Inhibitors of Anandamide Breakdown

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INTRODUCTION

^{9}-Tetrahydrocannabinol (THC), the psychoactive marijuana plant- derived cannabinoid, and numerous synthetic derivatives have been shown to bind to a specific brain receptor, cannabinoid receptor 1 (CB1) (Howlett et al. 1990; Matsuda et al. 1990; Herkenham et al. 1990; Mailleux and Vanderhagen 1992). Arachidonoyl ethanolamide (anandamide), homo--linolenyl ethanolamide, and docosatetraenyl ethanolamide are naturally occurring brain constituents that bind to CB1 and as a class are called the anandamides (Mechoulam et al. 1994; Devane et al. 1992; Hanus et al. 1993; Felder et al. 1993; Devane 1994). Anandamide behaves as a cannabimimetic compound in vitro, stimulating receptor-mediated signal transduction that leads to the inhibition of forskolin-stimulated adenylate cyclase (Vogel et al. 1993; Childers et al. 1993)[1]. In a neuroblastoma cell line, anandamide causes partial inhibition of N-type calcium currents via a pertussis toxin-sensitive guanosine triphosphate binding protein (G-protein) pathway, independently of cyclic adenosine monophosphate (cAMP) metabolism (Mackie et al. 1993). Using a series of behavioral tests to evaluate cannabinoid analogs, anandamide has been shown to be a cannabinoid receptor agonist exhibiting pharmacological activity in mice parallel to that of other psychotropic cannabinoids (Fride and Mechoulam, 1993; Crawley et al. 1993; Smith et al. 1994; Abadji et al. 1994).

Soon after the discovery of anandamide, enzymatic activities responsible for its degradation (an amidase, also called an amidohydrolase) and synthesis (synthase) were described (DiMarzo et al. 1994; Desarnaud et al. 1995; Ueda et al. 1995; Deutsch and Chin 1993; Kruszka and Gross 1994; Devane and Axelrod 1994). Phenylmethylsulfonyl fluoride (PMSF) was discovered to be a potent inhibitor of the enzymatic breakdown of arachidonoylethanolamide (Deutsch and Chin 1993). A series of anandamide analogs was synthesized (trifluoromethyl ketone, -keto ester, and -keto amide derivatives) and tested in vitro and in intact cells as amidase inhibitors (Koutek et al. 1994). The trifluoromethyl ketones (e.g., arachidonyltrifluoromethyl ketone) were found to be potent inhibitors in the low micromolar (M) range. Most recently, a potent irreversible inhibitor of anandamide hydrolysis (AM 374) has

been synthesized and found to be effective in the low nanomolar (nM) range.

ANANDAMIDE SYNTHESIS

An enzymatic activity has been identified thatcatalyzes the synthesis of arachidonoylethanolamide (Deutsch and Chin 1993) as shown below:

Arachidonic Acid + Ethanolamine _ Arachidonoyl Ethanolamide + H_2O

This catalytic activity for the biosynthesis of anandamide requires ethanolamine and arachidonic acid and is readily detected in incubations of rat brain homogenates. When [^{3}H]-arachidonic acid was employed as the label, addition of exogenous ethanolamine was necessary to observe anandamide synthesis, indicating that ethanolamine is limiting in the brain homogenate. It was found that anandamide synthesis increased with increasing amounts of ethanolamine so that 35 percent of the [^{3}H]-arachidonate counts were incorporated into anandamide with 7 millimolar (mM) unlabeled ethanolamine (Deutsch and Chin 1993). (Subsequently, one of the authors (DGD) found that synthesis may be observed with as little as 0.1 mM ethanolamine.) When labeled ethanolamine was employed, the apparent increased rate of anandamide synthesis, in the presence of PMSF, was an artifact caused by the reaction of PMSF with ethanolamine to form the corresponding ethanolamide, which had the same mobility on the thin layer chromatography (TLC) plate as anandamide (W. Devane, personal communication, July 1994). Interestingly, Devane and Axelrod (1994) observed that PMSF was a potent inhibitor of the synthase. Recently, it was postulated that the amidase and synthase are the same enzyme. This is based upon the observation that the amidase and synthase activities cochromatograph during purification and that they exhibit the same behavior towards inhibitors, in their pH dependence, and in their heat inactivation profiles (Ueda et al. 1995). Synthesis of anandamide has also been demonstrated in subcellular fractions. The highest activity occurred in the synaptic vesicles, myelin, and microsomal and synaptosomal membranes (Devane and Axelrod 1994). The reaction was selective for arachidonic acid as the aliphatic constituent and ethanolamine as the polar moiety. Furthermore, it was clearly demonstrated that this reaction occurs through an adenosine triphosphate (ATP)- and CoA-independent process (Kruszka and

Gross 1994). It is interesting to note that Bachur and Udenfriend (1966) described a rat liver microsomal system utilizing aliphatic fatty acids, but not arachidonate, and several amines as substrates for the synthesis of fatty acid amides.

Burstein and Hunter (1995) observed that THC stimulated the biosynthesis of anandamide in neuroblastoma cells employing either ethanolamine or arachidonic acid as the label. Anandamide biosynthesis has also been shown to occur in primary cultures of rat brain neurons labelled with ^{3}[H]-ethanolamine when stimulated with ionomycin, a Ca^{++} ionophore (Di Marzo et al. 1994). These authors proposed an alternate model for the biosynthesis of anandamide in which N-arachidonoyl phosphatidyl ethanolamine is cleaved by a phospholipase D activity to yield phosphatidic acid and ararchidonoylethanolamide. This model is based upon extensive studies undertaken by Schmid and collaborators (1990), who have shown that fatty acid ethanolamide formation results from the N-acylation of phosphatidyl ethanolamine by a transacylase to form N-acyl phosphatidylethanolamine. Possibly resulting from postmortem changes, this compound is subsequently hydrolyzed to the fatty acid ethanolamide and the corresponding phosphatide by a phosphodiesterase, phospholipase D.

