New Insights into Endocannabinoid Degradation and its Therapeutic Potential

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Abstract: Endocannabinoids are amides, esters and ethers of long chain polyunsaturated fatty acids, which act as new lipidic mediators. Anandamide (*N*-arachidonoylethanolamine; AEA) and 2-arachidonoylglycerol (2-AG) are the main endogenous agonists of cannabinoid receptors, able to mimic several pharmacological effects of (-)-Δ^9-tetrahydrocannabinol (THC), the active principle of *Cannabis sativa* preparations like hashish and marijuana. The activity of AEA and 2-AG at their receptors is limited by cellular uptake through an anandamide membrane transporter (AMT), followed by intracellular degradation. A fatty acid amide hydrolase (FAAH) is the main AEA hydrolase, whereas a monoacylglycerol lipase (MAGL) is critical in degrading 2-AG. Here, we will review growing evidence that demonstrates that these hydrolases are pivotal regulators of the endogenous levels of AEA and 2-AG *in vivo*, overall suggesting that specific inhibitors of AMT, FAAH or MAGL may serve as attractive therapeutic targets for the treatment of human disorders.

Recently, the *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), which synthesizes AEA from *N*-arachidonoylphosphatidylethanolamine (NArPE), and the diacylglycerol lipase (DAGL), which generates 2-AG from diacylglycerol (DAG) substrates, have been characterized. The role of these synthetic routes in maintaining the endocannabinoid tone *in vivo* will be discussed. Finally, the effects of inhibitors of endocannabinoid degradation in animal models of human disease will be reviewed, with an emphasis on their ongoing applications in anxiety, cancer and neurodegenerative disorders.

Keywords: Anandamide, 2-arachidonoylglycerol, fatty acid amide hydrolase, human disease, membrane transport, therapy.

INTRODUCTION

The majority of the actions of the psychoactive Cannabis principle, (-)-Δ^9-tetrahydrocannabinol (THC) [1] are thought to be mediated by two different subtypes of G-protein-coupled receptors (GPCRs), type-1 (CB1) [2] and type-2 (CB2) [3] cannabinoid receptors.

CB1 receptors (CB1R) are particularly abundant in central nervous system (CNS), and are also present in peripheral tissues and cells, including lung [4], male and female reproductive organs [5], vascular endothelial cells [6], and T-lymphocytes [7]. On the other hand, CB2 receptors (CB2R) are mainly associated with spleen, tonsils [8, 9], and immune cells [10], whereas they are thought to be absent in CNS. Yet, they have been recently found in the retina of adult rats [11] and in microglia cells [12].

The presence of cannabinoid receptors prompted an intense search of endogenous compounds, able to bind and activate them. The first to be identified as endogenous agonists of CBR were *N*-arachidonoylethanolamine, termed anandamide (AEA) from "ananda", the Sanskrit word for "bliss" [13], and 2-arachidonoylglycerol (2-AG) [14, 15] (Fig. **1**). Both were isolated from brain and peripheral tissues, and are members of a class of compounds called "endocannabinoids" [16]. These are lipidic mediators that include other endogenous fatty acid derivatives such as 2-arachidonoyl-glyceryl-ether (noladin ether) [17] and virodhamine, (Fig. **1**). The latter is an "inverted" AEA that consists of arachidonic acid and ethanolamine joined together by an ester bond, instead of the amide bond of AEA [18]. As reported, AEA seems to be able to produce full activation of CB1R, but only partial activation of CB2R, while 2-AG binds indistinctly to both receptors [3, 19].

Recent studies have suggested that AEA is also an "endovanilloid" [20] because, in addition to CB1R and CB2R, it binds the transient potential vanilloid receptor type-1 (TRPV1), a ligand-gated and non-selective cationic channel activated by capsaicin, the pungent component of hot peppers, and resinferatoxin [21]. Moreover, endocannabinoids were found to induce several effects not mediated by CBR or TRPV1, suggesting the presence of non-CB1, non-CB2, and non-vanilloid receptors [see for reviews 22- 26].

AEA and 2-AG activate their targets triggering several effects, such as modulation of ion channels, inhibition of gap junction communication in glial cells [27, 28], interaction with dopaminergic neurotransmission [29], and with glutamate-induced neurotoxicity [30, 31]. At the periphery, AEA and 2-AG may be involved in important biological processes, in particular they have been shown to exert cardiovascular actions [32, 33], playing a role as

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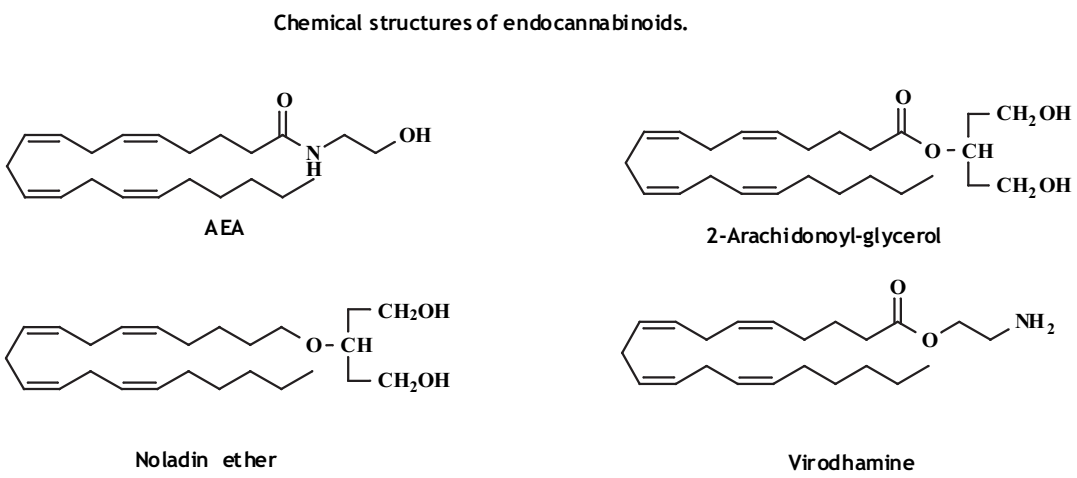


Fig. (1). Chemical structures of endocannabinoids. The most relevant endocannabinoids, AEA and 2-AG, and two recently proposed endogenous ligands of cannabinoid receptors.

vasorelaxant [34-37], and to be connected with impairment of pregnancy in mice [38] and in humans [39]. Furthermore, it has been suggested a protective role of cannabinoid receptors against apoptosis induced by AEA *via* vanilloid receptor [40], and then this concept has been largely confirmed in different experimental systems [41].

