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Review

Endocannabinoids and Related Compounds: Walking Back and Forth between Plant Natural Products and Animal Physiology

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Cannabis sativa has been known, used, and misused by mankind for centuries, and yet only over the last two decades has research stemming from the chemical constituents specific to this plant, the cannabinoids, started to provide fundamental insights into animal physiology and pathology, resulting in the development of new therapeutics. The discovery of the endocannabinoid system, and its targeting with two new pharmaceutical preparations now on the market in several countries, represent the most recent example of how studies on medicinal plants and on the mechanism of their biological effects can reveal, through a chain of breakthroughs, new systems of endogenous signals and physiological phenomena that can become the source of novel strategies for unmet therapeutic challenges.

Introduction

The cannabinoid CB_1 receptor antagonist, rimonabant, has been marketed since August 2006 in several EU countries under the commercial name of Acomplia as a pharmacological aid to exercise and calorie-intake reduction for the treatment of obesity and its metabolic complications, such as dyslipidemia and glucose intolerance [1]. About a year earlier, Sativex, a pharmaceutical preparation based on extracts of *Cannabis sativa*, and containing the psychotropic cannabinoid Δ^9-tetrahydrocannabinol (THC) together with the nonpsychotropic cannabidiol (Figure 1) in an ~1:1 ratio, was introduced in Canada for the treatment of neuropathic pain associated with multiple sclerosis [2]. These recent milestones of the exploitation of the plant with perhaps the oldest history of medicinal and recreational use by mankind are the result of four decades of intensive research. This process started with the identification and chemical synthesis of THC, the controlled study of its pharmacological properties, and the identification of its receptors in animal organisms and of their endogenous ligands, the endocannabinoids (Figure 1), and it continued with the progressive understanding of the physiological and pathological roles of this newly discovered signaling system and the appreciation of the potential therapeutic use not only of THC and endocannabinoids, but also of other plant cannabinoids. Despite this impressive sequence of achievements, whose aspects have been the subject of recent "historical" reviews [3–5], there is much more to the original understanding of the THC mechanism of action than the discovery of the endocannabinoid system. The purpose of this article is not merely to highlight the past contribution of chemical biology to our current understanding of endocannabinoid regulation and function, but, more importantly, to discuss the several other discoveries that stemmed, and are still likely to originate in the future, from the finding of cannabinoid receptors and their endogenous ligands.

From Plant Chemicals to Chemical Signals: Are Endocannabinoids Just Like Endorphins?

Thanks to the chemical synthesis of enantiomerically pure THC analogs (Figure 1), it became clear that the pharmacological actions of this compound were not merely due to its capability to alter membrane permeability in a nonspecific way, but rather to the interaction with specific binding sites. The same analogs, once chemically modified and radiolabelled, served as a tool for the identification of these "cannabinoid" receptors in the rat brain [6]. The finding of the first cannabinoid receptor, the CB_1, out of a series of previously cloned orphan G protein-coupled receptors (GPCRs), soon to be followed by the homology cloning of the second type of cannabinoid receptors, the CB_2, marked the beginning of the discovery of the endogenous cannabinoid signaling system and prompted the search for endogenous ligands, much in the same way as the identification of receptors for another plant natural product, morphine, had led to the identification of the endorphins two decades earlier. This search, however, might have lasted for several years had it not been guided by a chemical concept, i.e., that, by homology to the highly lipophilic THC, physiological cannabinoid receptor ligands were to be looked for among endogenous lipids rather than peptides like the endorphins. This idea was also supported by the observation that CB_1 receptors exhibit relatively high homology with the GPCRs for another family of lipid signals, the lysophosphatidic acids. The identification of the fatty acid ethanolamide anandamide (*N*-arachidonoylethanolamide) [7] and of its glycerol ester analog, 2-arachidonoyl-glycerol (2-AG) [8, 9], as

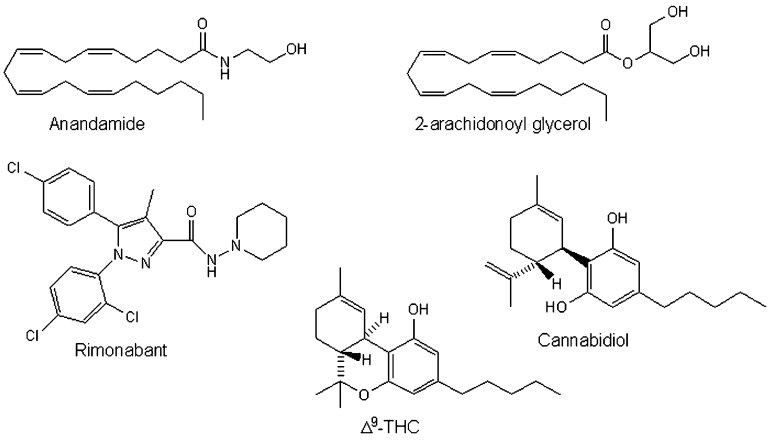


Figure 1. Chemical Structures of the Most-Studied Endocannabinoids, of a Cannabinoid Receptor Antagonist, and of Some Plant Cannabinoids

Chemical structures of (1) the two best-studied endocannabinoids, anandamide and 2-arachidonoyl glycerol [7–9]; (2) the CB_1 receptor antagonist/inverse agonist rimonabant, now on the market under the trade name Acomplia, as a pharmacological aid for the therapy of obesity and the metabolic syndrome [1]; (3) the two major plant cannabinoids, the psychotropic Δ^9-THC and the nonpsychoactive cannabidiol, which are also the major constituents, in a 1:1 ratio, of Sativex, currently marketed in Canada against neuropathic pain in patients with multiple sclerosis [2].

endogenous agonists of CB_1 and CB_2 receptors (Figure 1), and the subsequent finding of their metabolic pathways and enzymes (see [10] for an updated review; see Figure S1 in the Supplemental Data available with this article online), provided the basis for the study of the physiological and pathological role of this endogenous signaling apparatus.