ANANDAMIDE AMIDASE

The reaction for the degradation of anandamide to arachidonic acid and ethanolamine is shown below.

Arachidonoyl Ethanolamide + H_2O _ Arachidonic Acid + Ethanolamine

The distribution of this amidase activity was characterized in tissues from the rat using a TLC assay with [arachidonoyl 5,6,8,9,11,12,14,15- ^{3}H]-ethanolamide as the substrate (Deutsch and Chin 1993). This enzymatic reaction was expressed in homogenates from brain, liver, kidney, and lung (figure 1). Under these conditions, the main degradation product detected was arachidonate. Barely detectable activity was present in homogenates of rat heart and skeletal muscle (Deutsch and Chin 1993; Desarnaud et al. 1995). In addition to the main arachidonic acid degradation product, lung and liver produced an unknown second catabolite that may be an oxidized form of anandamide. Recently, it has been shown that anandamide may be hydroxylated by the cytochrome P450s (Bornheim et al. 1993) and this may account, in part, for the additional metabolite. When the ethanolamides of a series of fatty

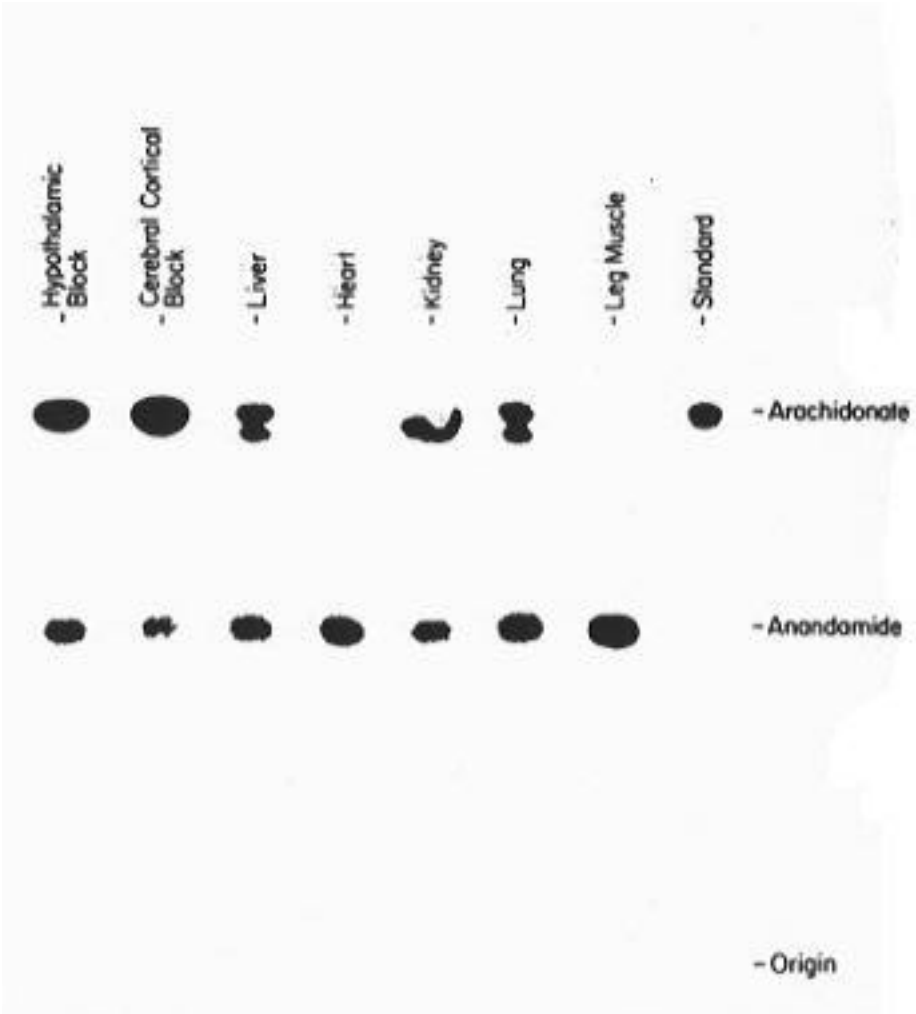

FIGURE 1. *Degradation of anandamide in homogenates of rat tissue. Incubations were at 37° for 45 min with 10 mg of tissue homogenate protein and 0.4 microcuries (μCi) [^{3}H]-anandamide in PBS.*

acids was tested as substrates for the amidase, the highest substrate specificity was for arachidonoyl ethanolamide (Desarnaud et al. 1995; Ueda et al. 1995).