Many experimental data have shown that the endocannabinoid system (ES), and in particular the proteins responsible for the degradation of AEA and 2-AG, are involved in several diseases of CNS and peripheral tissues. To put these data in a better perspective, we will briefly describe the major properties of the metabolism of AEA and 2-AG, including selective inhibitors that may be exploited in therapeutic strategies for the treatment of CNS and peripheral dysfunctions.

ANANDAMIDE SYNTHESIS AND DEGRADATION

AEA, as other endogenous *N*-acylethanolamines (NAE), is not stored in intracellular compartments, but is produced "on demand" by receptor-stimulated cleavage of a lipid precursor [42]. The synthesis of AEA is made up of two enzymatic steps involving the sequential action of a calcium dependent *N*-acyltransferase (NAT) and of a NAPE-specific

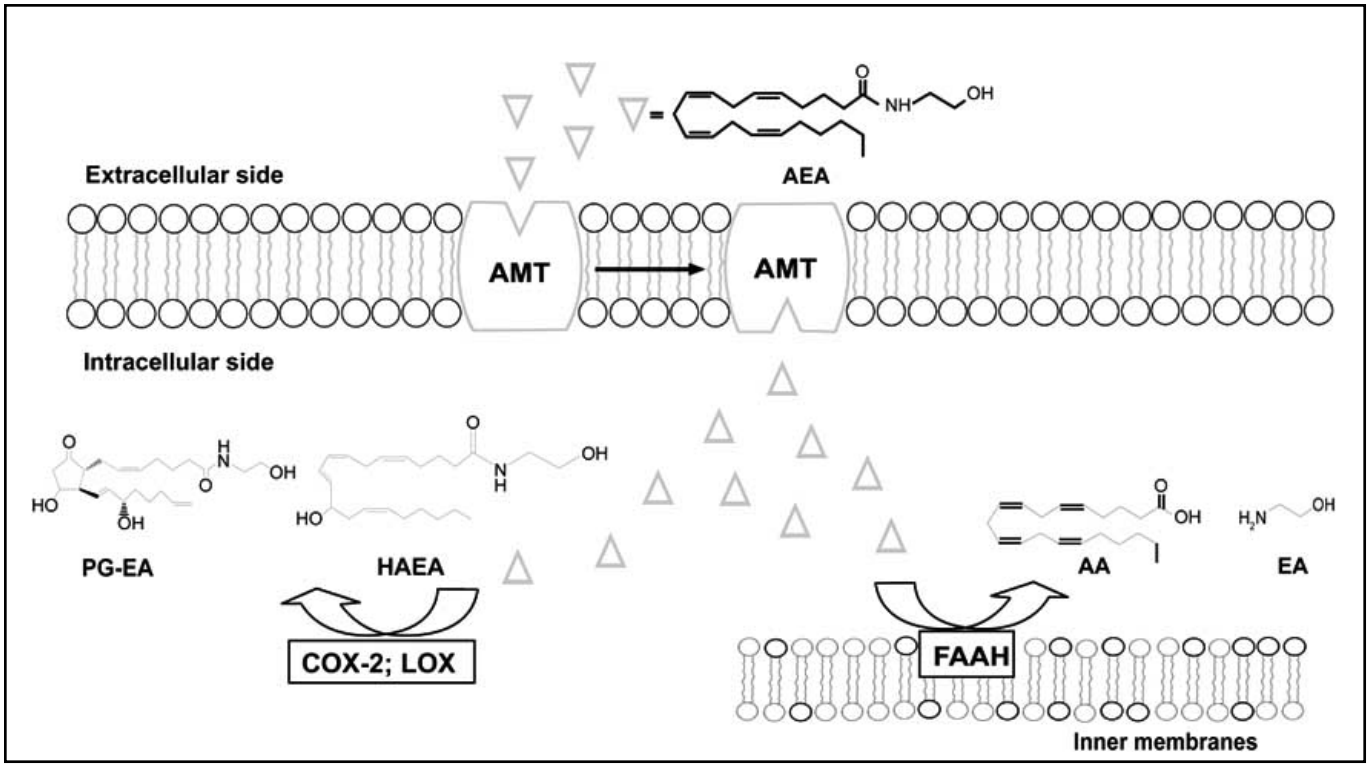


Fig. (2). Pathway for anandamide inactivation. Once uptaken, anandamide (AEA) is rapidly cleaved by membrane-bound FAAH, raleasing arachidonic acid (AA) and ethanolamine (EA). Alternatively, different lipoxygenases (LOXs) and cyclooxygenase-2 (COX-2) can metabolize AEA, generating hydroxy derivatives of AEA (HAEAs) and prostaglandin-ethanolamides (PG-EAs), respectively.

phospholipase D (NAPE-PLD; E.C. 3.1.4.4) [43]. In the first step, NAT catalyzes direct transfer of arachidonic acid from the *sn*-1 position of phosphatidylcholine (PC) to phosphatidylethanolamine (PE), generating NArPE, the AEA precursor. This biosynthetic pathway could explain the low amount of AEA with respect to other NAEs, because the arachidonic acid levels in position 1 of phospholipids are very low. In the last step, NArPE is hydrolyzed by NAPE-PLD which releases AEA and phosphatidic acid (PA). Recently, this enzyme has been cloned and purified from rat heart [43]. The same researchers suggested that several PLA_1/A_2 isozymes can generate *N*-arachidonoyl-lysoPE (NAr-lysoPE) from NArPE and that a lysoPLD releases AEA from NAr-lysoPE. This study shows that the sequential action of PLA_1/A_2 and lysoPLD may represent an alternative biosynthetic pathway for NAEs, including AEA [44].

The action of AEA at CBR is tightly controlled and relatively short, because of a rapid deactivation process. In fact, the endocannabinoid is taken up into cells by a membrane transporter, called AMT, and then hydrolyzed by FAAH, which breaks the amide bond and releases arachidonic acid and ethanolamine [45] (Fig. **2**) The protein responsible for the transport of AEA has not been cloned yet, even if its activity has been characterized in several neuronal [46, 47] and peripheral cells [48-50], as well as in *ex vivo* systems and synaptosomes from human, mouse and rat brain [51].