Beyond the similar history of their discoveries, the commonalities between opioid and cannabinoid receptors have been known even before the discovery of the latter, and they include similar inhibitory actions on nociception, gastrointestinal and cardiovascular function, anxiety and stress, and facilitatory actions on food intake and reward in laboratory animals (see [11–13] for recent reviews; Table S1). The coupling, via inhibitory $G_{i/o}$ proteins, of these two receptors to similar intracellular signaling pathways [14] underlies, to some extent, these similarities, whereas interactions between these pathways [13] probably explain why antagonists of each receptor type sometimes counteract the pharmacological effects induced by the stimulation of the other. However, one should not get the impression that the endocannabinoid system is a mere functional duplication of opioidergic signaling. The emerging scenario, in fact, distinguishes between the general strategies of endocannabinoid and endogenous opioid signaling based on the fact that, unlike endorphins, anandamide and 2-AG are lipophilic compounds produced from membrane phosphoglycerides via Ca^{2+}-sensitive biosynthetic pathways triggered on demand, rather than being prestored in secretory vesicles. Hence, because of their chemical nature, endocannabinoids do not typically function like hormones, but they instead act as local (autocrine or paracrine) mediators. This is clearly the case with endocannabinoid action in the brain, where the elevation of intracellular Ca^{2+} caused by postsynaptic neuron depolarization or stimulation of postsynaptic neurotransmitter receptors coupled to Ca^{2+} mobilization from intracellular stores, or both, stimulates enzymes catalyzing the biosynthesis of anandamide and, particularly, 2-AG. These compounds are then released from the neuron to activate presynaptic CB_1 receptors that, in most cases via inhibition of voltage-activated calcium channels, reduce the release of both excitatory (e.g., glutamate) and inhibitory (e.g., GABA) neurotransmitters, thereby producing various effects on neuronal synapses [15]. This ''retrograde'' mode for endocannabinoid action (Figure S1) has been implicated in several physiopathological functions of the brain, including the control of food intake, habit-forming and mnemonic processes, neuronal plasticity, and excitotoxicity. Since the biosynthetic precursors for endocannabinoids seem to be ubiquitous in membranes, it is the reciprocal pattern of expression of endocannabinoid biosynthesising enzymes and cannabinoid receptors that determines the specificity of endocannabinoid action, whereas the localization of the degrading enzymes sets its duration. Indeed, the *sn*-1-specific diacylglycerol lipase (DAGL)-α catalyzing 2-AG release from diacylglycerols [16] is expressed in postsynaptic dendritic ''spines'' that make synapses with axon terminals expressing CB_1 receptors [17, 18]. This allows a lipophilic molecule like 2-AG to reach its target in close proximity to its site of biosynthesis. Also, one of the enzymes responsible for 2-AG degradation, the monoacylglycerol lipase (MAGL), is located in presynaptic neurons, thus allowing for the immediate inactivation of the endocannabinoid signal [19]. Elegant experiments established the time and space frames for endocannabinoid retrograde action [20, 21], which can distinguish between neighboring CB_1-expressing glutamate- and GABA-releasing neurons as targets [22, 23]. The type and time coincidence of Ca^{2+}-mobilizing stimuli play a major role in determining the occurrence of retrograde signaling by endocannabinoids [24, 25].

Experiments are being performed to extend this mode of action also to CB_2 receptors or to nonneuronal cells, including lymphocytes, macrophages, and microglial cells (for the control of the immune and inflammatory responses) [26, 27]; endothelial cells (for the control of vascular tone and angiogenesis) [28]; adipocytes and pancreatic β cells (for the control of adipokine and insulin secretion) [29] or other endocrine cells [30]; and to reproductive tissues [31, 32]. For example, the tight control in time and space of endocannabinoid biosynthesis and degradation within the framework of the chemical

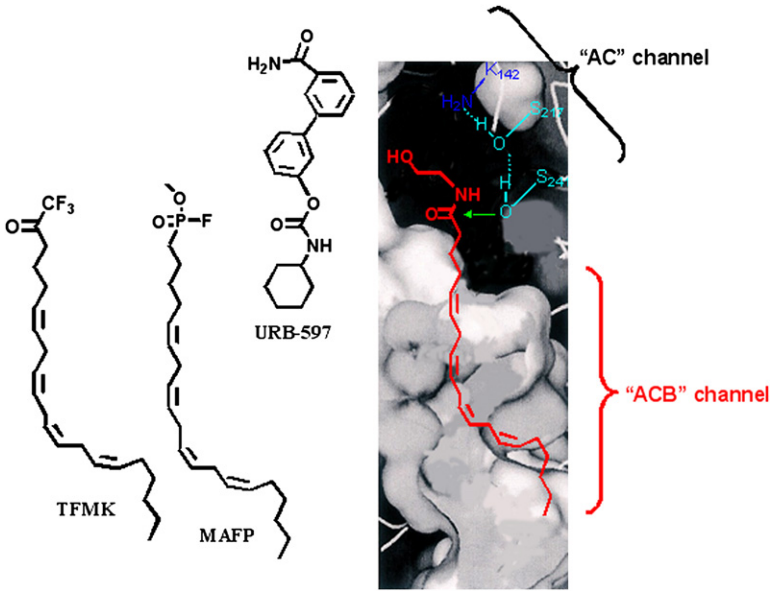


Figure 2. Schematic Representation of the Binding Site of Fatty Acid Amide Hydrolase

Schematic representation of the active site of fatty acid amide hydrolase (FAAH), with "hydrophobic" and "polar head" pockets ("ACB" and "AC" channels, respectively) to accommodate anandamide and other fatty acid amides, as deduced from crystallographic studies [39] and structure-activity relationship studies carried out with several analogs of FAAH inhibitors [43]. Note how the "AC" channel can theoretically accommodate bulkier groups than ethanolamine. Arachidonoyl-trifluoromethylketone (TFMK) and its analogs [34] were used for the affinity chromatography purification of FAAH [35], whereas methyl-arachidonoyl-fluoro-phosphonate (MAFP) was used to obtain crystals from a slightly modified and purified form of FAAH [39]. Carbamate inhibitors like URB597 (see also Table S2) were suggested to enter the active site in a way opposite that of the fatty acid derivatives, with the ester rather than the amide bond being cleaved by the catalytic action of Ser241 [43].

communication between the fertilized egg and cells of the oviduct and uterus is crucial for the correct implantation of the embryo [33]. Thus, although, like the endorphins, they exert a homeostatic function, the endocannabinoids, ultimately due to their chemical nature and peculiar biosynthetic pathways (and to the fact that constitutive CB_1 receptors are much more widely distributed than originally thought, whereas CB_2 receptors appear to be upregulated under several pathological conditions), play a local and yet more general function, seemingly regarding all aspects of animal health and disease (Table S1).

Faces and Facets of Endocannabinoid Metabolic Pathways and Enzymes

The interplay between chemistry and biology played a major role in the identification of the enzymes catalyzing endocannabinoid synthesis and degradation. The fatty acid amide hydrolase (FAAH), which catalyzes the hydrolysis of anandamide (and of other bioactive fatty acid amides, see below) and 2-AG, was isolated after the synthesis of inhibitors [34], which were then used for affinity chromatography purification of the enzyme. This eventually led to the cloning of FAAH [35], now considered to be a promising target for the development of new anxiolytic and analgesic drugs [36–38]. Also, the X-ray structure of FAAH was obtained thanks to the fact that a "modified version" of the purified enzyme yields good crystals only when covalently bound to an irreversible active-site inhibitor [39]. As to the cloning of the DAGL-α and -β catalyzing 2-AG formation from diacylglycerols, this was achieved by using a bioinformatic approach, i.e., starting from the gene encoding a DAGL from *Penicillium*, and then "fishing" for orthologs in the sequenced genomes of increasingly complex animal organisms, ending with the human genome [16].

