The anandamide membrane transporter and the therapeutic implications of its inhibition

Natalia Battista, Valeria Gasperi, Filomena Fezza & Mauro Maccarrone†

†Author for correspondence
University of Teramo, Department of Biomedical Sciences, Piazza A.Moro 45, 64100 Teramo, Italy
Tel.: +39 86 126 6875
Fax: +39 86 141 2583
Maccarrone@unite.it

Endocannabinoids are a new class of lipid mediators that include amides, esters and ethers of long chain polyunsaturated fatty acids. Anandamide (*N*-arachidonoylethanolamine), the most prominent member of this group together with 2-arachidonoyl glycerol, has several pharmacological actions, even if its life span is quite short because of the presence of an efficient mechanism of cellular removal which involves transport across the plasma membranes and hydrolysis by fatty acid amide hydrolase (FAAH). Here, we review evidence in favor or against the existence of a true anandamide membrane transporter (AMT) and discuss the structural properties of compounds that inhibit AMT without affecting other proteins of the endocannabinoid system, such as cannabinoid receptors or FAAH. Also the therapeutic implications of novel AMT inhibitors will be reviewed in the light of their potential exploitation for the treatment of neurodegenerative diseases and other human pathologies.

Keywords: AM1172, AM404, anandamide, arvanil, cannabinoid receptors, fatty acid amide hydrolase, membrane transport, neurodegenerative disease, O-2109, olvanil, OMDM1, OMDM2, therapy, UCM707, vanilloid receptor, VDM11, VDM13

The endocannabinoid system

Anandamide (*N*-arachidonoylethanolamine, AEA) is an endogenous cannabinoid agonist [1]. It binds to and activates the cannabinoid receptors, thus mimicking some pharmacological actions of Δ^9-tetrahydrocannabinol (THC) [2]. To date, two different subtypes of G-protein-coupled cannabinoid receptors have been described that are present in the CNS and in the periphery – CB1 and 2 [3,4], Besides acting as a true 'endocannabinoid', AEA is also considered an 'endovanilloid', as it binds to and activates the vanilloid receptor type-1 (now known as the transient receptor potential channel vanilloid receptor subunit 1, TRPV1). This is a capsaicin-sensitive, non-selective cation channel involved in inflammation, thermal pain, vasodilation, bronchoconstriction, smooth muscle tone modulation and nociception, as well as inhibition of tumor cell growth and induction of apoptosis [5–8]. As with other lipid mediators, AEA is released from cells on demand by stimulus-dependent cleavage of membrane phospolipid precursors through a transacylase–phosphodiesterase-mediated synthesis [6]. In fact, the AEA precursor is *N*-arachidonoylphosphatidylethanolamine enzyme (NArPE), which is believed to originate from the transfer of arachidonic acid from the sn-1 position of 1,2-sn-di-arachidonoylphosphatidylcholine to phosphatidylethanolamine, catalyzed by a calcium-dependent *N*-acyltransferase (NAT). NArPE is then cleaved by a phosphodiesterase enzyme of the phospholipase D (PLD) type, indicated as *N*-acylphosphatidylethanolamine (NAPE)-hydrolyzing PLD [9], which releases AEA and phosphatidic acid. Recently, this enzyme has been purified from rat heart and cloned [10].

The pharmacological effects of AEA at CB receptors depends on the life span of the lipid in the extracellular space, which is limited by a rapid transport through the membrane [6,11,12], followed by intracellular degradation of AEA to arachidonic acid and ethanolamine by the enzyme fatty-acid amide hydrolase (arachidonoylethanolamide amidohydrolase, EC 3.5.1.4; FAAH). Both components of the inactivation process are still the subject of investigation, even if they have been characterized in several cells.

FAAH is a membrane-bound enzyme found mainly in microsomal and mitochondrial fractions of rat brain and liver [13,14], and of porcine brain [15]. The enzyme exhibits a molecular weight of 64 kDa, works optimally at pH values around 9 [16] and has been recently crystallized and analyzed at 2.8Å resolution [17]. FAAH activity has been demonstrated and partially characterized in rat, porcine, and dog brains [18,19]. Furthermore, the FAAH gene has been cloned from rat, mouse and human cDNAs, allowing the disclosure of its regulation and substrate specificity [18,20,21].