Most of experimental studies have been focused on the analysis of AEA transport through AMT, although there is strong evidence that AMT is also the transporter for 2-AG [52-54]. As reported in literature, AEA transport is concentration-, time- and temperature-dependent and is influenced by second messengers [46, 47, 49, 55]. This process is independent of external Na^+ ions, ATP hydrolysis [46] and selectively inhibited by other fatty-acid-derived molecules, by AEA congeners (*N*-palmitoylethanolamine, PEA; *N*-oleoylethanolamine, OEA), THC and non-psychoactive cannabinoids, like cannabidiol (CBD) [56]. In the previous years, several models for AEA transport into the cells have been reported. The first theory considers that AEA transport occurs by a protein carrier that translocates the lipid from the external to the internal side, but can also act in the opposite direction [57, 58]. This mechanism is independent of FAAH, as demonstrated by the use of selective AMT inhibitors with no effect on FAAH activity [59, 60], or by the use of cells lacking FAAH as HeLa cells [61]. Recently, we have also shown, in human keratinocytes, that AMT is spatially separated from FAAH, strongly speaking in favour of a true transporter on the membrane surface [62].

Evidence against this model has been reported by Deutsch's group [63], who has proposed that FAAH drives the AEA accumulation by creating and maintaining an AEA gradient across the plasma membranes. Therefore, the transport would occur by simple diffusion as a consequence of FAAH-catalyzed hydrolysis. These data have been supported by using FAAH inhibitors structurally unrelated to (endo)cannabinoids, like phenylmethylsulfonyl fluoride (PMSF). In fact, this compound inhibited AEA uptake in RBL-2H3 cells and in FAAH-transfected HeLa cells, but not in wild-type HeLa cells. Therefore, Glaser and coworkers suggested that the inhibition of AEA accumulation at long time points (e.g., 5 min) depends on blockade of downstream components of the AEA transport, like FAAH. Later, a specific uptake inhibitor, UCM707, was found to decrease AEA transport in neurons isolated from FAAH wild-type and knock-out mice [64]. Interestingly, AEA transport was significantly reduced also by SR141716, a selective antagonist of CB1R, also suggesting an involvement of CB1 receptors [64].

Another mechanism of AEA accumulation suggests that the endocannabinoids might associate with specific compartments of the membrane or with intracellular binding proteins [65]. These may act as a saturable reserve for AEA, thus explaining the saturability of the accumulation process. Other authors have recently proposed that caveolae-mediated endocytic process might be responsible for AEA uptake. In fact, depletion of cholesterol followed by disruption of these special plasma membrane domains reduced AEA uptake by ≈ 50% [66]. Therefore, the presence of a binding protein can be proposed in the caveolae, that efficiently transports AEA inside the cell [66]. Recent data from our group seem to favour this "endocytic hypothesis" showing that disruption of lipid rafts reduced AMT activity in neuronal cells [67].

Once transported into the cell, AEA and the other congeners like PEA and OEA are rapidly metabolized by fatty acid amide hydrolase (FAAH; EC 3.5.1.4) [68]. This is a membrane-bound enzyme found mainly in the microsomal and mitochondrial fractions of tissue homogenates of various organs [69, 70, 71, 72]. cDNAs of FAAH have been cloned in rat, mouse, porcine and human tissues [45, 73, 74] by homology screening. All the coded proteins are composed of ≈ 600 aminoacids with a molecular weight of ≈ 64 kDa, and they work optimally at pH values around 9 [75, 76]. Furthermore, the protein sequences have homology higher than 80% in the considered species [73]. The sequence analysis of FAAH has shown the presence of a highly hydrophobic transmembrane domain at the N-terminus responsible for protein oligomerization, a serine- and glycine-rich domain, containing a typical "amidase signature" (amino acids 215-257), and a proline-rich domain, which is homologous to the class II SH3-binding domain [45]. FAAH has been recently crystallized and analyzed at 2.8 Å resolution [77], and has been shown to have a Ser^{241}-Ser^{217}-Lys^{142} catalytic triad [78].

Further experiments have demonstrated that mouse and human FAAH genes (on chromosomes 1 and 4 respectively) are composed of 15 exons [79]. As reported by Puffenbarger and coworkers [80], mouse FAAH promoter lacks a TATA-box, and it contains multiple transcription start sites. Moreover, through *in vitro* experiments using human neuronal and immune cells, our group has shown that there is a differential regulation of FAAH promoter in the neuro-immune axis [81]. In this line, Cravatt's group, by using a transgenic mouse model which selectively expresses FAAH in the nervous system (FAAH-NS mice), has reported that there is a functional dissociation of the central and peripheral AEA, as well as other NAEs, signaling systems [82].

In addition to hydrolysis by FAAH, experimental observations suggest that AEA, as 2-AG, can also be metabolized by a range of oxygenases including COX-2,

LOX and cytochrome P450 [83]. The same enzymes are involved in eicosanoid synthesis from AA. COXs catalyze the bis-dioxygenation of AA to provide PGH_2, the precursor of the primary prostanoids. Human COX-2, the inducible isoform of COX, has been shown to metabolize AEA generating several oxygenated products such as PGE_2-ethanolamide (PGE_2-EA) [84] (Fig. **2**). LOXs, non-heme iron-containing enzymes, add a single molecule of oxygen to polyunsaturated fatty acids, thus converting them into conjugated hydroperoxides. These products are rapidly reduced to the corresponding hydroxides in the cellular environment. Different LOX isozymes are present in different tissues and cells, and *in vitro* 12- and 15-LOX have been shown to convert AEA into 12- and 15-hydroxy-AEA (12- and 15-HAEA), respectively [47, 85] (Fig. **2**). Finally, cytochrome P450s also metabolize AEA producing several polar lipids [86].

