Within this latter domain, two

ENDOCANNABINOID MEMBRANE TRANSPORTER(S) Putative and elusive membrane protein(s) that has (have) been postulated to be capable of binding selectively to the endocannabinoids and to facilitate their transport across the plasma membrane according to concentration gradients .

Box 1 | **'Endocannabinoid enzymes' — state of the art**

The enzymes regulating endocannabinoid levels can be pharmacologically targeted to manipulate the concentration of endocannabinoid in tissues (see also TABLE 1). Five potential therapeutic enzymes have been identified to date: *N*-acylphosphatidyl-ethanolamine-selective phospholipase D (NAPE-PLD), involved in the conversion of *N*-arachidonoyl-phosphatidyl-ethanolamine to anandamide[85]; fatty acid amide hydrolase (FAAH), which catalyses anandamide hydrolysis and subsequent inactivation at cannabinoid receptors[104]; the *sn*-1-selective diacylglycerol lipase isozymes α and β (DAGL-α and DAGL-β), which hydrolyse diacylglycerols to 2-acylglycerols[88]; and monoacylglycerol lipase (MAGL), which catalyses the hydrolysis of 2-AG[109,110,156]. All these enzymes lack selectivity for one particular member of the families of their substrates, which means that an inhibitor of these enzymes will affect the levels of both cannabinoid receptor-active and -inactive *N*-acylethanolamines (NAEs) and MAGs. These enzymes are also located where their hydrophobic substrates are most likely to be partitioned (FIG. 4), which indicates different subcellular compartmentalization of anandamide and 2-AG, and therefore different functions for the two compounds — for example, in short- and long-term synaptic plasticity[157]. This hypothesis is also supported by the complementary cellular and subcellular localization of the two 'inactivating' enzymes in the adult central nervous system (FIG. 3)[88,110,158]. The endocannabinoid membrane transporter is an as yet uncharacterized and still controversial[103] membrane protein thought to be crucial in the regulation of the distribution of all endocannabinoids between the intracellular and extracellular milieu, and subsequently their ability to interact with cannabinoid receptors and degrading enzymes. Early and indirect evidence[94,102,103] indicates that endocannabinoid transport across the cell membrane is an active process and is not simply driven by intracellular hydrolysis, as suggested by some authors[99].

highly conserved amino-acid residues — Ser443 and Asp495, which normally participate in the catalytic triad of these enzymes — were shown to be necessary for DAGL activity. This opens up the possibilty of developing specific DAGL inhibitors on the basis of knowledge of Ser-hydrolase and lipase-3 inhibitors. DAGLs also contain four unusual hydrophobic, and possibly *trans*-membrane, domains that are probably responsible for DAGL localization to the plasma membrane[88]. Interestingly, although the α- and β-isoforms are preferentially, although not exclusively, expressed in the adult and developing brain, respectively, both enzymes experience a shift in their cellular localization during brain development. Although they exhibit axonal co-localization with CB_1 in the pre- and postnatal nervous system, they seem to be localized in postsynaptic neurons in the adult brain[88]. This shift reflects the proposed role for 2-AG as an autocrine messenger in axonal guidance[89,90], and as a retrograde messenger in the adult brain[22,23]. In both cases, the enzymes clearly use DAGs as substrates, and their action depends on that of other enzymes capable of producing these compounds — such as phospholipase C, for which inhibitors suitable for use *in vivo* have already been developed but are likely to also affect other pathways.

Both the NAPE-PLD and the DAGLs are significantly stimulated by high Ca^{2+} concentration, which explains, in part, why Ca^{2+} influx or its mobilization from intracellular stores triggers anandamide and 2-AG biosynthesis in intact cells. This observation, and the fact that endocannabinoid biosynthesis relies greatly on phospholipid-derived precursors, strongly indicates that these compounds are not stored in secretory vesicles, but are instead biosynthesized and released from cells only 'when and where needed'. This 'ON DEMAND' character of endocannabinoid production fits very well with the local modulatory role proposed for the endocannabinoid system under pathological conditions, which are normally accompanied by increases in intracellular Ca^{2+} to high (mM) concentrations.