Once released, extracellular AEA is rapidly taken up by cells through a mechanism that meets the criteria for carrier-mediated transport – fast rate, temperature dependence, saturability

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and substrate selectivity [11,12]. Unlike classical neurotransmitters, AEA reuptake was shown to be independent of external Na^+ ions or cellular ATP and to be unaffected by metabolic inhibitors [8]. Overall, the available evidence seems to suggest the existence of a carrier-facilitated diffusion of AEA through the plasma membranes [11,12,22]. However, in the last 2 years some laboratories have argued against this evidence.

Anandamide membrane transporter

Since the discovery and isolation of AEA, various studies focused on determining the nature and mechanism of AEA transport from the extracellular medium to cytoplasm across the plasma membranes. Unlike the FAAH area of research [18,20,21], no membrane transporter for endocannabinoids has yet been cloned and thus existence is based only on activity assays and pharmacological data. Therefore, the actual characteristics of AEA uptake are still a matter of debate. However, the kinetic constants of AEA uptake have been determined in several neuronal and peripheral cells and are summarized in **Table 1**. For instance, when radioactive AEA is added to the medium of intact neuroblastoma [23] and glioma cells [13], of brain slices [24] and synaptosomes [25,26], or to primary cultures of cerebellar granule neurones [11], it is possible to recover the radiolabelled compound in the intracellular compartment. The presence of an AEA transporter has also been investigated and well characterized in peripheral cells, such as human lymphoma (U937) and human umbilical vein endothelial cells (HUVECs) [23,27]. Interestingly, human mast cells (HMC-1), rat RBL-2H3 basophils and prostatic PC3 cells take up AEA with a saturable process and exhibit a clear, saturable transport of this lipid which is time and temperature sensitive [28–30]. This transport activity is independent of extracellular ion gradients and is inhibited selectively by other fatty acid-derived molecules, by AEA congeners or by psychoactive cannabinoids such as THC.

Table 1. Kinetic properties of anandamide membrane transporter (AMT) in neuronal and peripheral cells.

Cell type/tissue	Km §	V_{max}	Ref.
Lymphoma cells (U937)	0.13 ± 0.01	140 ± 15§§	[23]
Neuroblastoma cells (CHP100)	0.20 ± 0.02	30 ± 3§§	[23]
Cerebellar granule cells	41 ± 15	610 ± 4§§§	[11]
Rat basophilic leukemia cells (RBL-2H3)	11.4 ± 2.3	0.17 ± 0.02§§§	[29]
Prostate epithelial cells (PC3)	4.70 ± 0.02	3.3 ± 0.3§§§	[30]
Human platelets	0.20 ± 0.02	22 ± 2§§	[34]
Human endothelial cells (HUVEC)	0.19 ± 0.01	45 ± 3§§	[44]
Human mast cells (HMC-1)	0.20 ± 0.02	25 ± 3§§	[28]
Mouse brain	0.66 ± 0.08	370 ± 23§§	[26]
Human brain	0.48 ± 0.05	357 ± 18§§	[26]

§Expressed as μM.

§§Expressed as pmol/min per mg protein.

§§§Expressed as pmol/min per 10^6 cells.

The purported AEA membrane transporter (AMT) responsible for AEA uptake demonstrates conserved kinetic properties through different biological systems. The first characteristic is the time and temperature dependence of AEA accumulation. The second is the saturability of AMT at physiological micromolar concentrations of AEA [11]. In addition, a very intriguing feature of the movement of AEA across the membrane is its bidirectionality – both import and export of AEA are temperature-dependent processes and show similar kinetic profiles [11].

A further characteristic of AEA transport is the substrate specificity towards analogs with similar structures. For example, in cerebellar granule cells, only *N*-oleoylethanolamide (OEA), *N*-arachidonoylbenzylamine and *N*-arachidonoylpropylamine reduce the accumulation of AEA, whereas at concentrations in the low micromolar range, neither *N*-palmitoylethanolamine (PEA), nor arachidonic acid or AEA hydroxides oxidized at the 12 or 15 position inhibit this process [11]. However, there is strong evidence to suggest that instead, AMT is also the transporter for the other major endocannabinoid, 2-arachidonoyl glycerol (2-AG) – as reported in the literature and more recently by Hajos [31,32].

Recently, it has been reported that rabbit platelets [33], unlike human platelets, do not have a carrier-mediated mechanism for the transport of AEA into the cell [33–35] (**Table 1**). In fact, uptake of AEA in these cells is not temperature dependent nor is it saturable, suggesting that AEA enters rabbit platelets by simple diffusion [33].