FAAH is an unusual enzyme since, unlike most serine hydrolases which employ a Ser-His-Asp triad, its catalytic mechanism involves a Ser-Ser-Lys triad [40] (Figure 2). This is consistent with mutagenesis and enzymological studies indicating that Ser241 plays a critical role as both acid and base in the hydrolytic cycle, whereas Lys142 is the activator of Ser241 [41, 42]. Mutagenesis studies also involve Ser217, a residue conserved in all enzymes with an amidase signature sequence, in the catalytic mechanism of FAAH. This residue may act as a "bridge" that, through a proton shift, facilitates both the nucleophile attack and the exit of the leaving group. The FAAH crystal structure revealed the existence of an acyl chain-binding (ACB) channel for entry of hydrophobic substrates and a cytoplasmatic access (AC) channel for hydrophilic compounds, i.e., water for substrate hydrolysis and hydrophilic breakdown products. This latter channel is also likely to accommodate the polar head of anandamide and other fatty acid amides [40, 43] (Figure 2). Despite its atypical catalytic mechanism, FAAH is inhibited by most classical serine hydrolase inhibitors, including trifluoromethyl ketones, fluorophosphonates, carbamates, and α-ketoheterocycle compounds (Table S2). The inhibitory mechanism of these compounds is based on the presence in their structures of a strong electrophile that engages the Ser241 nucleophile in either a covalently reversible or irreversible manner. However, the systematic evaluation of the selectivity of these FAAH inhibitors against other Ser hydrolases is necessary to delineate their therapeutic potential. A chemical proteomic approach known as "activity-based protein profiling" (ABPP) has been used to assess the selectivity of several FAAH inhibitors [44]. ABPP methods, by using active site-directed chemical probes, allow for the screening of inhibitors against multiple enzymes in parallel, and consequently for the detection of possible off-target enzymes [45]. More recently, a multidimensional protein-identification technology (MudPIT) was employed to identify the major off-targets for several FAAH inhibitors [46]. Using

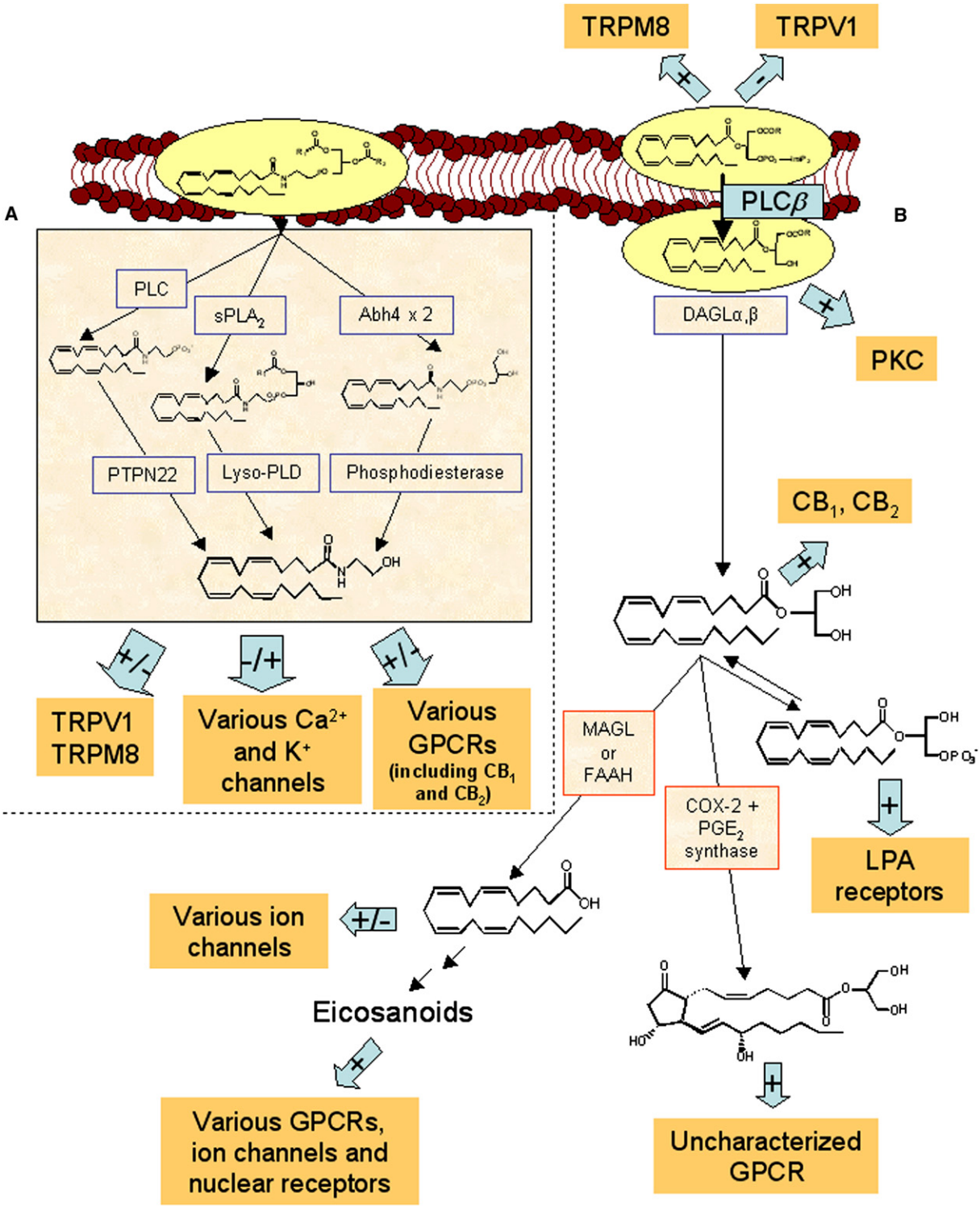


Figure 3. Redundancy, Functional Plasticity, and Target Multiplicity of Endocannabinoid Metabolic Pathways

(A) The three pathways proposed for *N*-arachidonoylphosphatidyl-ethanolamine conversion into anandamide as an alternative to the one represented in Figure S1 [50–52]. Although speculative, it is possible that the multiplicity of the biosynthetic pathways of anandamide reflects to some extent its capability to interact at a submicromolar concentration with several molecular targets [126], including, in addition to cannabinoid receptors, opposing actions on TRPV1 (activation) and TRPM8 (inhibition) [127] channels (see Figure 5).

(B) Biosynthesis and degradation of 2-arachidonoylglycerol seen as a sequence of different signals acting on different targets at different times. Apart from being converted, as discussed in the text, to arachidonic acid and to a COX-2 derivative with specific targets, 2-AG can be transformed into, and produced from, 2-arachidonoyl-lysophosphatidic acid, which activates LPA receptors with relatively high homology with CB_1 receptors [128].

this method, it was shown that the carbamate compounds known as URB-597 [36] and BMS-1 (but not SA-72 [47]), the carbamoyl tetrazole LY2077855, and the α-keto-heterocycle OL-135 [37] act on off-targets [44–46] (Table S2). Also, the recently developed irreversible inhibitors of DAGLs [48] are ideal candidates to be investigated with proteomics-type strategies. These compounds, known as O-3841 and O-3640, are fluorophosphonate derivatives of oleic acid and are at least selective against other proteins of the endocannabinoid system.