2-AG SYNTHESIS AND DEGRADATION

2-Arachidonoylglycerol isolated from canine gut and rat brain [14, 15], acts as potent and full agonist at both CB1 and CB2 receptors with K_i values from high nanomolar to low micromolar values [14, 15]. Like anandamide, 2-AG is not stored in intracellular compartments, but it is produced on demand by receptor-stimulated cleavage of lipid precursors. A pathway for 2-AG formation involves the hydrolysis of phosphatidylinositol (PI) by PLA_1 into lysoPI, followed by hydrolysis by phospholipase C (PLC) to produce 2-AG [87]. Another biosynthetic pathway provides for quick hydrolysis of inositol phospholipids by a specific PLC generating DAG [88, 89]. DAG is then converted to 2-AG by a *sn*-1-DAG lipase [90, 91].

Very recently, two *sn*-1-specific DAG lipases responsible for the synthesis of 2-AG have been cloned by comparing human genome with *Penicillium* DAGL sequence [91]. Both proteins were found to have four transmembrane domains, and are members of the serine-lipase family with Ser^{443} and Asp^{495} participating in the enzymatic catalytic triad. DAGLα (120 kDa) and β (70 kDa) have shown an optimum at pH 7 and Michaelis-Menten constants (K_m) of 155 μM and 74 μM, respectively. Both enzymes are influenced negatively by Ser/Cys hydrolase inhibitors, but not by PMSF, while they are activated by glutathione and Ca^{2+} and inhibited by RHC80267 [91]. The two DAGLs are differentially regulated in the brain depending on the area; for example, during the embryonic development they facilitate axonal growth [92]. In addition, the β isoform seems to be predominant in the adult brain, while the β isoform is expressed in developing brain [91].

Recently, it has been demonstrated that in primary cultures of mouse microglial cells and astrocytes, millimolar concentrations of ATP significantly increase 2-AG synthesis without acting on AEA production [93]. ATP enhances 2-AG levels in a time-dependent manner by activating purinergic $P2X_7$ receptors. These ionotropic receptors are permanently permeable to Ca^{2+} [94], confirming the relevant role of calcium in the biosynthesis of endocannabinoids [95]. Interestingly, DAGL activity may be enhanced in peripheral systems by the potent bioactive phospholipid, platelet-activating factor (PAF) [96].

2-AG induces several biological effects in tissues and cells, therefore the pharmacological effects of this lipid depend on its life span in the extracellular space, which is limited by a rapid transport through the membrane. It has been suggested that the 2-AG membrane transporter may be the same as the one used by anandamide, i.e. AMT [52, 53]. Human astrocytoma cells like primary neuronal cell cultures [54], have been shown to accumulate radioactive 2-AG through an Na^+- and ATP-independent process. This accumulation is tightly temperature- and concentration-dependent and is reduced by AM404 (*N*-(4-hydroxyphenyl)-arachidonamide), an AMT inhibitor, and indirectly by high concentrations of arachidonic acid [52]. The effect of AM404 is due to the inhibition of AMT and not to the blocking of FAAH activity, because using two strong FAAH inhibitors like URB597 and AM374, the concentration of 2-AG remained unaltered [54].

Once accumulated in the cell, 2-AG can be degraded by FAAH. In fact, AEA as well as FAAH inhibitors, including arachidonoyltrifluoromethyl ketone (ATFMK), blocked 2-AG hydrolysis by N18TG2 and RBL-2H3 membranes [97]; conversely, 2-AG is able to inhibit the hydrolysis of AEA [45]. These data have suggested that FAAH contributes to the hydrolysis of 2-AG to arachidonic acid and glycerol (Fig. **3**). Yet, it is not the main enzyme responsible for the metabolism of 2-AG. In fact, FAAH (-/-) mice are unable to metabolize AEA, but preserve the ability to hydrolyze 2-AG [98]. These data indicate that there is another enzyme responsible for 2-AG degradation, and indeed, the possible candidate for this role, MAGL, has been isolated from porcine brain [99]. MAGL has been cloned and characterized in rat [100] and human brain [101]. Rat brain MAGL is a 33 kDa protein showing 92% homology with mouse adipocyte MAGL [102], and keeps the catalytic triad residues commonly found in lipases, i.e. Ser^{122}, Asp^{239}, and His^{269} [102]. Additionally, it does not show posttranslational changes but includes consensus sequences for phosphorylation by Ca^{2+}/calmodulin kinase II and protein kinases A and C [102]. MAGL has been shown to be unable to metabolize AEA and PEA, but it hydrolyzes preferentially 2-acylglycerols such as 2-AG and 2-oleolylglycerol (2-OG) [100]. In contrast with FAAH, MAGL is localized in the cytosol (besides being membrane bound), has a pH optimum at 7-8 [103] and is discretely distributed throughout the CNS [100].

Finally, also 2-AG is metabolized by cyclooxygenase-2, but not by cyclooxygenase-1, into PGH_2-glycerol ester *in vitro* and in cultured macrophages [83] (Fig. **3**).

INHIBITORS OF AEA AND 2-AG INACTIVATION

AEA and 2-AG are involved in several diseases of CNS and periphery. As pointed out above, the enzymes responsible for the synthesis of AEA and 2-AG have been cloned only recently, but specific inhibitors suitable for use *in vitro* and *in vivo* have not been developed yet. Several studies have aimed at finding compounds able to inhibit AEA and 2-AG uptake or hydrolysis, in order to clarify the physiological functions of these endocannabinoids and to prolong their effects in those disease states in which they could act as anti-inflammatory, antioxidative, and protective