Based on the concept that FAAH drives the continuous AEA accumulation by creating and maintaining an AEA gradient across the plasma membranes [36], some researchers have argued against the existence of a genuine AMT and have affirmed that FAAH is indeed the one and only factor responsible for AEA degradation. In this view, the transport of AEA would occur by

simple diffusion as a consequence of FAAH-catalyzed hydrolysis. For instance, phenylmethylsulfonyl fluoride (PMSF), a FAAH inhibitor which is structurally unrelated to (endo)cannabinoids, has been reported to inhibit AEA uptake in RBL-2H3 cells and in FAAH-transfected HeLa cells, but not in wild-type HeLa cells which lack FAAH activity [37]. Furthermore, expression of FAAH in HeLa cells increased by twofold the maximum AEA transport compared with wild-type cells [37]. In addition, the length and temperature of AEA incubation during the uptake assay seem to be critical issues. In fact, it has been demonstrated that incubation times less than 1 min (at 37°C) are too short for FAAH to create and maintain any AEA concentration gradient across the plasma membranes and under these conditions, unsaturable AEA accumulation has been observed in both neuroblastoma and astrocytoma cells [38]. However, the great majority of the studies on AEA transport and its inhibition have used incubation times greater than 1 min (at 37°C), and were performed in cells and animals that do express FAAH. Therefore, it is highly probable that the effect of purported inhibitors of AMT may also depend on their capacity to block hydrolysis rather than the transport of AEA. Under this assumption, two observations seem to favor the hypothesis of a simple diffusion of this endocannabinoid. First, the saturability of AEA transport may be a direct consequence of its insolubility in aqueous media or limited solubility within cell membranes. Second, the temperature dependence is not a unique feature of carrier-mediated processes, in fact simple diffusion of hydrophobic molecules through membranes or lipid vescicles is also known to be temperature dependent [39].

In contrast to the data above, several other studies report in favor of the existence of a specific, saturable, pharmacologically inhabitable, and bidirectional facilitated diffusion of AEA across the plasma membranes [27,40]. They also show that this process is independent of FAAH activity. By using brain synaptosomes from FAAH (-/-) mice, Ligresti and colleagues have shown that FAAH contributes to AEA transport but that this process can still be operational in the absence of AEA hydrolysis [41]. In addition, the AEA accumulation is saturated by concentrations between 1 and 10 μM, that is, in the physiological range. The experiments performed by Ligresti and colleagues showed saturability of accumulation after 90 sec incubation [41], in contrast to Glaser and colleagues [38]. Finally, they demonstrated that AEA uptake is inhibited by specific compounds which block transport without affecting FAAH activity [41].

In keeping with the view that favors the existence of AMT, it has been demonstrated that brain neurons from FAAH (+/+) and FAAH (-/-) mice internalize AEA at the same rate [40]. Moreover, the administration of AM1172, a novel and metabolically stable inhibitor specific for AMT, was shown to inhibit the uptake but not the hydrolysis of AEA, suggesting that these processes are two different steps in AEA degradation [40].

A very recent report suggests that CB1 receptors may also be involved in the control of AEA transport. In fact, Ortega-Gutièrrez and colleagues have noted that after the treatment of neurons isolated from FAAH wild-type and knock-out mice with a specific uptake inhibitor – UCM 707 – AEA transport decreased in FAAH (-/-) animals [42]. These authors obtained the same results using SR141716A, a specific CB1 antagonist, suggesting the partial contribution of an additional, yet unknown, UCM707-sensitive protein [42].

To further support the existence of AMT, some experiments have addressed the issue of whether or not AEA can move bidirectionally across the membranes through the same transporter. These studies were based on both biochemical and electrophysiological experiments [11,27,43]. For instance, our group has found that by preloading HUVECs with radiolabelled AEA, the latter compound could be detected in the extracellular medium according to a saturable process showing the same kinetic properties as AEA uptake in the same cells [27].

Similarly, Gerdeman and colleagues have shown in subsequent studies a concentration-dependent loss of excitatory synaptic transmission after the administration of AEA to postsynaptic neurons [43]. These data confirm that AEA can be streamed outside and can act as a retrograde messenger to inhibit the glutamatergic release. Taken together, these data support the existence of a true membrane trasporter for the movement of AEA in and out of the cell.