In view of the "only when and where needed" character of their action, and of the increasing evidence that endocannabinoids participate in several pathological conditions [10], it can be foreseen that inhibitors of their biosynthesis or catabolism will be used for the pharmacological treatment of disorders ranging from neuropathic pain and psychiatric/neurological disorders to hypertension and obesity, possibly in a more selective way than with cannabinoid receptor agonists (see [49] for a comprehensive review). However, two peculiar aspects of endocannabinoid metabolic pathways should call for caution when it comes to their therapeutic exploitation (Figure 3): (1) the high degree of redundancy with which endocannabinoids are made and/or degraded—this means that, for example, inhibiting just one of the several pathways that have been suggested so far for anandamide biosynthesis from *N*-arachidonoyl-phosphatidylethanolamine [50–52], or only one of the several enzymes (FAAH, MAGLs, COX-2) that have been suggested to catalyze 2-AG hydrolysis [10, 53], might not be sufficient to manipulate anandamide or 2-AG tissue levels, respectively; and (2) the observation that endocannabinoids, as with other bioactive lipids, are part of a sequence of enzymatic reactions leading to a corresponding sequence of chemical signals with distinct molecular targets and biological outputs. 2-AG, for example, is at the same time the product of a family of fundamental intracellular signals, the diacylglycerols, and the precursor of prostaglandin glycerol esters (via COX-2), suggested to act at noncannabinoid receptors [54], or of arachidonic acid (via MAGL), the progenitor of a plethora of mediators and an intracellular mediator itself. Thus, the blockade of DAGLs might not only reduce endocannabinoid signaling, but also may enhance protein kinase C-mediated signals, whereas inhibition of MAGL might lead to the accumulation of prostaglandin glycerol esters. Although it is likely that cellular and subcellular compartmentalization intervenes to segregate these potentially concurring pathways, and perhaps also to ensure that only one pathway contributes to the formation or inactivation of anandamide and 2-AG acting at cannabinoid receptors in a certain cell or tissue (Figure 3), much work remains to validate the efficacy and selectivity in vivo of inhibitors of endocannabinoid metabolism.

Recent data have suggested, however, that some widely used pharmaceuticals might owe some of their therapeutic actions to the interaction with endocannabinoid-biosynthesizing or -inactivating proteins. In particular, some nonsteroidal anti-inflammatory drugs (NSAIDs) (Figure 4) possess this property. Indomethacin exerts anti-inflammatory actions that are partly antagonized by a CB_2 receptor antagonist [55], whereas ibuprofen enhances the analgesic actions of anandamide in a way sensitive to both CB_1 and CB_2 antagonists [56], and flurbiprofen inhibits the spinal release of proalgesic peptides in a way that is antagonized by a CB_1 antagonist [57]. Rofecoxib, a COX-2 inhibitor, synergizes with anandamide while elevating the tissue levels of anandamide and other analgesic fatty acid ethanolamides [58]. Indeed, one possible way to explain these findings is by suggesting that these NSAIDs also inhibit FAAH, as already shown by Fowler and collaborators for ibuprofen in 1997 [59]. More recently, the same group extended this property to other acidic NSAIDs, and they showed that it becomes more important when the extracellular pH is lower than 7, as during inflammatory conditions [60]. Meanwhile, other indomethacin derivatives, such as indomethacin methyl ester and indomethacin morpholinylamides (Figure 4), were also found to directly activate CB_1 and CB_2 receptors, respectively [61, 62]. More intriguingly, a recent study [63] showed that, in rats and mice, acetaminophen (paracetamol) is converted in vivo into *p*-aminophenol and then *N*-arachidonoyl-phenolamine (AM404) (Figure 4), a compound capable of inhibiting both FAAH and anandamide cellular reuptake and elevating anandamide tissue levels [64, 65]. The second reaction is catalyzed by FAAH (an enzyme that, under certain conditions, can facilitate the condensation of fatty acids with amines), thus opening the possibility that it might also occur with other aromatic amines used in the clinic and lead to aromatic amides capable of interacting with other proteins of the endocannabinoid system. Importantly, the analgesia on a hot plate of acetaminophen is blocked by CB_1 receptor antagonists, thus confirming that this widely used drug does also act through enhancement of the endocannabinoid system [66].

NSAIDs are not the only therapeutic drugs capable of interacting with endocannabinoid enzymes. The general anesthetic propofol (Figure 4) also inhibits FAAH, and a part of its sedating properties is due to the subsequent elevation of the brain levels of anandamide, which, via CB_1 receptors, can induce sleep [67]. More recently, the antiobesity drug tetrahydrolipstatin (orlistat) (Figure 4) was shown to inhibit DAGL-α and -β and, hence, to reduce 2-AG levels in intact cells, at concentrations lower than those necessary to inhibit other lipases [16, 48]. Although this compound inhibits obesity mostly by acting at the level of lipid assimilation, it is tempting to speculate that

Abbreviations: Abh4, α/β-hydrolase 4; CB_1, CB_2, cannabinoid receptors of type 1 and 2, respectively; COX-2, cycloxygenase-2; FAAH, fatty acid amide hydrolase; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; lyso-PLD, lyso-phospholipase D; MAGL, monoacylglycerol lipase; PKC, protein kinase C; $sPLA_2$, secretory phospholipase A_2, PLC, phospholipase C; PGE_2, prostaglandin E_2; PTPN22, protein tyrosine phosphatase N22; TRPM8, transient receptor potential melastatin type 8 channel; TRPV1, transient receptor potential vanilloid type 1 channel.

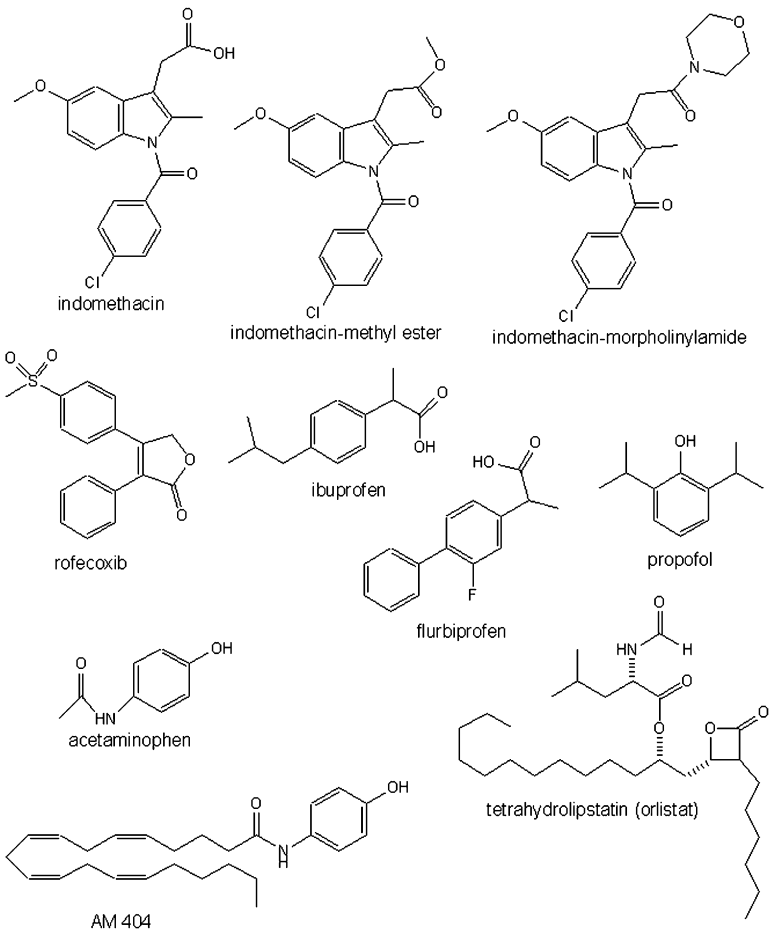


Figure 4. Chemical Structures of Widely Used Therapeutic Drugs that Have Been Reported to Interact with Proteins of the Endocannabnoid System