Once released from cells, and having activated their molecular targets, endocannabinoids need to be rapidly inactivated (FIG. 4). The importance of endocannabinoid inactivation mechanisms is underlined by a number of observations: low pain sensitivity and low susceptibility to developing colon inflammation[65,91] on the one hand, and pathological states such Parkinson's disease[45], premature abortion[67,68], stronger epileptic seizures[92] and, possibly, higher predisposition to drug abuse[93] on the other hand seem to be associated with impaired endocannabinoid (mostly anandamide) hydrolysis. To be hydrolysed, however, endocannabinoids need first to be rapidly cleared away from the receptor active site and, therefore, to be taken up by the cell.

This process occurs via rapid diffusion through the cell membrane; this is facilitated by intracellular degradation and — as suggested by indirect but robust data — at least one more selective mechanism[94]. This possibly relies on the presence of a membrane transporter[80,95–97], which would mediate a more rapid uptake of endocannabinoids according to their gradient of concentration across the plasma membrane (BOX 1). This putative endocannabinoid membrane transporter (EMT) also seems to be responsible for endocannabinoid release[95], because immediately after their biosynthesis endocannabinoids are more abundant inside compared with outside the cell. It must be emphasized, however, that there is no molecular evidence for the existence of the EMT, and that some authors have suggested that this process might uniquely depend on endocannabinoid intracellular hydrolysis[98,99].

Among the experimental data supporting the presence of a cellular uptake mechanism that is independent of enzymatic hydrolysis, the finding of synthetic substances that are capable of selectively inhibiting anandamide re-uptake over anandamide hydrolysis, and with extremely stringent chemical prerequisites, is certainly one the most convincing (FIG. 5)[94,100,101]. Moreover, genetic deletion of the major enzyme that catalyses the hydrolysis of anandamide does not seem to prevent its rapid and saturable cellular uptake[94,102].

It is possible that the process of endocannabinoid uptake and release is not as simple as originally proposed[80,103], and instead involves various organized forms of the plasma membrane as well as binding proteins. Clearly, further efforts will have to be dedicated to solving the controversial issue of the actual existence of a selective mechanism for endocannabinoid membrane transport; this could possibly be achieved by cloning, expressing and molecularly characterizing the specific protein(s) involved. This will facilitate the pharmacological targeting of endocannabinoid membrane transport, which will subsequently prolong the duration of

ANALYTICAL TECHNIQUES FOR ENDOCANNABINOID STUDIES
Methodologies for quantifying the levels of the endocannabinoids and of cannabinoid receptors, consisting mostly of isotope-dilution mass-spectrometric techniques for anandamide and 2-AG, polymerase chain reaction and *in situ* hybridization techniques for receptor and enzyme mRNAs, western immunoblotting and immunohistochemistry for receptor and enzyme proteins.

'ON DEMAND'
A typical property of the production of endocannabinoids, which are made in the organism only 'when and where needed'.

Table 1 | **Major features of 'endocannabinoid enzymes'***

Target	Cloned	Three-dimensional structure	Catalytic/active site	Selectivity	Tissue and cellular localization	Regulation	Knockout mice	Inhibitors promising in animal models of disorders
NAPE-PLD	Yes	Unknown	Unknown	All NAPEs tested	Unknown	Yes (+)	No	No
DAGLα	Yes	Unknown	Yes	All DAGs tested	Yes	Yes (+)	No	No
DAGLβ	Yes	Unknown	Yes	All DAGs tested	Yes	Yes (+)	No	No
FAAH	Yes	Yes	Yes	All long-chain NAEs and primary amides tested	Yes	Yes (+/–)	Yes	Yes
MAGL	Yes	Unknown	Yes	All MAGs tested	Yes	Yes (–)	No	No
EMT	No	Unknown	Unknown	Only polyunsaturated long-chain NAEs, 2-AG, and other endocannabinoids	Unknown	Yes (+/–)	No	Yes

*See also BOX 1. 2-AG, 2-arachidonoylglycerol; DAGL, *sn*-1-selective diacylglycerol lipase; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAE. *N*-acylethanolamine; NAPE-PLD, *N*-acylphosphatidylethanolamine-selective phospholipase D.

all endogenous cannabinoid signals identified so far. This presents potential therapeutic advantages in many disorders (see above): two examples include the inhibition of spasticity with few side effects in the CREAE model of multiple sclerosis, and blockade of diarrhoea after exposure to cholera toxin[46,63].