CELL CULTURE EXPERIMENTS AND ANANDAMIDE AMIDASE

When ^{3}[H]- anandamide ([arachidonoyl 5,6,8,9,11,12,14,15- ^{3}H]-ethanolamide) was incubated with neuroblastoma (N18TG2) or glioma (C6) cells, there was a time-dependent decrease of its levels in the media (Deutsch and Chin 1993). Anandamide was taken up by the cells immediately, but it did not accumulate since it was converted to arachidonate and other lipids containing arachidonate that migrate on TLC near the phospholipids, triglycerides, and cholesterol esters (figure 2). After 1 hour of incubation, only about 1 percent of the total radioactivity detected in all the cell fractions was from anandamide. Arachidonylethanolamide amidase was not expressed in all cell lines. Activity was found in neuroblastoma, glioma, and nonsmall-cell lung carcinoma cells, but not in HeLa cells, larynx epidermoid carcinoma (Hep2), and hepatocellular carcinoma (HepG2) cells (data not shown). The uptake and degradation of anandamide was recently confirmed in primary cultures of rat brain (Di Marzo et al. 1994).

SUBCELLULAR FRACTIONATION AND BRAIN LOCALIZATION

When the neuroblastoma and glioma cells were fractionated into membrane and soluble fractions, the enzymatic activity for the degradation of anandamide resided mainly in the mitochondrial and microsomal membrane fractions (figure 3). Within the central nervous system, the distribution of the amidase activity correlated well with the distribution of the cannabinoid receptor. The highest activity was found in the globus pallidus, hippocampus, substantia nigra, cerebral cortex, and cerebellum, and the lowest activity was found in the brain stem and medulla where cannabinoid receptors are sparse (Desarnaud et al. 1995; Hillard et al. 1995).

A RAPID ASSAY FOR ANANDAMIDE AMIDASE

The assays (see above) for arachidonoyl ethanolamide hydrolysis (anandamide amidase) employed ^{3}H-anandamide as the substrate

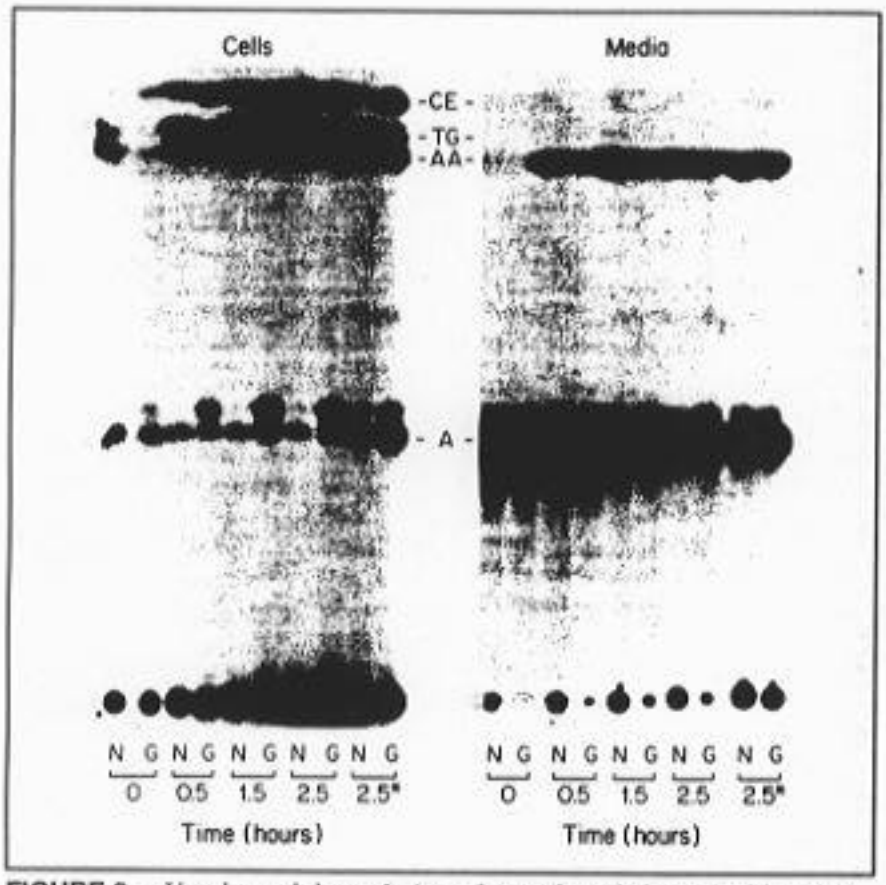

FIGURE 2. *Uptake and degradation of anandamide by neuroblastoma (N) and glioma (G) cells as a function of time. [^{3}H]-Anandamide (12 μCi) was added to the cell culture media. The radioactive products from the media and cells were analyzed by TLC and autoradiography to detect tritiated anandamide (A) and its putative metabolites: arachidonic acid (AA), triglyceride (TG), and cholesterol ester (CE). Phospholipids remained at the origin. In one set of experiments, 10^{-3} M emetine (*) was included in a 2.5-hr incubation.*