The cycloxygenase inhibitor indomethacin, and some of its derivatives, were suggested to directly bind to and activate CB_1 and/or CB_2 receptors [61, 62]. Despite the lack of chemical similarity with anandamide, the NSAIDs, rofecoxib, ibuprofen, and flurbiprofen, and the general anesthetic propofol were suggested to act, in part, by inhibiting FAAH-catalyzed anandamide degradation [56–60, 67]. The NSAID acetaminophen, instead, was shown to act, in part, through the conversion into AM404 and subsequent indirect activation of cannabinoid receptors [63, 66]. The antiobesity drug orlistat (tetrahydrolipstatin) was shown to potently inhibit the biosynthesis of 2-arachidonoylglycerol [16, 48].

part of its actions are due to inhibition of 2-AG-induced, and CB_1-mediated, gastrointestinal actions [68]. In summary, from these data the intriguing possibility emerges that we might already be using in the clinic, without knowing it, substances that act in part by manipulating endocannabinoid levels.

Back to Plants: Are Endocannabinoids Like Chili Peppers?

The chemical structure of anandamide is not so different from that of capsaicin (Figure 5A), the pungent fatty acid amide component of another plant with a long history of medicinal and nutritional use, the hot chili pepper *Capsicum annuum*. In fact, capsaicin is even more chemically similar to the aforementioned AM404 (Figure 4), which inhibits anandamide degradation [64, 65]. After the identification by Caterina and colleagues [69] of the *t*ransient *r*eceptor *p*otential *v*anilloid-type 1 channel (TRPV1, originally known as vanilloid VR1 receptor) as the molecular target of capsaicin (Figures 5B and 5C), we and others, based on these chemical considerations, began to investigate the possibility that this receptor and proteins of the endocannabinoid system share common ligands [70]. Compounds were synthesized that bind to CB_1 receptors, FAAH, or the putative anandamide membrane transporter (Figure S1), on the one hand, and to TRPV1, on the other hand [71, 72]. More importantly, it was possible to demonstrate that anandamide activates TRPV1 receptors [73, 74]. Later, other long-chain fatty acid ethanolamides, i.e., the naturally occurring homologs of anandamide (Figure 5A), and AM404 itself, were found to stimulate the activity of TRPV1 receptors [72, 75–78], and to share this property with other derivatives of arachidonic acid [79]. Molecular-modeling techniques were used to demonstrate that the preferential conformations of these compounds in solution overlap with those of capsaicin [78, 79] (Figure 5D). However, none of these endogenous compounds appeared to be nearly as potent as capsaicin at inducing TRPV1-mediated biological responses. Although we now know that the "vanilloid" activity of these compounds is dramatically increased by several regulatory factors [80], this observation, together with the original belief that vanilloid TRPV1 receptors, by being mostly expressed in sensory afferents, are used as receptors only for external painful stimuli (high temperature, low pH, plant toxins), seemed to rule out the existence of true "endovanilloids."

Meanwhile, however, increasing and conclusive evidence was obtained for the presence of TRPV1 receptors in the brain [81–83], where these proteins are unlikely to be

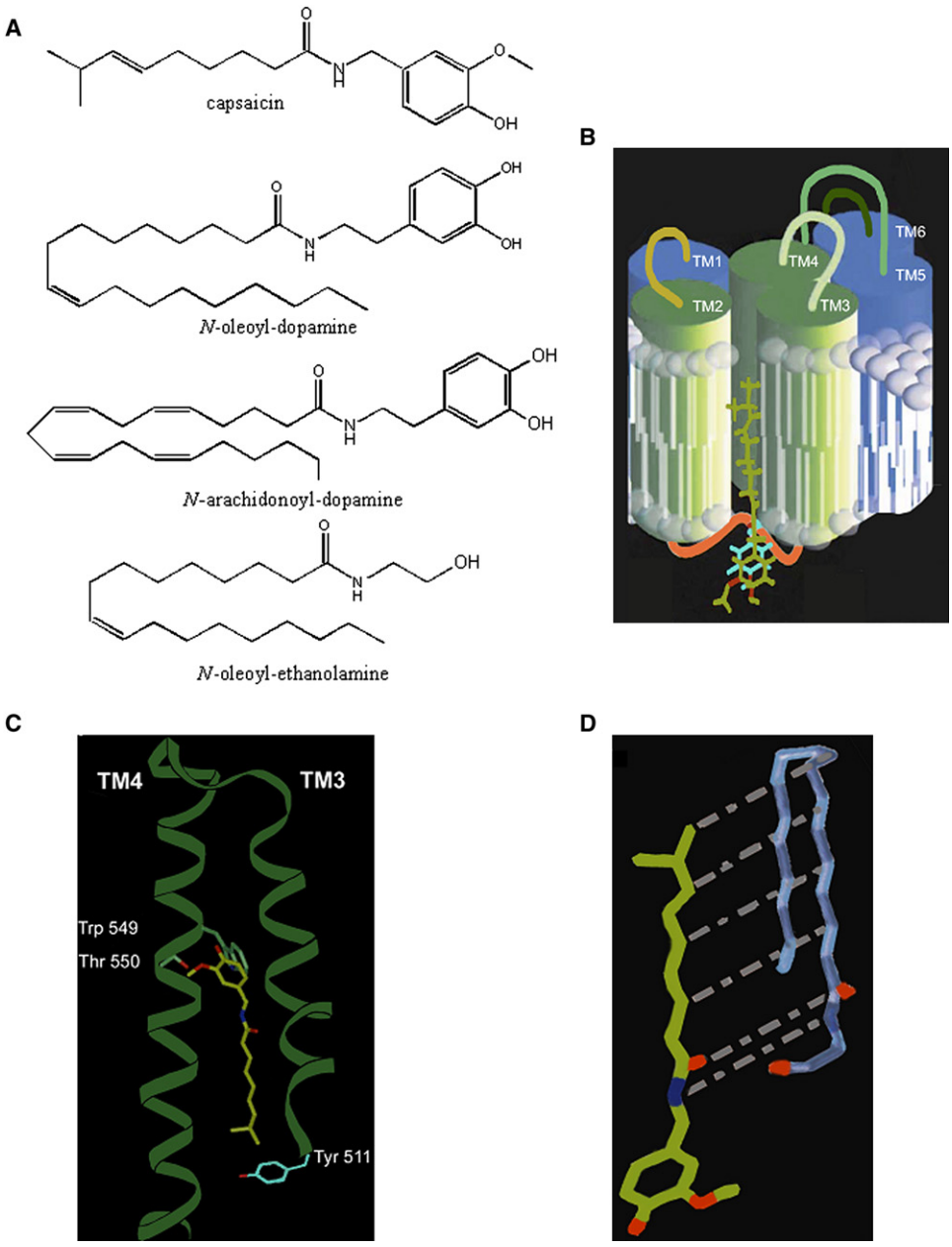


Figure 5. Vanilloid Receptors and Endovanilloids

(A) Chemical structures of *N*-acyldopamines and oleoylethanolamide, identified after the discovery of endocannabinoids and of the receptors for capsaicin and resiniferatoxin, the transient receptor potential vanilloid type 1 (TRPV1) channel.