As mentioned above, the intracellular metabolism of endocannabinoids occurs mostly, although not uniquely, through enzymatic hydrolysis. Indeed, the first 'endocannabinoid enzyme' to be cloned was the fatty acid amide hydrolase (FAAH)[104], which catalyses the hydrolysis of anandamide and other long-chain fatty acids and, under certain conditions, 2-AG (BOX 1; see REF. 79 for a review). Genetically engineered mice lacking this enzyme have also been developed[105] and seem to have 15-fold higher brain levels of anandamide (but not 2-AG), and a higher threshold sensitivity to nociceptive stimuli[91,105]. Accordingly, pharmacological inhibition of FAAH also leads to analgesic effects (although so far this has been assessed only in animal models of acute pain[106]), and to an antispasticity action in mice with CREAE[46].

Specific FAAH inhibitors have been developed that are able to significantly enhance anandamide levels in nervous tissues and to exhibit analgesic activity in rodents (FIG. 5). In one case, such compounds were shown to also elicit anxiolytic effects in two experimental models of anxiety[107]. An innovative proteomics approach has been also used to develop new inhibitors of FAAH, and led to the identification of nanomolar reversible inhibitors, and enabled promiscuous inhibitors to be discarded in favour of equally potent compounds with at least 500-fold selectivity for their targets[108]. With regard to 2-AG hydrolysis, there are, unfortunately, no available selective inhibitors of the enzyme principally responsible for this process — a monoacylglycerol lipase (MAGL) cloned in the 1990s[109] and recently shown to control 2-AG levels in isolated cells (BOX 1)[110]. A cocktail of cannabinoid-receptor-inactive monoacylglycerols is the only tool developed so far to specifically inhibit 2-AG degradation and enhance its pharmacological and therapeutically beneficial actions[43,111].

NON-PSYCHOTROPIC CANNABINOID
Any plant or synthetic cannabinoid-like compound that does not induce, in animal models and in humans, the central cannabimimetic effects typical of THC.

Developing endocannabinoid-based drugs

The therapeutic applications of cannabinoid CB_1-receptor agonists stem from anecdotal accounts of the medicinal use of *Cannabis*, as well as, most importantly, from undergoing controlled clinical trials (see below). However, the past decade of studies on plant cannabinoids and the endocannabinoid system have introduced the design of more innovative therapeutic strategies that are based on several approaches, each with its own advantages and disadvantages (TABLE 2).

CB_2-receptor agonists. The cloning of CB_2 receptors, and their virtual absence in the healthy brain, was welcomed as the opening of a possible gateway to the development of NON-PSYCHOTROPIC CANNABINOID drugs. However, research on the physiological and pathological function of CB_2 receptors is somehow lagging behind that of their cognate receptors, due to their apparent selective localization in immune cells as opposed to the seemingly more widespread distribution of CB_1 receptors. Nevertheless, we now know that at least three selective, non-psychotropic CB_2 agonists (HU-308, AM-1241 and JWH-133) (FIG. 6) hold promise in inflammatory and neuropathic pain[112,113] and, possibly, gliomas and malignant lymphomas[71,114].

CB_1-receptor antagonists (and inhibitors of biosynthesis). Antagonists are usually desirable compounds for drug companies to develop as therapeutics. Indeed, the most advanced clinical studies performed so far on a novel compound based on the endocannabinoid system are those on rimonabant (SR141716A; FIG. 6), a selective CB_1 antagonist/inverse agonist[115], which is being tested against obesity and tobacco dependence. Phase II clinical trials have been completed, with a successful outcome for both indications, although the dose used in the smoking cessation trials (40 mg per day) did cause some minor side effects. Most of the large-scale Phase III clinical trials will be completed in 2004, although the results of two of these have just been made public ('The endocannabinoid system: a new target for multi-risk management' presented at the Satellite Symposium at the American College of Cardiology meeting, New