AMT inhibitors

The possibility that the uptake of AEA may be mediated by a facilitated transport or by passive diffusion driven by FAAH, depending on the cell type used, has been difficult to investigate pharmacologically, since available compounds show little selectivity towards uptake and hydrolysis of AEA. In fact, many compounds capable of

blocking AEA internalization can also interact with FAAH, CBR or TRPV1. Thus, interest towards new and more selective 'bullets' has increased tremendously in the last few years.

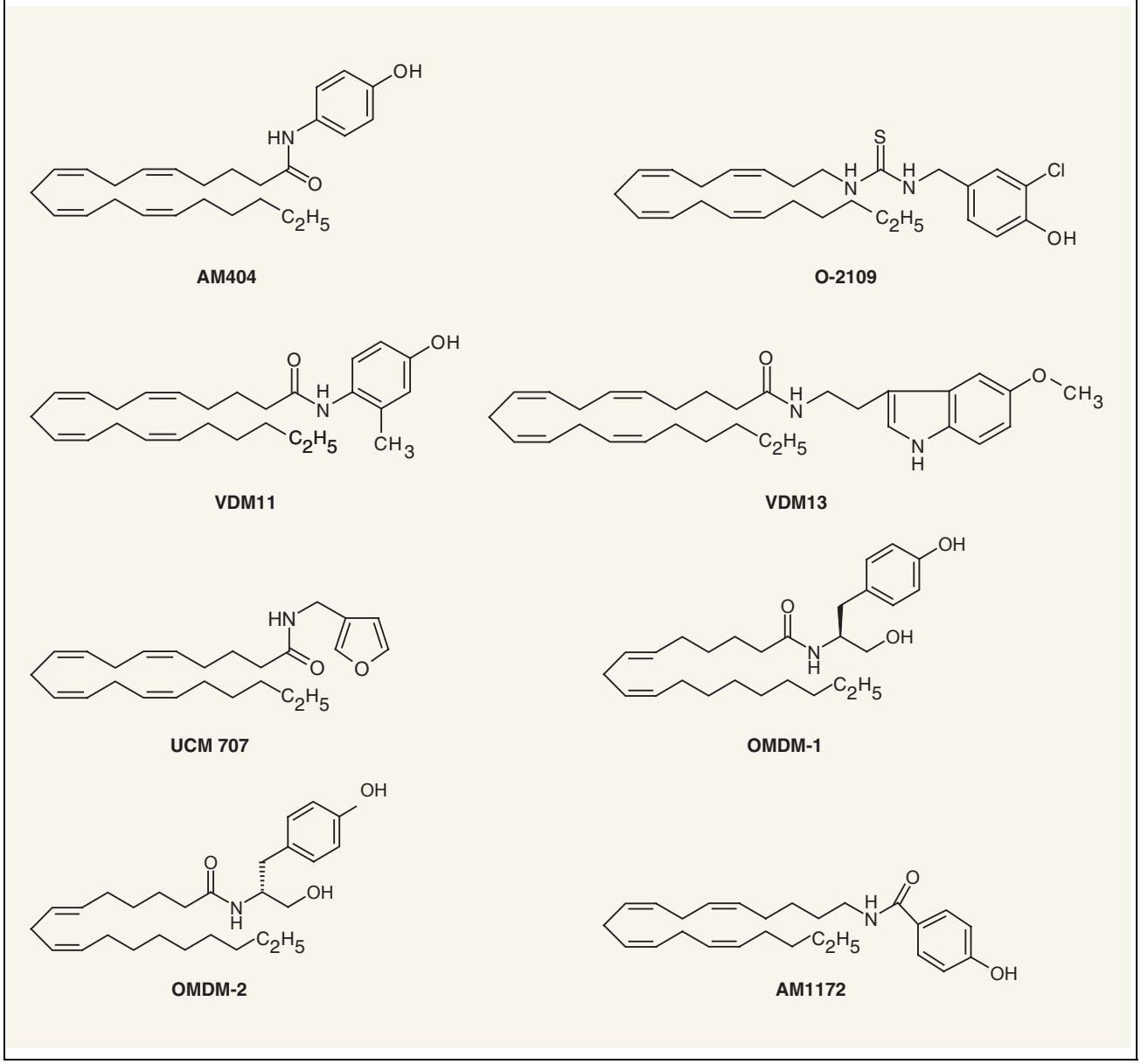
The first evidence of a 'natural' regulator selective for AEA uptake was reported by our group, showing that nitric oxide (NO) donors were capable of increasing AMT activity by approximately twofold without affecting the activity of FAAH in HUVECs [23,44]. Consistently, the NO scavenger hydroxocobalamin abolished the effect of NO donors on AEA transport, and depletion or enhancement of intracellular glutathione concentration potentiated or attenuated it, respectively [44]. Of interest is also the fact that peroxynitrite (NO_3^-), the product of NO and superoxide (O_2^-), caused an approximately fourfold activation of AEA uptake by HUVECs [44]. Overall, these results suggest that oxidative stress, mimicked by NO/NO_3^-, increases the movement of AEA through the plasma membranes, whereas the anti-oxidative defence by glutathione counteracts this effect. Yet, the pathophysiological implications of the interaction between NO and AEA degradation remain to be clarified.