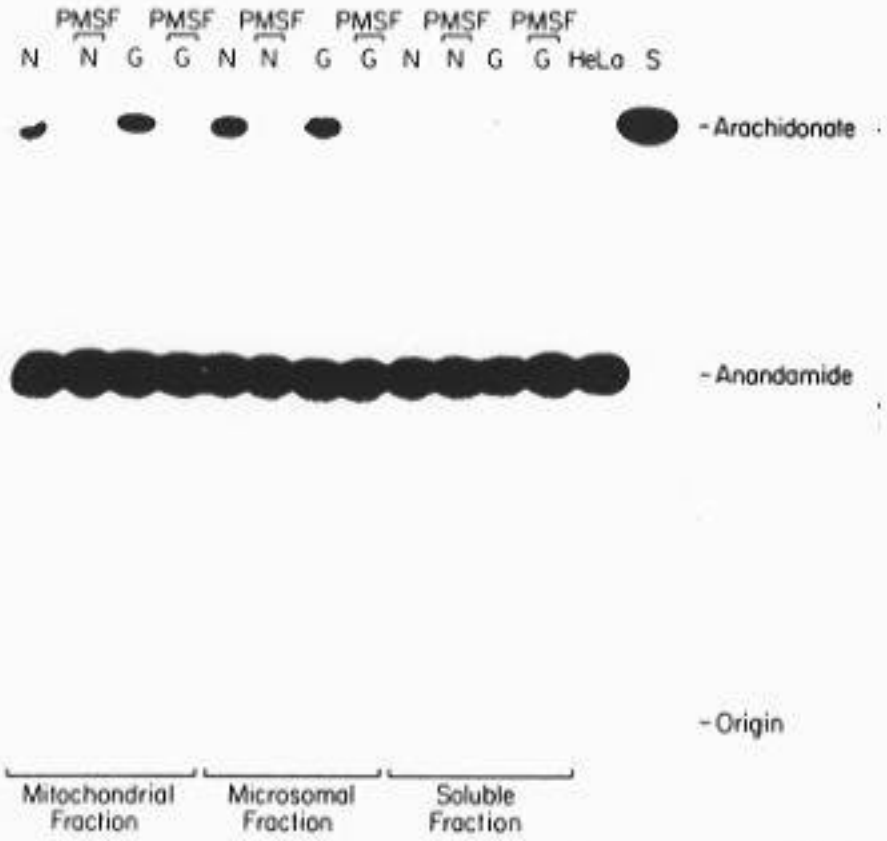

FIGURE 3. *Amidase activity in subcellular fractions of neuroblastoma (N) and glioma (G) cells. Incubations of whole fractions were for 45 min with 0.4 μCi [^{3}H]-anandamide in PBS at 37°. Where indicated, the fractions were incubated with 1.5 mM PMSF. The HeLa cells were assayed as a crude homogenate. The last sample on the plate is a [^{3}H]-arachidonic acid standard (0.16 μCi).*

(Deutsch and Chin 1993; Koutek et al. 1994). Using this assay, a TLC step is required to separate labeled arachidonic acid product from anandamide substrate. A new assay employing arachidonoyl ethanolamide labeled in the ethanolamine portion of anandamide (arachidonoyl ethanolamide-[1,2-^{14}C]) has recently been developed which obviates the need for the TLC step (Omeir et al. 1995). After incubation of the enzyme with this substrate, the reaction mixture is stopped by the addition of organic solvent. The radiolabeled anandamide substrate partitions into the organic phase while the product (ethanolamine- [1,2-^{14}C]) conveniently partitions into the aqueous phase which is subsequently measured by liquid scintillation counting. Employing this assay, it was found that the reaction proceeded linearly for at least 30 minutes. Anan-damide amidase exhibits maximal activity between pH 8 and pH 9 with a steep decline in activity at pH values below pH 6 and above pH 10. Arachidonoyl ethanolamide-[1,2-^{14}C] was found to be a good substrate to assay the amount of anandamide amidase from 10 to approximately 100 micrograms (g) of protein in brain homogenate (figure 4). Under the conditions of this assay, less than 10 percent of the substrate is hydrolyzed. Defatted serum albumin is included in the assays to bind the fatty acid product, thus preventing product inhibition. The results of a representative experiment for the initial velocity of anandamide degradation versus anandamide substrate concentration, when plotted as a rectangular hyperbole of the steady-state Michaelis-Menten equation, yields a Michaelis constant (K_m) of 30 Å 7 M and a maximal velocity (V_{max}) of 198 Å 13 nanomoles (nmoles) ethanolamine formed per hour per mg protein homogenate. The K_m and V_{max} values calculated from the saturating hyperbola must be considered approximate in view of the fact that the interfacial enzyme reaction occurs in an impure preparation, whose substrate and product have the potential to form micelles, which in turn may affect the enzyme activity. Recently Hilliard and colleagues (1995), Desarnaud and colleagues (1995), and Ueda and colleagues (1995) reported values of K_m and V_{max} for the hydrolysis of anandamine as follow: K_m 3.4, 12.7, and 60M and V_{max} 0.132, 0.337, and 28.8 moles/hr/mg protein, respectively.

The enzyme showed some specificity when two chiral methanandamide stereoisomers (Abadji et al. 1994) were tested as competitors of anandamide (figure 5). At low concentration the (R)-methanandamide stereoisomer did not affect the enzyme activity, while the (S)-meth-anandamide isomer interacted weakly. At higher concentrations this effect disappeared, with both compounds giving some inhibition of