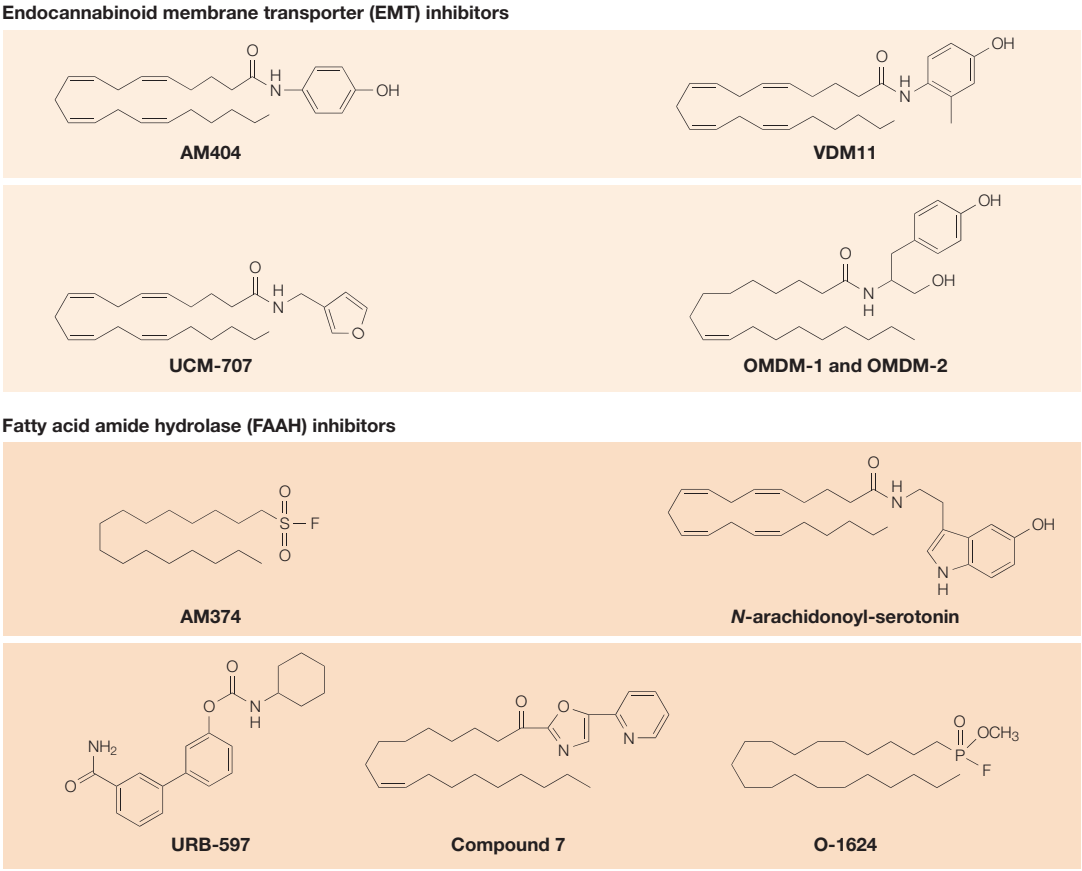

Figure 5 | **Inhibitors of endocannabinoid inactivation.** Inhibitors of both endocannabinoid cellular uptake and intracellular degradation by fatty acid amide hydrolase (FAAH) that have been tested *in vivo* are shown. Of the uptake inhibitors, AM404 was the first to be developed[96], but was not particularly selective. VDM-11, UCM-707 and the two OMDM isomers are more selective, but the last two compounds are more metabolically stable[100,101]. Of the FAAH inhibitors shown[106,107], *N*-arachidonoyl-serotonin[163] is the least potent *in vitro*, but is possibly more selective over cannabinoid receptors or phospholipase A_2. Compound 7 was developed by Cravatt and co-workers using a non-conventional proteomics approach[108]. No inhibitors have yet been developed for monoacylglycerol lipase.