In the perspective of exploiting AMT inhibitors, it is of interest to underline the structural characteristics that are essential for interaction with the AEA transporter. Structure–activity relationship (SAR) studies in human astrocytoma cells have shown that substrate recognition requires the presence of at least one *cis* double bond situated in the middle of the fatty acid carbon chain, indicating a preference for ligands whose hydrophobic tail can adopt a bent U-shaped (hairpin) conformation [22]. Further experiments, testing compounds with a different chain length and a different number of double bounds such as *N*-palmitoyethanolamine (PEA, [C16:0]), *N*-oleoyl & ethanolamine (OEA, [C18:1Δ9]), *N*-eicosaenoylethanolamine [C20:1Δ11], AEA ([C20:4Δ5,8,11.14]) and 2-arachidonoylglycerol, have demonstrated that a hairpin conformation is required for translocation. These SAR studies point to the conclusion that the carrier can distinguish among chemically similar molecules and that there is a specific protein-mediated process of accumulation. However, it is important to underline that the modification of functional groups may influence significantly the solubility of the test compounds and therefore it may confound these SAR studies. In any case, the change of the polar head group allowed the synthesis of the first uptake inhibitor – *N*-(4-hydroxyphenyl)arachidonoylamide (AM404) (**Figure 1**).

AM404 was shown to inhibit with high affinity *in vitro* the accumulation of AEA in rat neurons, astrocytes and RBL-2H3 cells, in human astrocytoma cells and in rat brain [12,23,24,29]. Even if it is structurally similar to AEA, AM404 administered alone is unable to elicit cannabimimetic responses, as a consequence of its low affinity for CB1 receptors. Therefore, the effects of AM404 result from an increase in endogenous AEA levels. Furthermore, the lack of effect of the nonspecific FAAH blocker PMSF confirms the idea that AM404 produces its effect by protecting endogenous AEA from transport-mediated inactivation. For example, by measuring the plasma level of AEA *in vivo* after a bolus injection of AM404 it was possible to observe an increase in the level of AEA at 60min after administration. Due to the fact that a decrease in OEA levels after treatment with AM404 has also been registered *in vivo*, it has been proposed that AM404 might inhibit the OEA transporter, which is different from the carrier of AEA [45]. In parallel with its ability to increase AEA levels in plasma, it was observed that AM404 also elicited a time-dependent inhibition of motor activity and this hypokinetic effect was reversed by the CB1 receptor antagonist SR141716A [45]. Recent studies carried out in FAAH knockout mice have formulated the hypothesis that instead of blocking AMT, AM404 could be a substrate of FAAH activity [38,42]. In fact brain membranes from wild-type mice degrade AM404, whereas those from FAAH knockout animals do not [42].

Further investigations have indicated that this compound is also capable of activating vanilloid receptors (TRPV1) at concentrations similar to or lower than those necessary to block AEA accumulation [46,47]. In fact, the vanilloid receptor ligands known as *N*-acyl-vanillyl amides (N-AVAMS), such as arvanil and olvanil were shown to inhibit AEA uptake [7,48]. Arvanil is a structural hybrid between AEA and capsaicin. This compound is derived from the link of arachidonic acid and the polar head of the ligand of TRPV1 [7]. Arvanil was found to be one of the most potent ligands of TRPV1 receptors and a very effective inhibitor of AMT [49]. Olvanil is the condensation product of oleic acid and the polar head of capsaicin [7]. In the study by De Petrocellis and colleagues olvanil blocked AEA-facilitated transport more potently than other inhibitors such as phloretin, AM404 or

Figure 1. Chemical structures of classical inhibitors of the anandamide membrane transporter.



oleoylethanolamide [50]. Conversely, it was a poor inhibitor of AEA hydrolysis by RBL-2H3 and N18TG2 cells, suggesting that the inhibitory effect on AEA breakdown observed in intact cells was actually due to the inhibition of AEA uptake. Olvanil was stable to enzymatic hydrolysis and displaced the binding of high affinity cannabinoid receptor ligands to membrane preparations from N18TG2 cells and guinea pig forebrain [49].