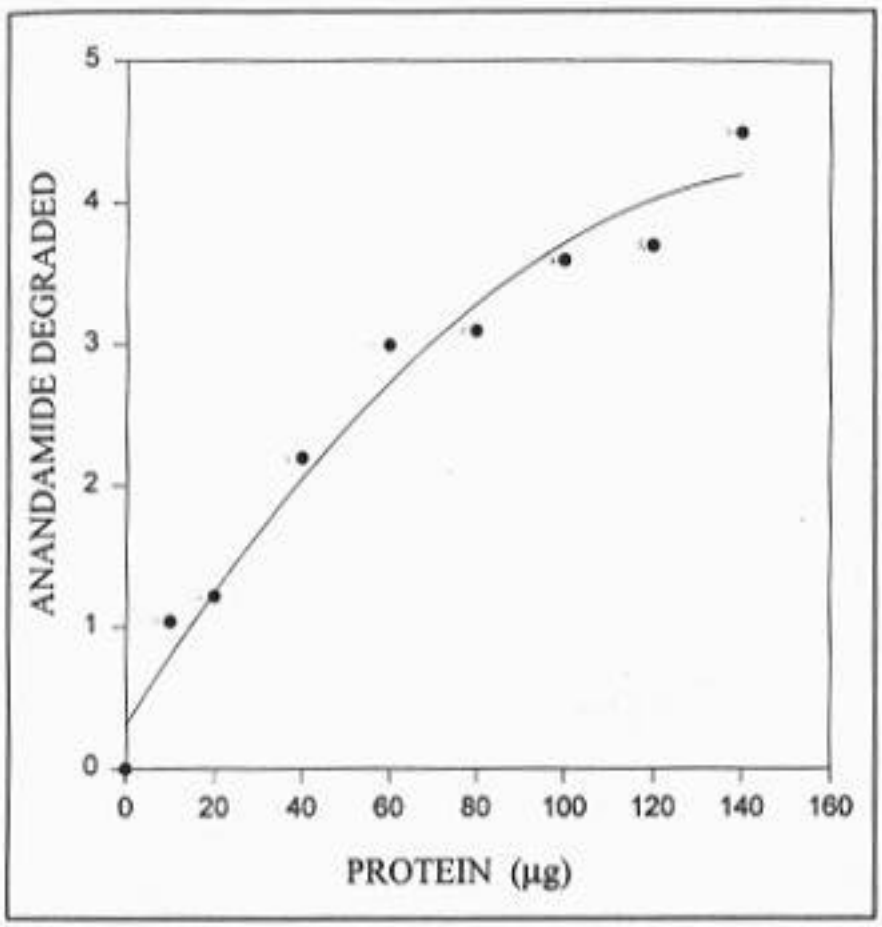

FIGURE 4. *Assay of anandamide amidase activity in crude protein brain homogentate. The amount of brain protein indicated above was incubated for 0.5 hr in a 0.2 milliliter (mL) incubation volume. Anandamide degraded is in units of nmoles/hr.*

the anandamide-amidase reaction. These results may be interpreted in terms of one stereoisomer (at low concentrations) being more effective at the substrate binding site on the enzyme.

Interestingly, the enzymatic activities found for anandamide amidase may be related to those reported in the literature for the hydrolysis of other fatty acid amides. Bachur and Udenfriend (1966) and Schmid and colleagues (1990) described a rat liver microsomal enzyme that hydrolyzed fatty acid ethanolamides. Natarajan and colleagues (1984) described activity in a dog brain microsomal fraction that hydrolyzed

palmitoylethanolamide with an apparent K_m of 53 M and a V_{max} of 667 nmole/h/mg.

PUTATIVE TRANSITION-STATE INHIBITORS OF ANANDAMIDE AMIDASE

As shown in figure 3, PMSF was discovered to be a potent inhibitor of the enzymatic breakdown of arachidonoylethanolamide in neuroblastoma and glioma cellular fractions (Deutsch and Chin 1993). It was also found that 1.5 mM PMSF (which was originally added to prevent proteolytic degradation of the enzymes in the subcellular fractions) completely abolished the amidase activity in rat brain and other tissue homogenates. This is consistent with the observation that inclusion of PMSF in receptor binding assays increased the apparent potency of anandamide and cogeners susceptible to the amidase (Abadji et al. 1994; Childers et al. 1994). The anandamide amidase activity was not inhibited by aprotinin, benzamidine, leupeptin, chymostatin, or pepstatin, suggesting that it is distinct from some of these common proteases that are susceptible to the tested inhibitors (Deutsch and Chin 1993). The amidohydrolase did not hydrolyze substrates for plasmin, aminopeptidase, elastase, or chymotrypsin (Ueda et al. 1995). The detailed mechanism by which PMSF inhibited this activity remains to be elucidated, although PMSF is known to inhibit serine proteases, some thiol proteases, and nonprotease enzymes such as erythrocyte acetylcholinesterease. Based on the behavior of anandamide with receptor preparations in the presence of PMSF, Childers and colleagues (1994) postulated that the enzyme mechanism may involve an active-site serine hydroxyl.

To further explore the question of the mechanism of anandamide amidase inhibition, analogs of anandamide were synthesized (Koutek et al. 1994). These anandamide analogs (figure 6) represent three classes of putative transition-state inhibitors: trifluoromethyl ketone (4), -keto ester (3), and -keto amide derivatives (2). The general strategy of this study was based upon the hypothesis that polarized carbonyls, such as those in trifluoromethyl ketones and -keto carboxylate derivatives, may form stabilized hydrates or enzyme adducts that mimic the tetrahedral