Orleans, 9 March 2004). Rimonabant (20 mg per day) was efficacious in reducing body weight and waist circumference in obese people after 1 year of treatment (with more than 44% of the treated patients having lost more than 10% of their weight), and in ameliorating most of the symptoms of their metabolic syndrome. In another study, the same dose of rimonabant almost doubled the abstinence rate in smokers during 4 weeks of treatment, with no change in body weight in normal smokers and a significant loss of weight in overweight smokers. Interestingly, the minor and occasional adverse events observed with the 40-mg-per-day dose of rimonabant (particularly diarrhoea and nausea) in the Phase II trials were exactly what would have been expected from previous knowledge of those functions that are clearly tonically regulated by the endocannabinoid/CB_1 system in animals — specifically colon motility[116] and emesis[117,118]. These findings in humans indicate that the appropriate dose of rimonabant specifically counteracts that part of endocannabinoid signalling that becomes overactive and participates in some pathological states. Other possible applications of CB_1 antagonists/inverse agonists, based on observations carried out in animal models, might be in the palliative care of Parkinson's and Alzheimer's diseases, and of premature spontaneous abortion. Similarly, yet-to-be-developed selective inhibitors of endocannabinoid biosynthesis might, in the future, be used in these same disorders.

Inhibitors of metabolism. As pointed out above, endocannabinoids seem to be produced 'on demand', and in several cases fulfil a protective role 'when and where needed'. This protection, however, is rendered incomplete by the rapid degradation of endocannabinoids *in vivo*. Therefore, one possible therapeutic approach would be to retard the inactivation of endocannabinoids when they are being produced with a protective function — for example, at the onset of some neurological, cardiovascular and intestinal disorders, or during anxious states. Promising results in preclinical studies have already been published with inhibitors of endocannabinoid metabolism (FIG. 5) in experimental models of acute pain[106], epilepsy[35], multiple sclerosis[46], Parkinson's disease[45], anxiety[107] and diarrhoea[63]. This approach, as opposed to the direct stimulation of cannabinoid

Table 2 | **Therapeutic strategies from the endocannabinoid system***

Strategy	Available	Routes of administration tested	Advantages	Disadvantages	Clinical trials complete?	Indications tested clinically or preclinically
CB_1 agonists	Yes	Oral, suppository	Wide range of applications	Psychotropic effects, tolerance	Yes	Nausea, Tourette's, Parkinson's disease, pain‡, cachexia, MS, glaucoma, cancer, diarrhoea, stroke
CB_2 agonists	Yes	Oral	No psychotropic effect	Limited range of applications	No	Pain, gliomas, lymphomas, inflammation
Partial agonists	Yes	None	Unlikely development of tolerance	Limited efficacy	No	Pain
'Soft' agonists and agonists unable to cross the BBB	No	None tested	No psychotropic effect	Applications limited to 'peripheral disorders'§	N/A	N/A
CB_1 antagonists	Yes	Oral	No psychotropic effect, very few side effects	Limited range of applications	Yes	Obesity, nicotine and alcohol dependence, ileus
Inhibitors of biosynthesis	No	None tested	No psychotropic effect, very few side effects	Limited range of applications	N/A	N/A
Inhibitors of inactivation	Yes	None	Higher selectivity, wide range of applications	Residual side effects	No	Pain, anxiety, diarrhoea, Parkinson's disease
Multi-target preparations and 'hybrid' agonists	Yes	Oral, mixed	Higher efficacy, low tolerance	Limited range of applications	Yes	Pain, spasticity in MS
Cannabinoid receptor-inactive cannabinoids	Yes	Oral	No psychotropic effect; very few side effects	Unknown mechanism of action	Yes	Pain, head injury, rheumatoid arthritis
Cannabis extracts	Yes	Sublingual spray	Toxicology well investigated	Initial side effects	Yes	Pain, spasticity in MS

*See text for details and references. ‡'Pain' denotes chronic, neuropathic, inflammatory, MS-related and post-oprative pain. §'Peripheral disorders' denote those disorders that occur in peripheral organs or tissues as opposed to those developing in the central nervous system. BBB, blood–brain barrier; MS, multiple sclerosis and its animal model (allergic experimental encephalomyelitis); N/A, not applicable.

receptors with systemically administered agonists, is likely to influence endocannabinoid levels principally in those tissues in which there is an ongoing production of otherwise 'silent' endocannabinoids, and therefore should produce fewer side effects.