The subsequent chemical modifications on the vanillyl group and the introduction of thiourea led to the synthesis of the O-2109 compound, which shows low affinity for FAAH, while it is equally potent as TRPV1 agonist and AMT inhibitor [48] (**Figure 1**).

Next, two novel AEA derivatives were synthesized by Di Marzo's group –VDM11 and 13 – which inhibit AMT as potently as AM404 and have low binding activity at TRPV1 receptors [49] (**Figure 1**). VDM11 ([5Z,8Z,11Z,14Z]-*N*-[4-hydroxy-2-methylphenyl]-5,8,11,14-eicosatetraenamide) presents an *O*-methyl group on the vanillyl structure, while VDM13 contains hindering aromatic moieties. Since both compounds were weak inhibitors of FAAH and poor ligands of CB1 and CB2 receptors, they were considered selective inhibitors of AMT [49,50].

Later, a group of new inhibitors derived from arachidonic acid were synthesized – the UCM family [51]. The common characteristic of these compounds is the replacement of ethanolamine with a fragment containing a five-membered ring with one heteroatom. Most are able to inhibit AEA uptake at very low concentrations. In general, these substances have weak interactions with CB1, CB2 and TRPV1 receptors and there is no relationship between their potency as inhibitors of AEA uptake and their ability to inhibit AEA hydrolysis [51]. UCM707 (*N*-[3-furylmethyl]eicosa-5,8,11,14-tetraenamide) demonstrates the highest potency and selectivity *in vitro* as an inhibitor of the AMT transporter (**Figure 1**). Unfortunately, detailed information on the behavioural effects of this compound are not yet available, apart from the hypomotility and antinociception typical of (endo)cannabinoids. The *in vivo* activity of UCM707 has been challenged in open-field and hot-plate tests [52]. It has been found that UCM707 used alone produced small responses at high doses and that these effects were not statistically significant compared with those of AM404 [52]. Nonetheless, the administration of this compound with AEA produced a larger decrease in exploratory activity and ambulation, along with an increase in the time spent in inactivity and in the latency to respond to a painful stimulus, overall demonstrating that it could be a potent inhibitor of AMT without direct effects on CB receptors [52].

Recently, novel compounds and analogs of AEA and OEA have been produced and named OMDM-1, OMDM-2 (**Figure1**), OMDM-3 and OMDM-4 [53]. Changes introduced in the chemical structure of these molecules were made to create metabolically stable, potent and selective inhibitors with negligible effects on CB1 and TRPV1 receptors on FAAH. They contain an aromatic group in the ethanolamine head allowing for strong interaction with AMT through aromatic stacking. This modification abolishes the capacity of interaction with TRPV1 receptors and on FAAH and warrants the selectivity towards AMT. Their efficacy has been tested in RBL-2H3 cells and in rat brain and spleen membranes, demonstrating their low affinity for CB1 and CB2 receptors [53]. In addition, they were found to be more effective on AMT than VDM11 and VDM13, more selective than arvanil and AM404, and extremely stable to enzymatic hydrolysis [53].

The newest compound which was shown to block efficiently the accumulation of AEA in rodent cortical neurons and human astrocytoma cells, without acting as a FAAH substrate or inhibitor, is AM1172 (*N*-[5Z,8Z,11Z,14Z eicosatetraenyl]-4-hydroxybenzamide) (**Figure 1**). This substance contains a reverse amide moiety with respect to AM404 and has high metabolic stability. Binding experiments have shown that AM1172 has a partial affinity for CB1 and CB2 receptors, but none for TRPV1 [40].

Therapeutic applications

Many studies have demonstrated that the endocannabinoid system is involved in several disorders of CNS and peripheral tissues. Therefore, alteration of the activity of one component of this system, such as AMT, could have therapeutic value for the treatment of a number of human diseases. Analogously, it is known that by blocking the reuptake of serotonin and norepinephrine, it is possible to prolong the action of these neurotransmitters. This discovery allowed the synthesis of new drugs for the treatment of neuropsychiatric diseases widespread in the human population, such as depression, anxiety and bipolar disorder [54].