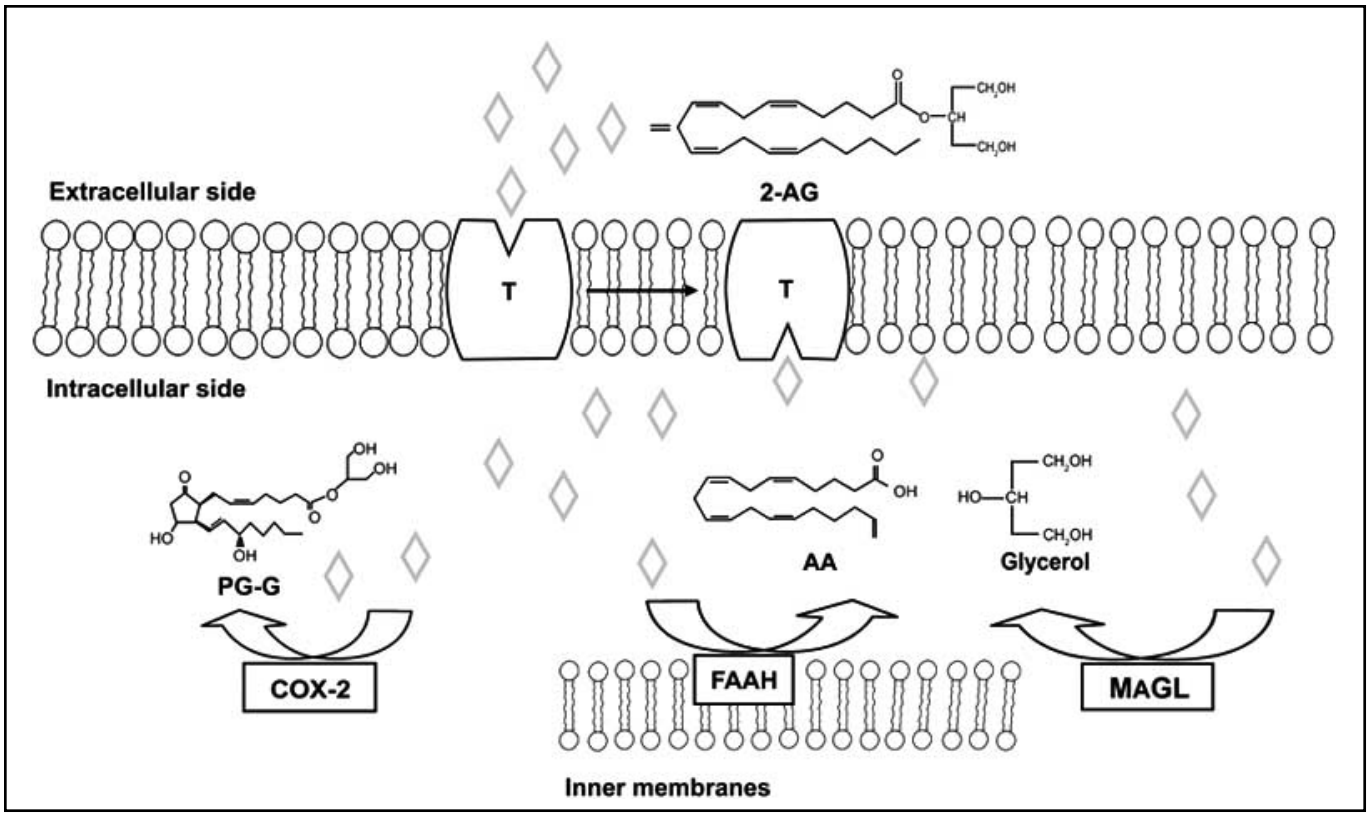


Fig. (3). Pathway for 2-arachidonoylglycerol inactivation. Once uptaken, 2-arachidonoylglycerol (2-AG) is rapidly cleaved by monoacylglycerol lipase (MAGL) releasing arachidonic acid (AA) and glycerol. Alternatively, 2-AG can be hydrolized by membrane-bound FAAH into the same products. Moreover, 2-AG can be oxygenated by cyclooxygenase-2 (COX-2), generating prostaglandin-glycerol esters (PG-Gs).

agents without the undesired effects of CB receptor agonists [104]. To date available compounds show little selectivity towards uptake and hydrolysis of AEA, and in fact many compounds able to block AEA internalization can also interact with FAAH, CBR or TRPV1. Thus, interest to find new and more selective "bullets" has increased tremendously in the last few years.

Our group has reported the first evidence of a "natural" regulator selective for AEA uptake demonstrating that nitric oxide (NO) donors double AMT activity without affecting FAAH in human endothelial cells (HUVEC). This effect was abolished by hydroxocobalamin, a NO scavenger [49].

Moreover depletion or enhancement of intracellular glutathione concentration potentiated or attenuated AEA accumulation, respectively. Interestingly, also the product of the condensation of NO and superoxide (O_2^-), peroxynitrite ($ONOO^-$), caused a ~4-fold activation of AEA uptake by HUVECs, suggesting that oxidative stress, mimicked by $NO/ONOO^-$, increases the movement of AEA through the plasma membranes. Conversely, the antioxidative defense by glutathione counteracts this effect [49].

Another natural modulator of AEA uptake is cannabidiol (CBD), a non psychotropic component of Cannabis. This compound is considered an anti-inflammatory and antioxidative agent and this view is supported by the the analysis of its chemical structure. In fact, CBD contains the phenolic structure typical of many antioxidants isolated from the plants. CBD and one of its synthetic derivatives, the 5'-DMH-CBD, were able to inhibit AEA uptake in RBL-2H3 cells without interacting with CBRs [56].

Extensive structure-activity relationship (SAR) studies have indicated that the carrier can distinguish among structurally related compounds, confirming that there is a specific protein-mediated process of accumulation. Furthermore, the modification of functional groups may significantly influence the solubility of the tested compounds, thus confounding SAR results. One of the first synthetic uptake inhibitors was AM404, which inhibits with high affinity the accumulation of AEA and 2AG both *in vitro* and *in vivo* [52, 105, 106]. AM404 does not interact with CB1R. As shown by recent experiments, besides blocking AMT [63], AM404 could be a substrate of FAAH [107]. In fact, brain membranes from wild-type mice degrade AM404, whereas those from FAAH knock-out animals do not [107]. Moreover, this compound also activates TRPV1 at concentrations similar to or lower than those necessary to block AEA accumulation [108, 109]. This research has shown that also vanilloid receptor ligands named *N*-vanillylacylamides, such as arvanil and olvanil, were able to inhibit AEA uptake at micromolar concentrations [110-113]. Arvanil is a structural "hybrid" between AEA and capsaicin, derived from arachidonic acid and the polar head of the ligand of TRPV1 [112]. This compound was found to be one of the most potent ligands of TRPV1 and a very effective inhibitor of AMT (IC_{50} = 3.6 μM in RBL-2H3 cells) [112]. Olvanil, instead, is the condensation product of oleic acid and the polar head of capsaicin [111]. The latter

ligand of TRPV1 blocked AEA uptake more potently than AM404 (IC_{50} = 9 μM in RBL-2H3 cells) without influencing FAAH activity, even if it has an affinity for CB1R slightly lower than that reported for vanilloid receptors [111].