'Dual target' strategies. Synergistic actions of cannabinoid CB_1-receptor agonists with substances activating other receptors have recently been explored. In particular, two strategies could result in the potential development of either new drugs or new therapeutic treatments. The first approach is through the exploitation of synergistic analgesic effects of CB_1 and μ- or δ- opioid-receptor agonists, which, through the co-administration (for example, of THC and morphine), produces analgesic actions stronger and longer-lasting (through the avoidance of the development of morphine tolerance) than those obtained with each agonist alone[119,120]. The second approach builds on the observation that CB_1 agonists and substances that activate the receptor for capsaicin (the vanilloid transient receptor potential cation channel-1 (TRPV1) receptor) show partly overlapping medicinal actions; this fact could subsequently guide the development of 'hybrid' CB_1/TRPV1 agonists, such as the prototypical arvanil, which has promising analgesic/anti-inflammatory[121–123], antispastic[123] and antiproliferative actions[124].

Soft drugs, pro-drugs, partial agonists and others. These new strategies for the development of non-psychotropic cannabinoids with therapeutic value have been much discussed, but have, to date, made little progress. More effort needs to be directed to several areas. First, the development of compounds that although unable to cross the blood–brain barrier are still able to activate peripheral CB_1 receptors. Second, the development of cannabinoid pro-drugs. And last, the development of drugs with a short half-life and that can activate only peripheral receptors after local administration (soft-drugs). These strategies are recommended to solve the problem of the unwanted psychotropic effects with cannabinoid-based therapies, a problem that is perhaps principally of social, rather than pharmaceutical, importance. On the other hand, the possible use of PARTIAL AGONISTS of CB_1, which should not cause tolerance and dependence phenomena, particularly after dosage uptitration, seems to be an easier option, as evidenced by a few examples of such compounds that already exist[125–127].

Cannabis extracts and non-psychotropic plant cannabinoids. Cannabinoids of either plant or synthetic origin that are non-psychotropic because they are only weakly active on cannabinoid receptors have been studied. The most promising of the plant compounds

PARTIAL AGONIST
Any receptor agonist that does not induce a full functional response in a given functional assay of receptor activation.