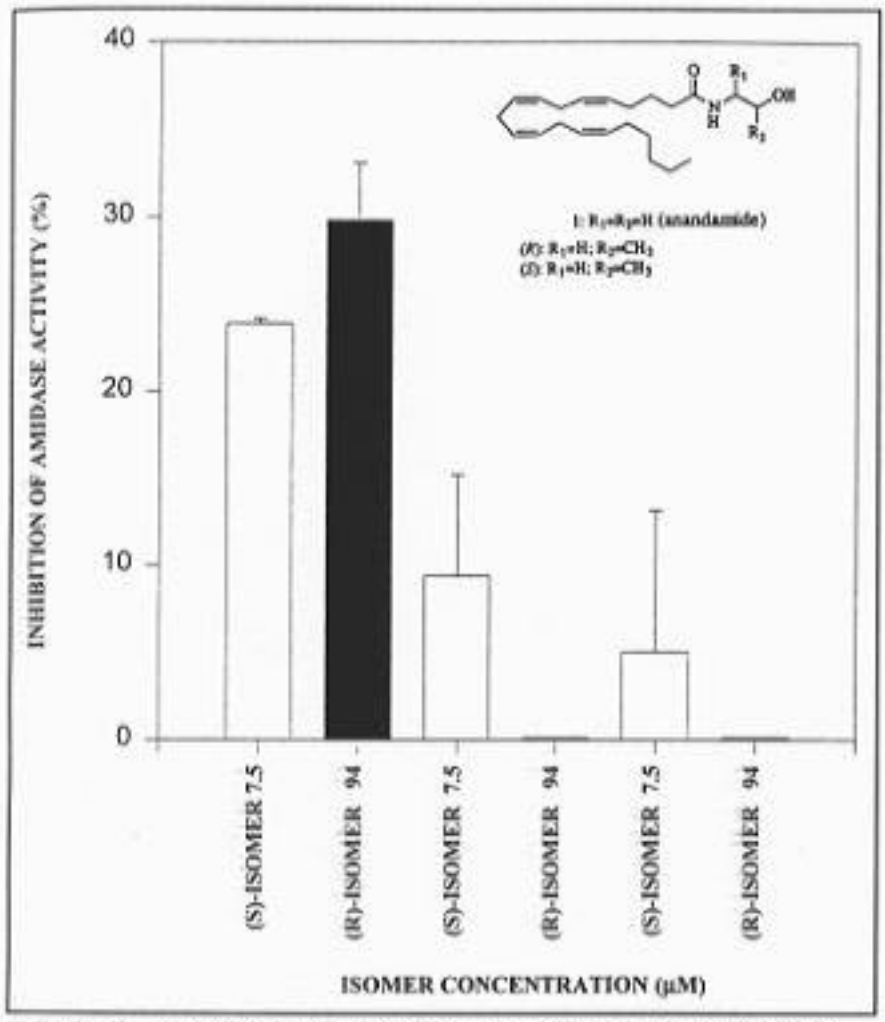

FIGURE 5. *Two chiral congeners (R and S methanandamide isomers) were tested as competitive inhibitors of anandamide amidase at 94, 7.5, and 5 μM. The percentage of inhibition was calculated by [(rate of control-rate of experimental)x100]/ rate of control. The anandamide concentration was 27.7 μM.*

intermediates formed during the reaction between the nucleophilic residue (e.g., an active-site serine hydroxyl in the hydrolytic enzyme) and the carbonyl group of anandamide (figure 7). They were tested for inhibition of anandamide hydrolysis in vitro, in cell culture, and as ligands for CB1 (Koutek et al. 1994). When tested for their ability to inhibit the hydrolysis of anandamide in vitro, the most effective classes of compounds were the trifluoromethyl ketones (4a-e) and -keto esters (3a-c). The trifluoromethyl ketones and -keto esters showed nearly 100 percent inhibition of anandamide hydrolysis in vitro in the presence of 7.5 M inhibitor and 27.7 M anandamide. Arachidonyl trifluoromethyl ketone (4a) and ethyl 2-oxostearate (3b) were the most active members of these groups, yielding nearly 100 percent inhibition of the enzyme. The inhibition of anandamide amidase by arachidonyl trifluoromethyl ketone was reversible with increasing concentrations of anandamide. Arachidonyl trifluoromethyl ketone is also a potent inhibitor of the synthase (Ueda et al. 1995). The least potent inhibitors were the -keto amides (2a-c) and the saturated analogs of anandamide (1b-d). When incubated with neuroblastoma (N18TG2) cells, anandamide is taken up by the cells and rapidly hydrolyzed to arachidonate, which is then incorporated into other lipids containing arachidonate (figure 8). However, in the presence of arachidonyl trifluoromethyl ketone (4a), there is an approximately fivefold increase of anandamide levels at 7.8 M arachidonyl trifluoromethyl ketone (figure 8). The amount of anandamide in the experimental cells increases to a twelvefold maximum, relative to the control cells, at approximately 12 M arachidonyl trifluoromethyl ketone (figure 8, inset). The mechanism apparently involves inhibition of the amidase rather than increased uptake of anandamide, since preloading the cells with labeled anandamide and then treating with 4a also resulted in a dramatic increase in anandamide levels in the cells.

The series of fatty acid derivatives was each tested, at 10 M, for their ability to displace [^{3}H]CP-55940 ([1, 2 (*R*), 5] - (-) - (1,1-dimethyl- heptyl) -2 - [5-hydroxypropylcyclohexyl] - phenol)) binding to the THC receptor in rat brain membranes (CB1). Arachidonoyl trifluoromethyl ketone was the only synthetic compound in this series of fatty acid derivatives to significantly displace [^{3}H]CP-55940 binding to CB1 with an inhibition constant (K_i) of 0.65 M. This represents approximately a fortyfold lower affinity from that of arachidonoyl ethanolamide.