Later on, two other novel AEA derivatives, which inhibit AMT with the same potency as AM404 and show low binding activity at TRPV1, were synthesized and called VDM11 (*N*-(4-hydroxy-2-methylphenyl)-arachidonamide) and VDM13 (*N*-(5-methoxytryptamine)-arachidonamide) (K_i = 9.0 μM and 9.7 μM in RBL-2H3 cells, respectively) [113]. Since both compounds were weak inhibitors of FAAH and poor ligands of CB1R and CB2R, they were considered selective inhibitors of AMT [113].

New arachidonic acid derivatives have been synthesized and identified as highly potent and selective endocannabinoid transporter inhibitors [114]. Among them, UCM707 (*N*-(3-furylmethyl)-arachidonamide) was found to be the most powerful and selective inhibitor of AMT *in vitro* (IC_{50} = 0.8 μM in U937 cells) [114], and, as the other components of the UCM class, has weak interactions with CBR and TRPV1 [114]. Furthermore, it inhibits AEA uptake in both FAAH (+/+) and (-/-) mice [64] and although in this model its effect on FAAH has not been investigated, previous studies reported the inhibition of the hydrolase only at high concentrations (IC_{50} = 30 μM in rat brain omogenates) compared to those necessary to inhibit AMT [115]. Moreover, this compound was able to potentiate, *in vivo*, the action of AEA, thus supporting the notion of a selective effect on the endocannabinoid transporter [116].

Recently, two novel compounds, (*R*)- and (*S*)-1'-4-hydroxybenzyl derivatives of OEA, have been developed and named OMDM-1, and OMDM-2 (K_i towards AMT = 2.4 μM and 3 μM in RBL-2H3 cells, respectively) [117]. The changes introduced in the structure of OEA made these molecules metabolically stable, potent and selective inhibitors of AMT, unable to interact with CB1R, TRPV1, and FAAH. *In vitro* experiments have shown that they were more effective on AMT than VDM11 and VDM13, and more selective than arvanil and AM404 [117].

The newest compound tested as AMT inhibitor is AM1172 (*N*-(5Z,8Z,11Z,14Z eicosatetraenyl)-4-hydroxybenzamide), which contains a reverse amide moiety compared with AM404. It efficiently blocks the accumulation of AEA in rodent cortical neurons and human astrocytoma cells *in vitro* (IC_{50} = 2.1 μM and 2.5 μM, respectively), and is highly metabolically stable. In addition, it does not act as FAAH substrate or inhibitor, has a partial affinity for CB1R and CB2R, and does not interact at all with TRPV1 [107].

Further studies have been focused on natural and synthetic inhibitors of FAAH. Our group has found that oxidative metabolites of AEA generated by various LOXs are powerful inhibitors of FAAH [47, 118], and the simple addition of oxygen at specific positions of AEA makes these compounds as selective as URB597 [119]. In fact, all hydroxy-AEAs (HAEAs) inhibited FAAH with K_i values in the nanomolar-low-micromolar range. Instead, they did not inhibit AEA transport by AMT (with the exception of 12(*S*)-HAEA), nor did they bind to CBR [47, 118]. Unlike the most powerful synthetic inhibitors, all HAEAs are reversible competitive inhibitors of FAAH and they seem to be suitable tools for a flexible modulation of the endocannabinoid system *in vivo*. Moreover, other endocannabinoid-like compounds, like *N*-arachidonoylglycine, have been shown to inhibit FAAH at concentrations around 10 μM [120].

The majority of synthetic FAAH inhibitors utilizes electrophilic carbonyls, e.g. fluorophosphonates, sulfonyl fluorides, trifluoromethyl ketones and α-ketoheterocycles. These compounds are not selective for FAAH due to their ability to interact with other proteins. At first known as cytosolic phospholipase A_2 inhibitor, MAFP (methyl-arachidonyl-fluorophosphonate) has been found to be a very potent irreversible inhibitor of FAAH with an IC_{50} of 1-3 nM [121, 122], although it produces CB1R-dependent effects [104]. One of fatty acid sulfonyl fluorides is AM374 [123], which irreversibly inhibits FAAH at nanomolar concentrations, but it acts also on other targets [124]. The α-keto-N4-oxazolopyridine derivatives of Δ^9-octadecenoic acid have been tested and found to be powerful FAAH inhibitors, even if they might interact with other targets like CB1R or cytosolic phospholipase A_2 [125, 126].

Two other derivatives of AA, arachidonoyl diazomethyl ketone [121, 127], and *N*-arachidonoyl-serotonin (AA-5HT) [124], are mixed inhibitors of FAAH (IC_{50} = 0.5-6.0 μM and 1.5-12 μM, respectively). The former also inhibits 5-lipoxygenase [18], whereas the latter, although active on CB1R and cytosolic PLA_2, is more selective for FAAH [128]. Recently, Tarzia and coworkers identified a new class of highly selective *O*-arylcarbamate inhibitors of intracellular FAAH activity [129, 130]. A representative example of this class is URB597 (IC_{50} = 4.6 nM), which is effective both *in vitro* and *in vivo*. It was found to exclusively inhibit FAAH, by acylating an active site catalytic serine without affecting other serine hydrolases, like MAGL. It did not modulate AMT or CBR either [131]. More recently, other compounds have been synthesized and tested as FAAH inhibitors. They include heterocyclic sulfoxide and sulfone molecules, which were less potent than the corresponding α-ketoheterocycles [132]. Successively, the addition to these compounds of a 2-pyridyl C5 substituent yielded novel FAAH inhibitors, selective and with an exceptional K_i < 300 pM [133].

2-AG degradation by MAGL and its inhibition have been studied in rat cerebellar membranes [134], testing common serine hydrolase inhibitors. PMSF was unable to inhibit 2-AG hydrolysis, whereas HDSF (hexadecylsulphonyl fluoride), ATFMK and MAFP did inhibit MAGL with IC_{50} values of 241 nM, 66 μM and 2.2 nM, respectively [134]. Instead, AA-5HT was able to inhibit 2-AG degradation only at very high concentrations, versus an IC_{50} value of 12 μM for FAAH activity. OL53, a very potent α-ketoheterocycle compound, characterized by a picomolar K_i value for FAAH [125], was not able to inhibit 2-AG hydrolysis even at 1 mM concentration. In the same line, URB597 also poorly inhibited 2-AG degradation [134].