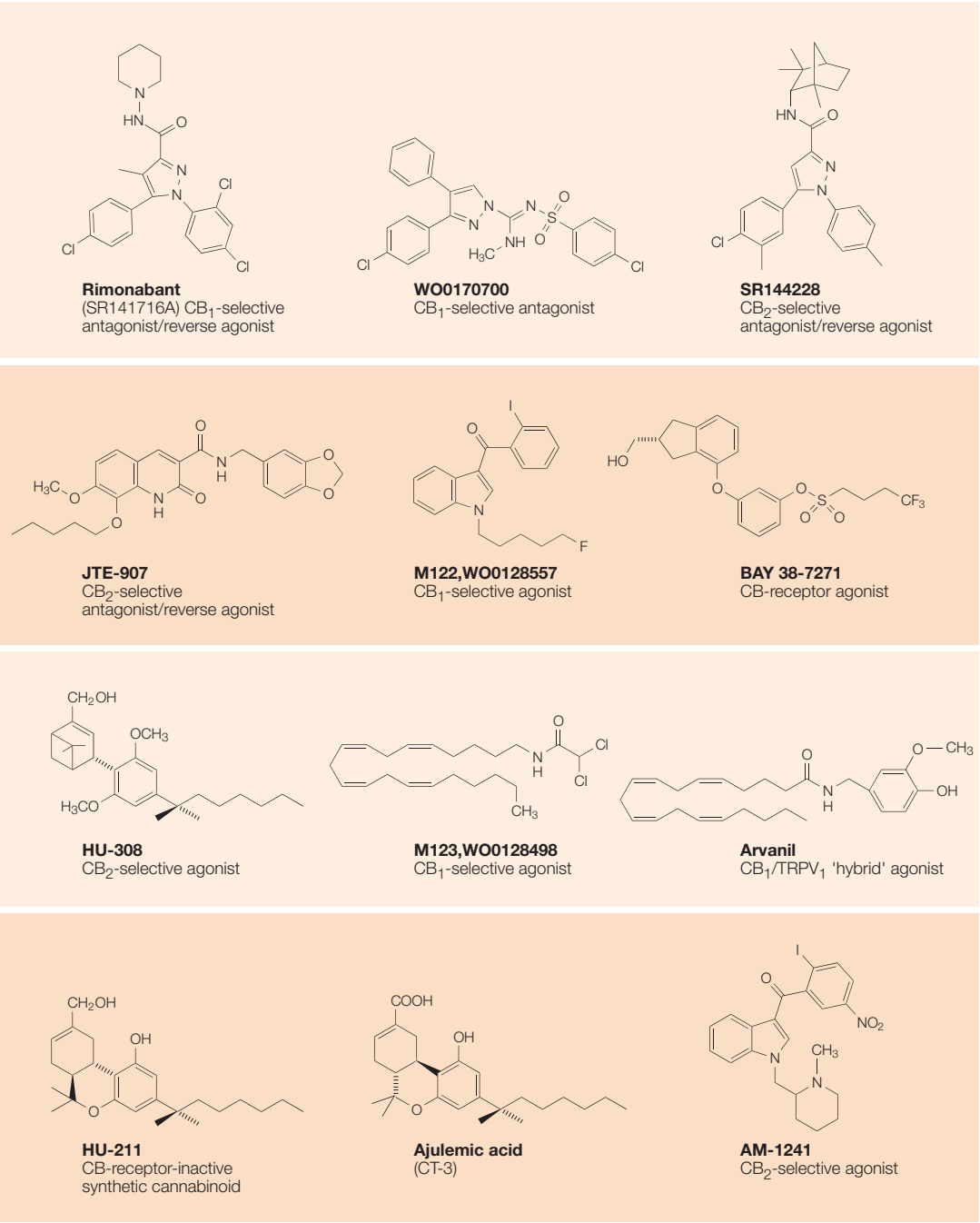

Figure 6 | **Chemical structures of some therapeutically promising, patented drugs based on the endocannabinoid system.** The most important feature of each compound is shown (collated from REFS 112–115,124,131,132,137–142). Arvanil activates both CB_1 and vanilloid ($TRPV_1$) receptors, and for this reason is defined as 'hybrid'.

is cannabidiol, for which interesting anti-inflammatory, anti-emetic, neuroprotective and anticancer actions have been reported[128]. The wide range of beneficial effects of cannabidiol and of other cannabinoids from *Cannabis* (FIG. 1) prompted the therapeutic use of cannabinoid-rich extracts of the dried flowers of this plant[129]. The genetic selection of *Cannabis* strains with exactly reproducible ratios of THC/cannabidiol allowed the preparation of such extracts and their testing in at least five Phase III clinical trials, the results of which are due soon[130]. These trials could open the way to the marketing of Sativex, a sublingual spray developed by GW Pharm and licensed to Bayer for possible future distribution in European countries. Several synthetic cannabinoids have been developed from plant cannabinoids: HU-211 (dexanabinol), a neuroprotective compound developed by Pharmos, now undergoing Phase III clinical trials for severe head injury[131]; ajulemic acid (CT3), a THC-11-oic acid analogue with potent analgesic and anti-inflammatory

activity[132]; and HU-320, a cannabidiol analogue recently shown to have interesting anti-inflammatory activity[133]. Cannabidiol weakly interacts with TRPV1 receptors and some endocannabinoid-degrading enzymes[134], HU-211 inhibits NMDA (*N*-methyl-D-aspartate) glutamate receptors[135], and CT3 activates peroxisome-proliferative activated receptor-γ (PPAR-γ)[136]. However, the molecular mode of action of these compounds is still open to investigation.