Arachidonyl trifluoromethyl ketone was reported to be a slow tight-binding inhibitor of a novel 85 kilodalton-(kDa) cytosolic human

phospholipase A_2 (Street et al. 1993; Trimble et al. 1993). Some -keto acid derivatives have been shown to act as inhibitors of serine and cysteine proteinases (Ocain et al. 1992; Peet et al. 1990), and effective inhibition of cathepsin B and papain by peptidyl -keto esters, -keto amides, -diketones, and -keto acids has also been demonstrated (Hu and Abeles 1990). The disparity between the activity of ethyl 2-oxo-stearate 3b in vitro and in cell culture may be due to its susceptibility to enzymatic degradation in cell culture. It is not known if arachidonyl trifluoromethyl ketone is metabolized in the neuroblastoma cells employed in this study, but when incubated at 10 M with monocytic cells in culture for 10 min, 10 percent is converted to the corresponding alcohol (Riendeau et al. 1994).

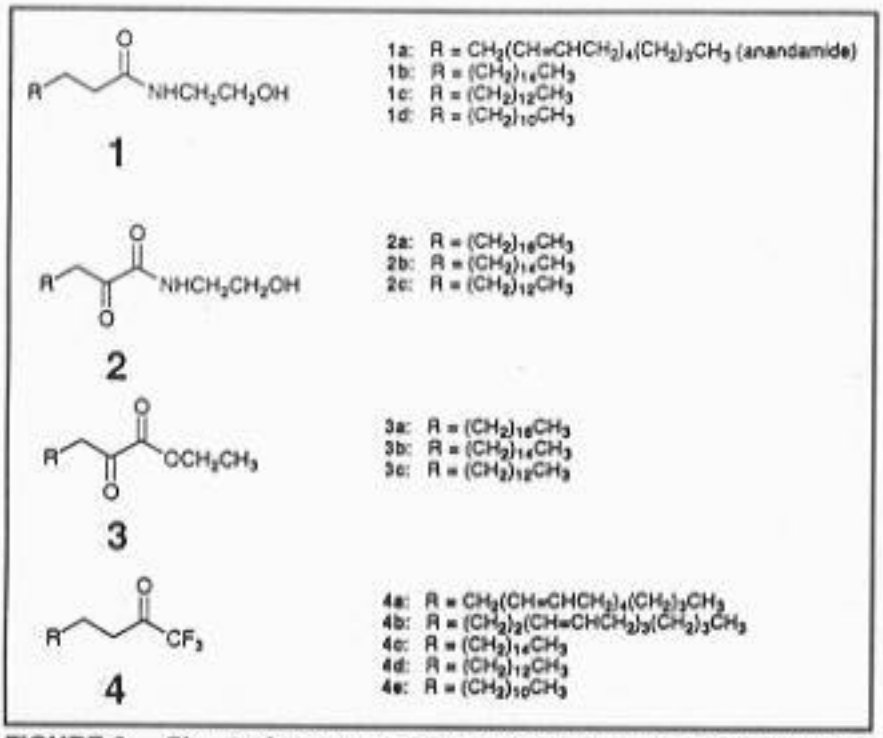

FIGURE 6. *Chemical structures of synthetic compounds. Four classes of compounds were synthesized: fatty acyl ethanolamides (1), α-keto ethanolamides (2), α-keto ethyl esters (3), and trifluoromethyl ketones (4) as described.*

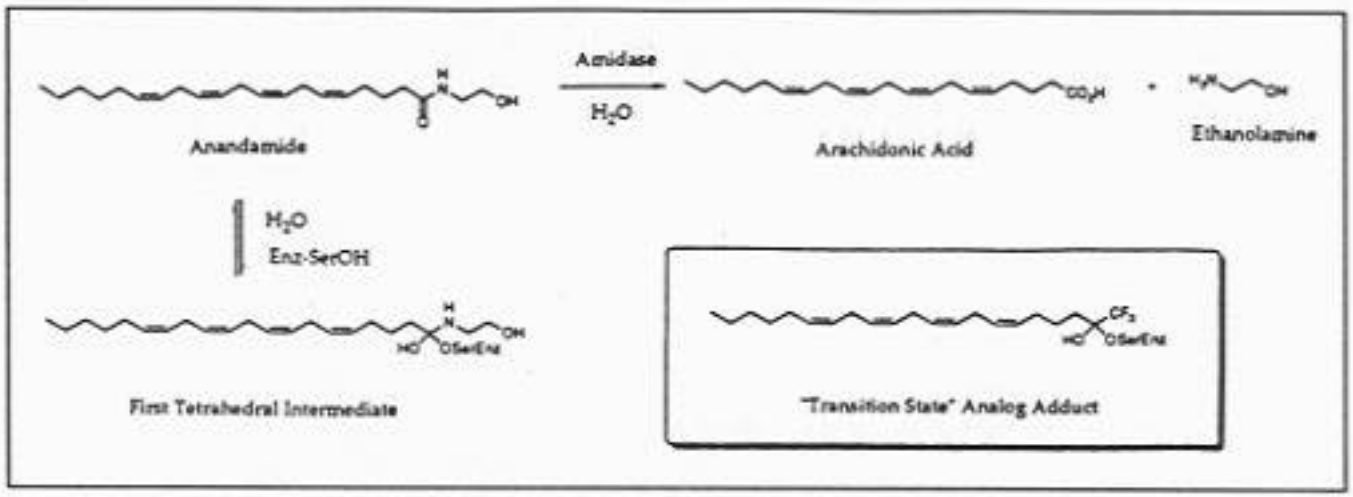

FIGURE 7. *Postulated mechanism of amidase inhibition by polarized carbonyls. Enzymatic hydrolysis of arachidonyl ethanolamide (anandamide, 1a) is postulated to proceed by a tetrahedral intermediate which can be mimicked by the adduct formed from trifluoromethyl ketone and an active-site serine residue (boxed).*