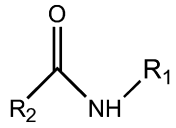
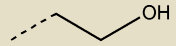
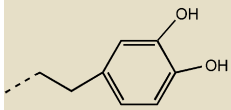
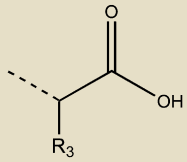
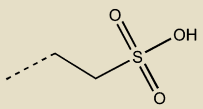
(B) Three-dimensional representation of a TRPV1 monomer with the binding site for capsaicin according to Jordt and Julius [129]. According to this model, aromatic stacking of the vanillyl moiety of capsaicin (as well as anandamide) with Tyr511 in the intracellular T2-T3 linker region stabilizes the binding of the aliphatic chain with a transmembrane site created by the T3 and T4 domains. The observation that the T2-T3 linker domain plays a crucial role in ligand recognition in both TRPV1 receptor and the menthol and "cold"-receptor, TRPM8, led to the identification of anandamide and NADA as TRPM8 antagonists [127].

(C) Model proposed by Gavva and colleagues according to which capsaicin, anandamide, and *N*-arachidonoyldopamine (NADA) bind to Tyr511 through hydrophobic binding with their aliphatic chain, whereas residues Trp549 and Thr550 are involved in binding with the vanillyl or cathecolamine moieties of capsaicin and NADA, respectively [130].

(D) Overlap of capsaicin and oleoylethanolamide conformations in solution, according to the molecular model proposed by Movahed et al. [78].

reached by exogenous stimuli, thus strongly suggesting the existence of endovanilloid ligands [84]. Considerations on the structure-activity relationships of synthetic vanilloid TRPV1 agonists, which indicated the necessity of (1) a long, unsaturated alkyl chain, (2) a secondary amide group, and (3) 3-hydroxy-4-methoxy substituents on the aromatic moiety, in order to achieve optimal interaction with the receptors [85, 86] (Figure 5C), together with the

Table 1. Five Families of Fatty Acid Amides

	Potential Combinations (and Compounds Identified So Far)	Possible Target(s) with a Known Molecular Structure	FAAH Substrate	Biosynthesis
General Structure				
R_1				
	16 (12)	CB_1, CB_2 (C20:4 > 20:3 > C22:4 > C18:3) [131]; TRPV1 (C20:4 > C18:2 > C18;1) [73, 74, 77]; TRPM8 (C20:4, which inhibits) [127]; PPARγ (C20:4) [132]; PPARα (C18:1 > C16:0) [103]; GPR55 (C16:0, C20:4) [107]; GPR119 (C18:1) [106]	Yes [35]	Same as anandamide (see Figure 3 and Figure S1) [50]
	16 (4)	CB_1 (C20:4) [87]; TRPV1 (C20:4 = C18:1) [88, 89]; TRPM8 (C20:4, which inhibits) [127]	No. Inactivated by COMT [88]	Condensation between dopamine and fatty acids [88]?
	336 (~44)	GPR18 (R_3 = Gly) [106] (unknown for other members)	Yes (but not with all amino acids) [93]	Condensation between amino acids and fatty acids [91]?
	16 (9)	TRPV1 (all of those tested) [104]; TRPV4 (all of those tested) [104]	Yes [97]	Condensation between taurine and acylCoAs [104]
H	16 (5)	Several proteins (but only at micromolar concentrations)	Yes [35]	Studied only for C18:1 [133, 134]

The possible combinations of amino acids or biogenic amines and the most abundant fatty acids found in mammalian tissues yield new potential endogenous mediators: the *N*-acyl-ethanolamines, *N*-acyl-dopamines, *N*-acyl-amino acids, and *N*-acyl-taurines [97–100]. Fatty acid primary amides have also been identified [135, 136]. The targets and metabolism of only a few of the several hundred possible compounds have been determined to date [103–107] and are summarized here. R_2 = C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:3, C20:4, C22:0, C22:4, C22:5, C22:6, C24:0, C24:1 alkyl chains; R_3 = residues of amino acids or GABA. COMT, catecholamine-*O*-methyl-transferase; FAAH, fatty acid amide hydrolase; PPAR, peroxisome-proliferator-activated receptor; TRPV, transient receptor potential vanilloid-type.

realization that these prerequisites could be met, to some extent, only by putative endogenous amides of dopamine with long, unsaturated fatty acids, led first to the synthesis [87] and then the isolation from the brain of *N*-arachidonoyl- and *N*-oleoyl-dopamine [88, 89]. These compounds (Figure 5A; Table 1) are the most potent and efficacious endovanilloids identified to date and are most abundant in brain regions rich in dopamine, in agreement with their potential role as endogenous TRPV1 activators and with their possible biosynthetic origin from the *N*-acylation of dopamine. They are accompanied by saturated homologs that, although inactive per se on TRPV1, are capable of enhancing the activity of other endovanilloids [90]. Thus, once again, the meeting of chemical and biological minds led to the finding of endogenous counterparts of a plant natural product. The importance of the discovery of

N-acyl-dopamines, however, went beyond that of finding potent endogenous ligands for TRPV1 receptors, since it supported the hypothesis that animal tissues might make, and use as chemical signals, any combination between fatty acids and biogenic amines.

Bioactive Fatty Acid Amides: When Animal Cells Play Combinatorial Chemistry

That cells could "combine" different fatty acids and biogenic amines to make several possible permutations of different fatty acid amides had been deduced already from the discovery in rodents of *N*-acyl-amino acids such as *N*-arachidonoyl-glycine (NAGly) and *N*-arachidonoyl-alanine (Table 1), which share with fatty acid ethanolamides like anandamide a potent analgesic and anti-inflammatory activity and the property of being recognized by FAAH as substrates [91–93]. This finding raised the possibility that any long-chain fatty acid available in tissues could be amidated with any of the ~20 amino acids and their naturally occurring derivatives. This would lead to the origination of hundreds of potential endogenous mediators with functions and molecular targets to be discovered. This possibility seems to be confirmed by the recent identification of *N*-arachidonoyl-serine, a brain constituent with vasodilatory and anti-inflammatory activity [94], and by increasing evidence for the presence in rodent tissues of ~40 other postulated fatty acyl amides [95, 96].