Further studies have analyzed the effect of 2-AG analogues on FAAH and MAGL activity [135]. Although they did not identify a MAGL-selective compound, three molecules called α-Me-1-AG (α-methyl-1-

arachidonoylglycerol), O-2203 (1-(20-cyano-16,16-dimethyl-eicosa-5,8,11,14-tetraenoyl)glycerol), and O2204 (2-(20-hydroxy-16,16-dimethyl-eicosa - 5,8,11,14-tetraenoyl)glycerol) were found to be useful as lead compounds for the development of selective MAGL inhibitors. Interestingly these compounds did not interact with CB1 receptors and inhibited FAAH only at high concentrations [135].

THERAPEUTIC APPLICATIONS OF INHIBITORS AND FUTURE PERSPECTIVES

Altered levels of endocannabinoids are associated with several pathological conditions, therefore the role of the endocannabinoid system is currently the subject of intense investigation. Experimental observations suggest that endocannabinoids might play a role as neuromodulators or as neurotransmitters, and can act as neuroprotective agents [136-138]. Several lines of evidence reveal a connection between endocannabinoid signaling and excitotoxicity [139], brain injury [140], pain [141], and stressful stimuli [142, 143]. Moreover, endocannabinoid levels are higher than normal in rat blood during hemorrhagic and septic shock [144, 145], in gastrointestinal disorders [146, 147], and in several human cancer tissues [148, 149]. Finally, our group has reported that high levels of AEA in pregnant women, due to a defective FAAH, correlate with premature miscarriage [39], or failure of *in vitro* fertilization [150]. Therefore, selective compounds able to reduce the degradation of endocannabinoids might represent novel drugs for human disease, including infertility [151].

Promising results have already been published with inhibitors of AMT in experimental models of various pathologies, as reported in (Table **1**). The importance of AM404 in some neurological disorders derives from its ability to produce motor inhibition [152]. This action has been recently shown also in a rat model of Huntington's disease (HD), generated by bilateral intrastriatal injections of 3-nitropropionic acid [153]. Treatment with AM404 alleviated the hyperkinetic signs and neurochemical deficits during the hyperkinetic state of neurodegeneration, but this action is due to the synthesis of new AEA following the direct activation of TRPV1, rather than to AMT inhibition [154, 155].

Also our group has recently reported data on the reduced activity of AMT in 6-OHDA (6-hydroxydopamine)-lesioned rats, a model of Parkinson's disease (PD) [156], and the reversal of this activity back to control values after chronic L-DOPA treatment [138]. Administration of AM404 or VDM11 significantly reduced the frequency of spontaneous glutamatergic activity recorded from striatal spiny neurons in all experimental groups. In the same line, the systemic administration of AM404 improved akinesia and sensorimotor orientation, which are anti-parkinsonian effects [157]. Indeed, it was found that the use of AM404 and VDM11 in mice suffering from chronic relapsing experimental allergic encephalomyelitis (CREAE), a model of multiple sclerosis (MS), markedly ameliorated spasticity [137].

As shown by Fernandez-Ruiz's group [158], other AMT inhibitors, like UCM707, produced beneficial effects in an animal model of neurological disease. In fact, UCM707 treatment alleviates hyperkinesia in malonate lesioned rats (Huntington's disease model) [158]. In addition, a recent report has demonstrated that UCM707 significantly protected mice against the excitotoxin kainic acid (KA) [139], confirming the role of endocannabinoids in neuroprotection. In the same line, a recent study reported that CBD exerts a combination of neuroprotective, antioxidative and anti-apoptotic effects against β–amyloid peptide (Aβ) accumulation, a pivotal event in the pathogenesis of Alzheimer's disease (AD) [159]. Moreover, AEA and noladin ether, through a CB1-dependent mechanism, have been shown to protect the cells against Aβ–induced injury *in vitro* [160]. As noted above, CBD enhanced the action of AEA at CB1 receptors by blocking its uptake [56]. These data indicate the possible therapeutic application of CBD in neurodegenerative diseases as inhibitor of AMT. Interestingly, CBD has also been shown to have anti-proliferative effects in human glioma cells [161, 162].

Some studies have shown that AEA dose-dependently decreases systemic blood pressure *in vivo*, and that this action is significantly potentiated and prolonged by AM404 and olvanil [163, 164]. Thus, endocannabinoid oriented drugs can also represent a therapeutic benefit as cardiovascular regulators.

Interestingly, it seems that (endo)cannabinoids might also be involved in the protection against tumor cell growth [149, 165, 166], representing an alternative therapeutic approach to the treatment of cancer. For example, by using VDM11, a block of the thyroid carcinoma growth has been observed in rats, following the increase of 2-AG levels [166].

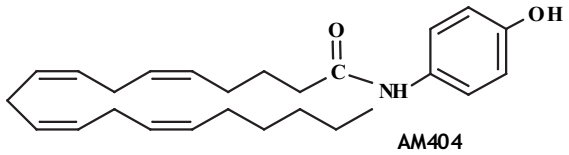
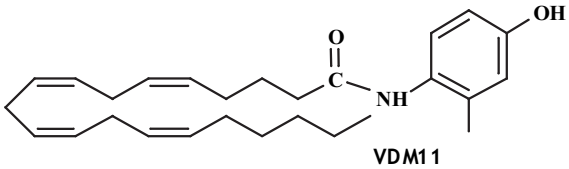
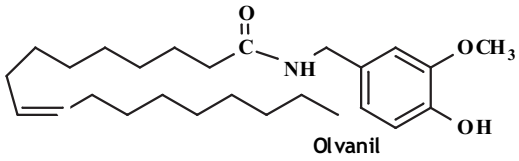
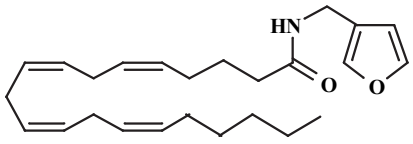
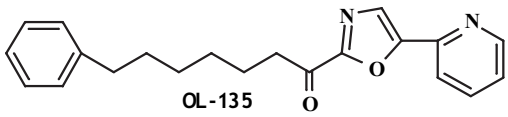
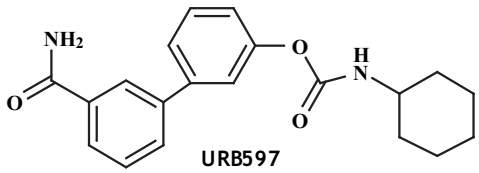
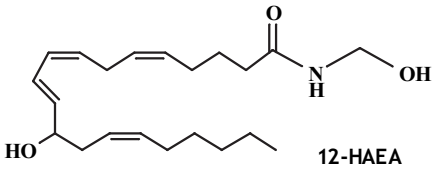
Moreover, several studies have provided strong evidence that FAAH, due to its broad distribution, represents an attractive therapeutic target for the treatment of many diseases in both the CNS and the periphery, as reported in (Table **1**).