In this line, THC is known to be involved in the control of emotional states both in humans and laboratory animals through the activation of brain CB1 receptors [54]. AEA seems to work on the same targets as THC, therefore it is conceivable that the pharmacological inhibition of AEA uptake or hydrolysis could be beneficial and devoid of the side effects typical of direct CBR agonists [54]. In fact, the biological efficacy of selective inhibitors of AMT is based on the concept that, by blocking the uptake of AEA, its endogenous levels are elevated, thus enhancing the binding to CB1, CB2, activating or not the TRPV1 receptors [55,56] and increasing the cellular effects.

Many experimental data has been obtained *in vivo* by administration of AM404 and, as seen below, we have to consider that many actions of this molecule are mainly due to its effect on TRPV1 receptors [56] or/and to blocking of FAAH activity. Indeed, the increase in AEA levels does not depend on the inhibition of AMT, but rather on the FAAH inhibition and enhancement of synthesis of this endocannabinoid, following the activation of the vanilloid receptor [56, 57].

It has been reported that AM404 can induce a remarkable decrease in prolactin and gonadotropin levels in plasma, without affecting the levels of luteinizing hormone (LH) during neuroendocrine regulation [58]. Moreover, the increase in

AEA levels as a consequence of the administration of AM404 was accompanied by an increase in the activity of tyrosine hydroxylase in the medial basal hypothalamus [58].

There are also studies which underline the importance of AM404 in some neurological disorders such as Huntington's disease (HD) and Parkinson's disease (PD). In fact, this compound produces a motor inhibition [59]. Lastres-Becker and colleagues have documented a role for AM404 in an animal model of HD, simulated by injecting 3-nitropropionic acid in rats [59]. Treatment with this inhibitor alleviated the hyperkinetic signs and neurochemical deficits during the hyperkinetic state of neurodegeneration [59] even if, as demonstrated recently, its action is due to a direct activation of vanilloid receptor [57]. Additionally, during the last congress of the International Cannabinoid Research Society, Fernandez-Ruiz's group demonstrated that AMT inhibitors with TRPV1 affinity, such as UCM707, behave as anti-hyperkinetic drugs [60]. In fact, it was possible to observe that treatment with UCM707 produced beneficial effects in rat models of neurological diseases such as HD, PD and also multiple sclerosis (MS) [60]. Indeed, it was found that the use of AM404 and VDM11 (i.v. 10 mg/kg) in mice suffering from chronic relapsing experimental allergic encephalomyelitis (CREAE) markedly ameliorated spasticity [61].

In addition, our group has recently reported data on the reduced activity of AMT in 6-OHDA-lesioned rats, a model of PD [62], and the reversal of this activity back to control values after chronic L-DOPA treatment [63]. Moreover, the application 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, recent data have indicated that the systemic administration of AM404 can induce significant anti-Parkinsonian effects, as revealed by the improvement of akinesia and sensorimotor orientation, as well as by the reduction of drug-induced turning [64].

In juvenile spontaneously hypertensive rats (SHR) – a putative model of the attention deficit hyperactivity disorder –the primary role of the endocannabinoid system in the regulation of psychomotor activity has been underlined again [24]. In particular, AM404 reduced the stimulation of motor behaviors elicited by the selective D2 family receptor agonist quinpirole [24].

Endogenous cannabinoids have also been recognized as immune and cardiovascular regulators [65]. In fact, some studies demonstrated that AEA decreases systemic blood pressure dose dependently *in vivo*, and that the vasodepressor responses elicited by AEA are significantly potentiated and prolonged by AM404 [66]. As indicated in HD studies [57], the cardiovascular effects of AM404 are mainly dependent on vasodilatation following activation of the vanilloid receptor.

It has been reported in the literature that natural and synthetic cannabinoids are involved in neuroprotection against exitotoxicity [67–70] and many studies have reported an increase in the synthesis of endocannabinoids following neuronal depolarization [71]. As reported by Marsicano in mice treated at different times with the excitotoxin kainic acid (KA), the tissue concentration of AEA, but not of 2-AG and PEA, reached a marked increase 20 min after KA injection [72]. The researchers observed that pre-treatment with 3 mg/kg of UCM707 significantly protected the mice against toxicity induced by 30 mg/kg of KA, confirming the role of endocannabinoids in neuroprotection and the importance of the inhibition of AEA transport in this neuronal damage.