Clinical trials: work in progress

The cloning of cannabinoid receptors and the identification of their endogenous ligands has stimulated an ever-increasing effort by pharmaceutical companies to develop novel, potent, selective and possibly water-soluble CB_1- and CB_2-receptor agonists and antagonists, and several relevant patents have been filed since the mid-1990s. Some compounds, shown in FIG. 6, are particularly noteworthy because they either exhibit novel features (such as that of being selective agonists at CB_1 or CB_2 receptors, or being water-soluble), or they are being tested in humans, or both[137–143]. Indeed, apart from the above-mentioned great expectations raised from rimonabant and Sativex, clinical trials have mostly been performed so far with oral THC and non-psychotropic cannabinoids. The results of a recent, multi-centre, randomized placebo-controlled study, including more than 600 patients with multiple sclerosis, were recently published[144]. Although no benefit on spasticity was found with a low oral dose of THC (5 mg per day), when this symptom was assessed using the Ashworth scale, more than 60% of the treated patients exhibited objective improvements in mobility and reported subjective improvements in pain, with an overall very low degree of adverse events[144].

Other promising results were obtained in randomized, double-blind, placebo-controlled crossover trials on the alleviation of tics in Tourette's syndrome[145] and on levo-3,4(OH)$_2$-phenylalanine (L-DOPA)-induced dyskinesia in Parkinson's disease[146], with THC and nabilone, respectively. Nabilone, however, was ineffective in reducing generalized and segmental primary dystonia in Parkinson's disease patients[147]. The topical administration of the synthetic cannabinoid receptor agonist WIN55212-2 was also very effective in reducing intraocular pressure in glaucoma patients[148]. When co-administered orally with morphine (30 mg) to humans, THC (20 mg) exhibited an analgesic effect only slightly synergistic with that of the opiate[120], and, at the dose of 5 mg, it did not significantly reduce post-operative pain in hysterectomized women[149]. The emerging picture of the side effects of oral THC administration, however, seems to be reassuring — only minor events were reported in most cases, and there were few, if any, of the serious immune-suppressive actions and neurocognitive deficits feared in the past[150,151]. Therefore, these current clinical trials raise the hope that, in addition to the traditional indications against nausea/vomiting and weight loss in cancer and AIDS patients (for which, however, there is still no consensus[152,153]), other therapeutic uses might arise in the future from the direct activation of cannabinoid receptors by either THC, its synthetic analogues or *Cannabis* extracts, particularly if higher doses (that is, at the upper threshold for side effects), or alternative routes to oral administration (which leads to poor bioavailability of the active principle) are used.

Clinical trials have also been carried out with CT-3 (40 mg per day), which in a randomized, double-blind, placebo-controlled crossover trial was effective against neuropathic pain[154], and with dexanabinol (48–150 mg), which in a similar, multi-centre study led to more rapid recovery from serious head injury[155]. These trials underline again, for substances developed from plant cannabinoids, the reasonably good agreement found so far between the results of preclinical studies in animal models and the outcome of clinical trials.

Conclusions

From the literature reviewed in this article it is possible to conclude that there is high potential for the opening of new therapeutic avenues from research on the endocannabinoid system, particularly for some disorders for which no satisfactory treatment exists to date. Some strategies aimed at obtaining new pharmaceuticals from THC and *Cannabis* extracts, although partly hampered by the unavoidable social implications concerning the use of psychotropic compounds and by preconceived ideas supported by little scientific evidence, are currently being explored in controlled trials. If successful, these trials — which build on millennia of anecdotal observations of the effects of *Cannabis* on man — will soon be translated into medicinal preparations readily available through medical prescription.

Other strategies, such as the use of synthetic receptor antagonists and agonists, now seem to be possible thanks to the enterprising energy of pioneering drug companies who have invested in this idea — in some cases before cannabinoid receptors were even discovered. More indications might be suggested for these compounds as our knowledge of the pathological role of the endocannabinoid system improves and as more efficacious routes for their administration are devised.

Finally, the realization of the most innovative approaches, such as the use in the clinic of inhibitors of endocannabinoid biosynthesis or inactivation, will require even further enthusiasm and investment, but might prove in the end more gratifying than the strategies currently explored, in terms of safety, efficacy, selectivity and exact knowledge of the mechanism of action of the new drugs developed. In conclusion, only the overturning of old taboos, the understanding that cannabinoids are not 'just for fun', and further basic and preclinical research involving more than a handful of pharmaceutical companies and often underfunded scientists will reveal whether all the promises held by the endocannabinoid system can be realized in the future.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

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