How is chemical-biological research going to cope with this "invasion" of new small-molecule mediators? How to discover and measure their levels in tissues, reveal their metabolic pathways, understand their physiological and pathological roles and, most importantly, identify their molecular targets? Clearly, the employment of the most modern "omic" approaches for gene, protein, and metabolite profiling will be required to find a function and a "parent receptor" to these orphan mediators, as well as to guide their discovery. Some progress in this direction has already been made. For example, based on the assumption that at least some of these compounds would be FAAH substrates, Cravatt and collaborators used an original global metabolite-profiling strategy, consisting of comparing, by the use of liquid chromatography-electrospray-mass spectrometric (LC-ESI-MS) techniques, the lipid components of tissues from wild-type and FAAH null mice, to identify new fatty amides. This led to the identification of *N*-acyl-taurines (Table 1) as new endogenous amides whose levels are controlled by this enzyme [97, 98]. A similar approach, using FAAH null mice treated with anandamide, led to the identification of a potential alternative pathway for the inactivation of this compound and other acylethanolamides through the formation of the corresponding *O*-phosphorylcholine derivatives [99]. Other comparisons between lipid profiles, for example those of distinct brain areas from mice at different phases of their estrous cycle [100], can be used to understand the function of the analytes. Multidimensional (tandem) mass spectrometric techniques applied to other types of lipidomic-like approaches [101, 102] also seem to be very promising for the discovery of even trace quantities of fatty acid amides belonging to several families, or for the analysis of already discovered compounds [95, 100]. Thanks to the use of "common fragment" methods, all of the naturally occurring members of the same family of fatty acid amides with different acyl chains but yielding common fragments after MS-MS (Table 1) can be analyzed in the same run (see Figure S2). Furthermore, the use of the optimal chromatographic conditions and of LC-ESI-MS-MS allows for the analysis of several fatty acid amide classes at the same time.

Progress is also being made in the identification of receptors for bioactive fatty acid amides. Some serendipitous but nevertheless important discoveries, i.e., that oleoylethanolamide binds to and activates peroxisome proliferator-activated receptor α [103], or that *N*-arachidonoyl-serine activates an as-yet-unidentified endothelial cannabinoid receptor [94], were prompted by the pharmacological actions observed for these compounds. *N*-acyl-taurines, like acylethanolamides and acyldopamines, activate TRPV1 receptors, but they also gate a related plasma membrane cation channel, the TRPV4 receptor [104]. Also, the finding of two GPCRs, GPR119 and GPR18, as high-affinity receptors for oleoylethanolamide and NAGly, respectively [105, 106], was the result of targeted approaches. The implications of industrial patents claiming that GPR55 might be a high-affinity target for palmitoylethanolamide have also been recently discussed [107]. However, in view of the observation that bioactive fatty acid amides can be promiscuous in their targets, and that the latter, as with other lipid mediators, encompass all types of receptors (i.e., GPCRs, nuclear receptors, ion channels [Figure 3]), it can be foreseen that more systematic approaches, such as the use of biospecific interaction analysis (BIAcore), protein or mRNA arrays, and even of virtual screening methodologies, will have to be used in the future for the "deorphanization" of these novel compounds and, at the same time, of the ~150 G protein-coupled and ~24 nuclear "orphan" receptors still awaiting assignment of endogenous ligands. These techniques, together with the MS methods mentioned above, will have to be applied to also investigate if other amides of fatty acids, for example with serotonin, histamine, adrenaline, and noradrenaline, as well as with trace amines, occur in mammalian tissues. Some of these compounds (e.g., *N*-arachidonoyl-serotonin, Figure 6) have already been synthesized and exhibit interesting pharmacological properties (see below).

Think Like a Tinker: Making Endocannabinoid-Based Drugs with Nature's Bits and Pieces

If nature can combine chemical moieties to make small, multitarget chemical signals via dedicated metabolic pathways, the synthetic chemist can also put together naturally occurring building blocks to make drugs that can interact with multiple receptor types, thus obtaining, in theory, more efficacious and therapeutically useful pharmacological tools. Given the overlap between the potential clinical applications (pain, inflammation, emesis, cancer, etc.) and binding recognition properties of

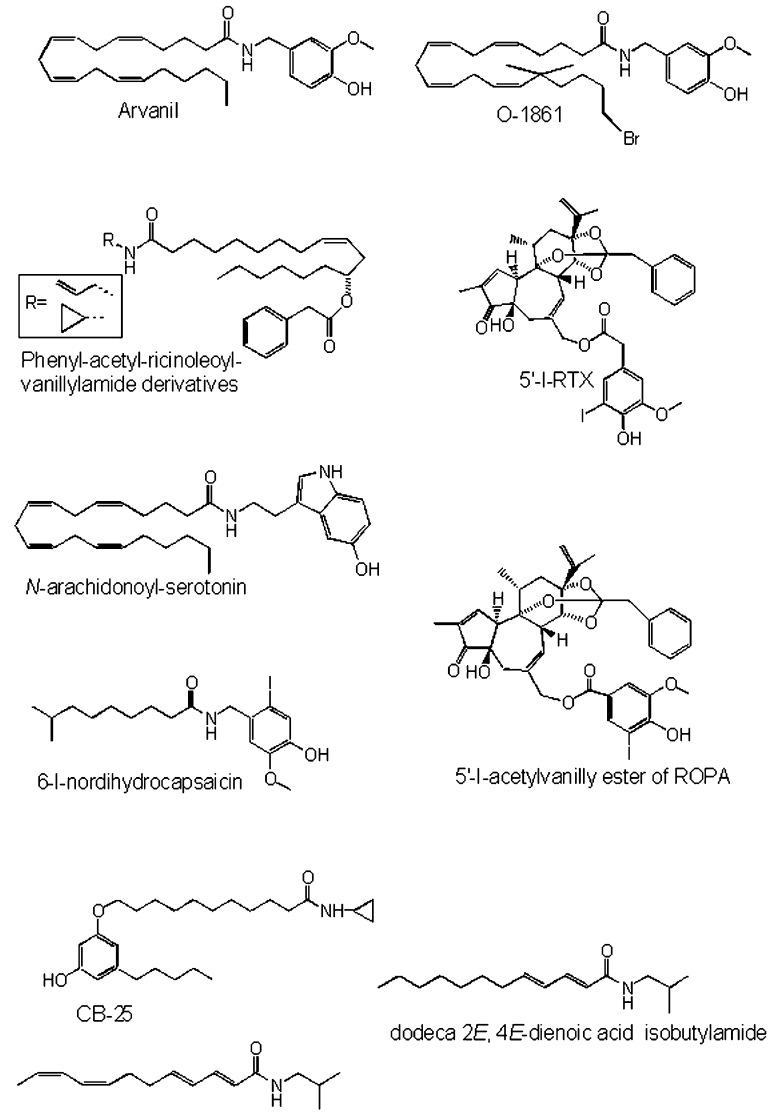


Figure 6. "Tinkering" with Nature's Building Blocks

Combining the vanillyl moiety typical of several plant natural products with arachidonic acid or ricinoleic acid, and the further modification of the chemical structures, yields compounds with mixed activity at cannabinoid and vanilloid TRPV1 receptors, i.e., arvanil, O-1861, and phenyl-acetyl-ricinoleoyl-cyclopropylamide [71, 108, 109]. The synthetic *N*-arachidonoyl-serotonin mimics Nature's capability to make amides between long-chain fatty acids and biogenic amines, and turned out to be a selective inhibitor of fatty acid amide hydrolase (Table S2) and a potent antagonist at TRPV1 channels [38, 110]. Iodination of the vanillyl moiety of a natural compound like resiniferatoxin (RTX), or of synthetic natural product derivatives, such as the acetylvanillyl ester of resiniferol orthophenylacetate (ROPA) and nor-dihydrocapsaicin, can produce opposing effects on the activity at TRPV1 channels [111–114]. The combination of a natural product like olivetol and a long-chain fatty acid yielded CB-25, a potent cannabinoid receptor ligand with "protean" agonist activity in vitro and in vivo [115–117]. Isobutyl analogs of anandamide are more potent than the parent compound at cannabinoid receptors, and this is perhaps the reason why the *Echinacea* components dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid- and dodeca-2*E*,4*E*-dienoic acid-isobutylamide are the only plant natural products apart from cannabinoids that activate cannabinoid CB_2 receptors quite potently [118–121].