Of interest is the fact that in the last few years, attention has been focused on the role of AMT inhibitors in ophthalmic diseases such as glaucoma, disclosing new targets under AMT control [66]. AM404 and olvanil, when applied topically, can affect the intraocular pressure (IOP) of normotensive rabbits. Clinical pilot treatments using topical AM404 provoked an initial ocular hypertension, followed by a significant decrease in IOP in treated eyes. In addition, olvanil caused an important reduction of IOP without provoking an initial hypertensive phase. Further, the co-administration of AM404 and AEA had no significant effect on the IOP profile of exogenous AEA alone, suggesting that only the effect of the endogenous tone of AEA on ocular hypotension was under the control of the AMT [66].

As a result of gastrointestinal studies, an increase in AEA levels and expression of CB1 receptor mRNA in the intestinal fluid accumulation following administration of cholera toxin (CT) has been registered in mice. SR141716A, but not SR144528, and capsazepine reversed this effect, indicating that fluid accumulation was induced through the CB1-dependent mechanism. Therefore excluding the involvement of TRPV1, the avoidance of CT-induced fluid accumulation by administration of VDM11 has suggested that

drugs inhibiting AMT could have an antisecretory role in the small intestine [73].

Finally oncological studies involving the administration of the metabolically stable AEA analog Met-F-AEA have significantly inhibited the growth and size of thyroid tumor xenografts induced in rats. Therefore, using inhibitors of AMT selected for their inactivity at cannabinoid receptors, such as VDM11, researchers have been able to observe the blockade of carcinoma cell growth even if for the increase of 2-AG levels but not for AEA levels [74].

Expert opinion & outlook

In this review we have summarized the properties of the endocannabinoid system. Taken together, the results from our group and others suggest the existence of a complex network of proteins and lipids. It is this complexity that is keeping alive the question of the presence of a true AEA membrane transporter. In fact, as discussed in this review, on one hand there are groups that favor the hypothesis that AEA uptake is a process of simple diffusion. This hypothesis is apparently corroborated by results demonstrating that numerous compounds used as inhibitors of the intracellular accumulation of AEA, such us AM404, arvanil and olvanil, can indeed also inhibit FAAH – the AEA-hydrolase which is downstream from the transmembrane transport. As a consequence, the continuous hydrolysis of AEA by FAAH would create and maintain the concentration gradient necessary to drive the process of simple diffusion. On the other hand however, other groups, including ours, have demonstrated that the intracellular accumulation of AEA is a process correlated to, but not necessarily dependent upon FAAH. In particular, the observation that the movement of AEA across the membrane is bidirectional seems to strongly point in favor of a true AMT capable of importing or exporting AEA depending only on the concentration gradient at both sides. In fact, it would be rather difficult to explain AEA export from the cell as a consequence of FAAH-catalyzed hydrolysis.

In conclusion, only the cloning and characterization of the protein responsible for AEA transport will provide a definitive answer to the question of the identity and properties of AMT. To this end, SAR studies of the most selective and effective AMT inhibitors described in this review will help to develop better tools to try to identify the AMT and to be used as novel and highly selective drugs for a number of human diseases. Neurodegenerative pathologies such as Huntington's disease, Parkinson's disease and multiple sclerosis may benefit from AMT-oriented therapeutics. In addition, AMT inhibitors alone or in synergy with AEA may ameliorate the symptoms of neuroendocrine pathologies of cardiovascular diseases, of anxiety and of glaucoma.

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Affiliations

Natalia Battista
University of Teramo,
Department of Biomedical Sciences,
Piazza A. Moro 45, 64100 Teramo, Italy
Tel.: +39 086 126 6875
Fax: +39 086 141 2583
N.Battista@med.uniroma2.it

Valeria Gasperi
University of Rome "Tor Vergata",
Department of Experimental Medicine and Biochemical Sciences, Via Montpellier 1,
00133 Rome, Italy
Tel.: +39 067 259 6385
gasperi@Med.uniroma2.it

Filomena Fezza
University of Rome "Tor Vergata",
Department of Experimental Medicine and Biochemical Sciences, Via Montpellier 1,
00133 Rome, Italy
Tel.: +39 067 259 6385
fezza@med.uniroma2.it

Mauro Maccarrone, PhD
University of Teramo, Department of Biomedical Sciences, Piazza A.Moro 45,
64100 Teramo, Italy
Tel.: +39 086 126 6875
Fax: +39 086 141 2583
Maccarrone@unite.it