It seems noteworthy that URB597, which augments endogenous brain levels of AEA, produces anxiolytic and analgesic effects through a CB1R-dependent mechanism [131]. Pre-clinical studies in rats have demonstrated that this FAAH inhibitor exhibited benzodiazepine-like properties in the elevated zero-maze test, and in isolation-induced ultrasonic vocalizations [131]. The advantage of URB597 is that it did not induce common side effects of typical CBR agonists, like hypomotility, catalepsy or hypothermia. In fact, it does not interact with cannabinoid receptors. Additional data have suggested that URB597 elicited anti-nociceptive effects in a model of acute pain [131].

Furthermore, a selective and powerful FAAH inhibitor used for the treatment of pathological states, is OL135, a reversible α-keto heterocyclic inhibitor that enhances AEA-induced analgesia *in vivo* [165]. Previously, FAAH (-/-) mice have been reported to exhibit high levels of AEA and a CBR-mediated phenotypic hypoalgesia [167, 168]. Taken together, these data suggest that FAAH inhibitors could be developed as new pain-killers.

Of interest is also the fact that cells are able to produce natural FAAH inhibitors, such as the HAEAs discussed above. Natural FAAH inhibitors appear to be simpler than

Table 1. Chemical Structures of Some Inhibitors of Endocannabinoid Degradation

Compound	Disorder	Therapeutic Effect
 AM404	Huntington's disease Parkinson's disease	Improvement of Hyperkinesia and Sensorimotor Orientation
	Multiple Sclerosis	Amelioration of Spasticity
	Hypertension	Decrease of Blood Pressure
 VDM11	Parkinson's disease	Improvement of Hyperkinesia and Sensorimotor Orientation
	Multiple Sclerosis	Amelioration of Spasticity
	Cancer	Inhibition of Cellular Proliferation
 Olvanil	Hypertension	Decrease of Blood Pressure
 UCM707	Huntington's disease Parkinson's disease	Improvement of Hyperkinesia and Sensorimotor Orientation
	Multiple Sclerosis	Amelioration of Spasticity
 OL-135	Hyperalgesia	Decrease of Pain Sensation
 URB597	Hyperalgesia	Decrease of Pain Sensation
 12-HAEA	Cytotoxic Edema	Reduction of Acute Cellular Swelling

those synthesized in the laboratory like URB597 (Table **1**). Remarkably, 12-HAEA, the 12-lipoxygenase metabolite of AEA, reduced *in vivo* cytotoxic edema formation through a CB1R-independent mechanism, whereas the 15-lipoxygenase metabolite, 15-HAEA, potentiated the neuroprotective effect of AEA [169].

Although psychotropic side effects of cannabinoids and public opinion about these plant extracts have limited their therapeutic applications, the discovery of endocannabinoids and related proteins has led to the development of compounds more and more selective, specific and able to modulate human pathophysiology. In this light, the endocannabinoid system represents an intriguing and novel target for therapeutics of next generation, potentially useful for the management of a vast array of human disorders.

ABBREVIATIONS

2-AG	=	2-arachidonoylglycerol
2-OG	=	2-oleolylglycerol
AEA	=	*N*-arachidonoylethanolamine
AM374	=	Palmitylsulfonylfluoride
AM404	=	*N*-(4-hydroxyphenyl)-arachidonamide
AMT	=	Anandamide membrane transporter
ATFMK	=	Arachidonoyltrifluoromethyl ketone
CB1R CB2R	=	Type 1 and type 2 of cannabinoid receptors
CBD	=	Cannabidiol
PEA	=	*N*-palmitoylethanolamine
COX-2	=	Cyclooxygenase-2
DAG	=	Diacylglycerol
DAGL	=	Diacylglycerol lipase
ES	=	Endocannabinoid system
FAAH	=	Fatty acid amide hydrolase
FAK	=	Focal adhesion kinase
GPCRs	=	G-protein-coupled receptors
LOX	=	Lipoxygenase
lysoPLD	=	Lysophospholipase D
MAG	=	Monoacylglycerol
MAGL	=	Monoacylglycerol lipase
MAPK	=	Mitogen-activated protein kinase
NAE	=	*N*-acylethanolamines
NAPE	=	*N*-acyl-phosphatidylethanolamine
PLD	=	Phospholipase D
N-Ar-lysoPE	= -	*N*-arachidonoyl-lysophosphatidylethanolamine
NArPE	=	*N*-arachidonoylphosphatidylethanolamine
NAT	=	*N*-acyltransferase
NOS	=	Nitric oxide synthase
OEA	=	*N*-oleoylethanolamine
PA	=	Phosphatidic acid
PAF	=	Platelet-activating factor
PC	=	Phosphatidylcholine
PE	=	Phosphatidylethanolamine
PGE_2-EA	=	PGE_2-ethanolamide
PGH_2	=	Prostaglandin-H_2
PI	=	Phosphatidylinositol
PLC	=	Phospholipase C
PMSF	=	Phenylmethylsulfonyl fluoride
TRPV1	=	Transient potential vanilloid receptor type
Δ^9-THC	=	(-)-Δ^9-tetrahydrocannabinol
UCM707	=	*N*-(3-furylmethyl)-arachidonamide
URB597	=	Cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl-ester
VDM11	=	*N*-(4-hydroxy-2-methylphenyl)-arachidonamide
VDM13	=	*N*-(5-methoxytryptamine)-arachidonamide

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