proteins of the endocannabinoid system and TRPV1 channels, one strategy to develop hybrid therapeutic drugs was to put together fatty acids and the vanillyl moiety of capsaicin, each opportunely modified in order to maximize the interaction of the end product with each postulated target. *N*-arachidonoyl-vanyllamide (arvanil) and its analog, O-1861 (Figure 6), were synthesized with this idea in mind, and they proved to be capable of potently activating TRPV1 receptors while acting as agonists at CB_1 receptors and inhibitors of the cellular reuptake of anandamide [71, 108]. Both compounds turned out to be more potent and efficacious analgesics than pure cannabinoid or vanilloid receptor agonists with comparable affinities for their respective receptors. The derivatization of the hydroxy group of ricinoleic acid, followed by amidation with chemically modified ethylamine "heads," yielded compounds with antagonist/inverse agonist activity at CB_2 receptors and agonist activity at TRPV1 receptors and, hence, potential application against inflammation [109]. The aforementioned *N*-arachidonoyl-serotonin not only inhibits FAAH, thus elevating endocannabinoid levels and acting as an indirect cannabinoid receptor agonist [110], but it is also a potent TRPV1 antagonist in vitro and in vivo, thus exhibiting equivalent activity against neuropathic pain as other compounds that are more potent at FAAH [38].

However, tinkering with natural products can also lead to unexpected results. For example, 5′-iodination of resiniferatoxin on its vanillyl moiety (Figure 6) transforms the ultrapotent agonist activity at TRPV1 channels of this plant toxin into the capability to antagonize these receptors, whereas 6′-iodination only weakens its agonist activity [111]. By converse, 6′-, rather than 5′-, iodination of the

much less hindered nordihydrocapsaicin (the saturated analog of capsaicin) and of other vanillamide TRPV1 ligands is required to transform their agonist activity into potent TRPV1 antagonists [112], without decreasing the capability of some of these compounds to directly inhibit NF-kB activity, thus yielding dual target anti-inflammatory and proapoptotic agents [113]. Even more intriguingly, 5′-iodination of the acetylvanillyl ester of resiniferol orthophenylacetate, a resiniferatoxin analog (Figure 6), yields a compound that, instead of behaving as an antagonist (as in the case of 5′-I-resiniferatoxin) or of losing activity (as in the case of 6′-I-resiniferatoxin), is 5-fold more potent as an agonist than the noniodinated analog [114]. On the "cannabinoid side," attaching a C-11 alkylamide "head" to olivetol, a plant-derived compound, led to compounds that, despite their limited conformational freedom compared to endocannabinoids, bind with higher affinity to both CB_1 and CB_2 receptors [115]. In this case, the surprise came from the dramatically different behavior of one these compounds (CB-25, Figure 6) in vitro and in vivo, where it can act as a CB_1 partial agonist and CB_2 "silent" antagonist or as a CB_1 antagonist, respectively [116], thus belonging to the ever growing family of receptor "protean agonists" (i.e., compounds that can exhibit agonist, antagonist, or inverse agonist activities depending on the constitutive "tone" of their receptors [117]).

If man can imitate nature by making hybrid drugs, Nature can also imitate the human body by making endocannabinoid-like natural products that potently bind to cannabinoid receptors. This is the case for the alkylisobutylamides from the roots of *Echinacea angustifolia* [118–120]. These compounds, and in particular dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid- and dodeca-2*E*,4*E*-dienoic acid-isobutylamide (Figure 6), bind to CB_2 receptors with higher affinity than endocannabinoids, although this property explains only part of their strong immunomodulatory and anti-inflammatory properties [121]. The crosstalk between man and Nature extends even further since $C_{18:1}$ and $C_{18:2}$ fatty acid ethanolamides and their corresponding phospholipid precursors are also found in higher plant seeds and leaves [122, 123], in which a functional homolog of the mammalian FAAH has been cloned [124]. These anandamide congeners are inactive at cannabinoid receptors but play a role in the control of plant growth via unidentified molecular targets [124, 125]. These chemical, but not necessarily functional, similarities between plant metabolites and mammalian mediators underscore the global importance of certain metabolic pathways in eukaryotes, and they emphasize how cells from phylogenetically distant species can exploit similar building blocks and anabolic/catabolic strategies to respond to different biological demands.

Take Home Messages and Open Questions

From the data reviewed in this article it is clear that biological and pharmacological studies carried out with a chemical mind can reveal new physiological and pathological mechanisms and, eventually, solve clinical problems. The exciting walk from the psychotropic constituent of *Cannabis* to the understanding of the mechanism of its biological actions led to the discovery of the endocannabinoid system, a signaling apparatus whose function goes well beyond what could have been imagined from pharmacological studies on THC. The route from the endocannabinoids to the *Capsicum* pungent principle then led to the finding of endovanilloids, endogenous mediators whose role is yet to be fully understood. The discovery of endocannabinoids and endovanilloids paved the way for the finding of several other lipid signals, and it prompted, on the one hand, new strategies for the chemical synthesis of compounds with multiple pharmacological and therapeutic targets, and, on the other hand, the search for chemically similar compounds back in plants. Many open questions to which the meeting of chemistry and biology can provide an answer still remain. Just to name a few, these questions involve the understanding of the mechanisms and exact physiological meaning of the target and metabolic pathway promiscuity of fatty acid ethanolamides; the identification of the elusive protein(s) facilitating endocannabinoid membrane transport; the receptor "deorphanization" of the several tens of bioactive fatty acid amides that have been discovered, and their testing on the several TRP channels that have been identified so far, many of which are, like TRPV1, activated by plant natural products; the determination of the exact function and molecular targets of fatty acid ethanolamides in plants; and the understanding of the mechanism of action in animals of other phytocannabinoids, like the therapeutically promising cannabidiol. Thus, the exciting promenade from plant natural products to animal physiology and back might soon become a true treasure hunt.

Supplemental Data

Supplemental Data include two tables and two figures, with relative references, on the physiological and pathological functions of the endocannabinoid system, the mechanism of endocannabinoid retrograde signaling, the chemical structures and selectivity profiles of FAAH inhibitors, and the metabolomic profiling of bioactive fatty acid amides by spectrometric techniques and are available at http://www.chembiol.com/cgi/content/full/14/7/741/DC1/.

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