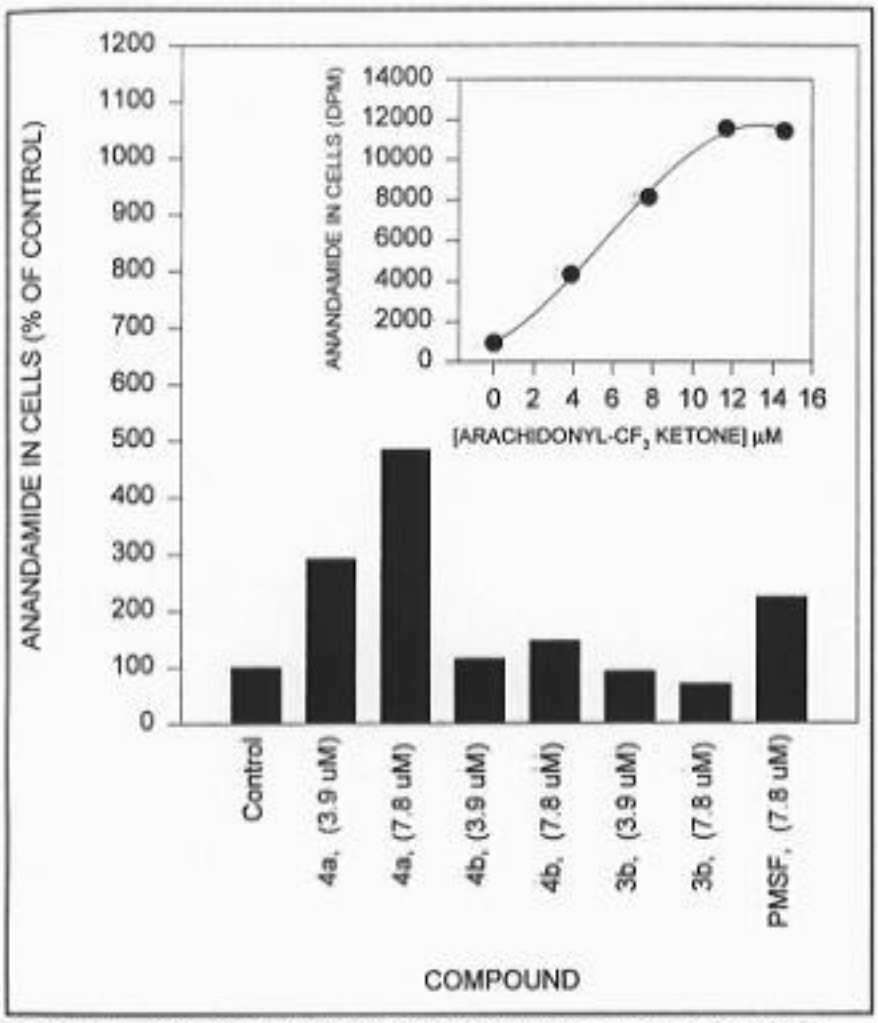

FIGURE 8. *The effect of amidase inhibitors on anandamide levels in neuroblastoma cells (N18TG2). The amount of [^{3}H]-anandamide was determined in the control and experimental cultures containing 1 x 10^6 cells, after separation by TLC and analysis of the silica gel sample scraped from the plate, by liquid scintillation counting. One hundred percent anandamide in the control cells corresponds to 1.3 percent of the total radioactivity detected in all the fractions analyzed. The inset shows the effect of increasing arachidonyl trifluoromethyl ketone concentration upon [^{3}H]-anandamide levels. Each incubation contained 4 x 10^6 cells.*

A POTENT IRREVERSIBLE INHIBITOR OF ANANDAMIDE AMIDASE

Very recently the authors developed a novel anandamide amidase inhibitor, AM374, whose potency in vitro and in neuroblastoma cells significantly exceeds that of other compounds developed to date as well as PMSF. In intact neuroblastoma cells, AM374 was found to dramatically increase the level of undegraded anandamide 55-fold at 10 nM. Interestingly, its affinity for the CB1 receptor was approximately tenfold weaker than anandamide (Deutsch and Makriyannis, unpublished data).

Ideally, a selective amidase inhibitor should antagonize the enzyme at concentrations that fail to appreciably bind to cannabinoid receptors. Furthermore, unlike PMSF, an inhibitor should not be toxic to the cells. Many of the synthetic compounds in this study fulfill these criteria; they do not bind significantly to CB1 at concentrations that inhibit amidase activity by greater than 90 percent in cell-free preparations and appear to have low toxicity toward the cells. The role that these inhibitors play in different tissues such as spleen, where a peripheral receptor (CB2) exists (Munro et al. 1993), or as inhibitors of the cytosolic phospholipase A_2 in brain and N18TG2 cells, remains to be elucidated. Furthermore, the most successful inhibitors will be subjected to in vivo testing. The development of inhibitors that block the breakdown of anandamide may be of value in any of the therapeutic applications in which THC (Mechoulam 1986) or anandamide (Crawley et al. 1993; Fride and Mechoulam 1993; Smith et al. 1994) has been shown to be potentially useful including analgesia, mood elevation, nausea, appetite, sedation, locomotion, glaucoma, and immune function.

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