Substrate Specificity and Stereoselectivity of Rat Brain Microsomal Anandamide Amidohydrolase

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Anandamide amidohydrolase (AAH) catalyzes the hydrolysis of arachidonylethanolamide (anandamide), an endogenous cannabinoid receptor ligand. To delineate the structural requirements of AAH substrates, rat brain microsomal AAH hydrolysis of a series of anandamide congeners was studied using two reverse-phase high-performance liquid chromatography (RP-HPLC) assays developed in our laboratory. Arachidonamide (**1**) was found to be the best substrate with an apparent K_m of 2.34 mM and a V_{max} of 2.89 nmol/min/mg of protein. Although anandamide (**2**) has a similar K_m value, its V_{max} is approximately one-half that of arachidonamide. *N*,*N*-Bis(2-hydroxyethyl)arachidonamide (**3**) was not hydrolyzed, suggesting specificity for unsubstituted or mono-*N*-substituted arachidonamides. Analogues with a methyl group at the 1′-position of the ethanolamido headgroup were also found to have greater resistance to enzymatic turnover and therefore increased metabolic stability. The enzyme exhibited high stereoselectivity as the rate of hydrolysis of (*R*)-α-methanandamide (2.4%) (anandamide = 100%) was about 10-fold lower than that of its (*S*)-enantiomer (23%). In contrast, (*R*)-*β*-methanandamide was 6-times more susceptible (121%) than the (*S*)-*β*-enantiomer (21%). Interestingly, an inverse correlation was shown between AAH stereoselectivity and the brain cannabinoid receptor affinity as the enantiomers with high receptor affinity displayed low susceptibility to hydrolysis by AAH. Metabolic stability is also imparted to analogues with a short hydrocarbon headgroup as well as to those possessing 2-monomethyl or 2,2-dimethyl substituents. 2-Arachidonylglycerol and racemic 1-arachidonylglycerol were shown to be excellent AAH substrates. To identify AAH inhibitors, hydrolysis of anandamide was also studied in the presence of a select group of cannabimimetics. Of these, (−)-Δ^8-THC and SR141716A, a biarylpyrazole CB1 antagonist, were found to inhibit enzymatic activity. These newly defined enzyme recognition parameters should provide a foundation for the rational development of stable, therapeutically useful anandamide analogues with high receptor affinity.

Introduction

Anandamide, originally isolated from porcine[1] and bovine[2] brains, has been demonstrated to bind[3] to the central cannabinoid receptor CB1 and to inhibit both forskolin-stimulated adenylyl cyclase activity[4,5] and voltage-dependent N-type calcium channels.[6] In addition, this endogenous cannabinoid, which was characterized as the ethanolamide of arachidonic acid, produces the characteristic in vivo effects of cannabinoids such as antinociception, hypothermia, analgesia, and catalepsy in mice[7−9] and rats.[10] These findings strongly suggest a role for anandamide as a neurotransmitter in the modulation of behavior, memory, cognition, and pain perception. Shortly after the discovery of anandamide, two other endogenous ethanolamides of polyunsaturated fatty acids were also isolated and shown to be cannabinoid receptor agonists.[11]

Enzymatic activity catalyzing the hydrolysis of anandamide has been demonstrated in rat brain homogenates and intact neurons.[12,13] This enzyme, known as anandamide amidase, anandamide amidohydrolase (AAH), or fatty acid amidohydrolase (FAAH), has been characterized and partially purified from mammalian brains[13−15] and cultured neuroblastoma cells.[16] In brain, the highest enzymatic activity was found to be in the globus pallidus and hippocampus, two regions with the highest density of cannabinoid receptors.[13b] There is evidence suggesting that AAH, which exhibits substrate selectivity for anandamide when compared to other unsaturated fatty acid ethanolamides, is an intracellular enzyme which may play a role in terminating the biological activity of anandamide.[13,15] Phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor also inhibits AAH in vitro.[4] When added to cannabinoid receptor binding experiments or in cellular assays, it has been shown to enhance the apparent potency of anandamide analogues by blocking their enzymatic degradation.[4,12] A number of amidohydrolase inhibitors have been reported,[18,19] the most potent of which are sulfonylfluoride analogues developed in our laboratory.[17] Recently, an amidohydrolase was cloned[20] and shown to be a membrane-bound protein of 60−65 kDa. Also recently

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Table 1. Kinetic Parameters for Hydrolysis of Representative Arachidonyl Amides by Microsomal AAH

substrate	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (mL/min/mg)
arachidonamide (**1**)	2.34 ± 0.28	2.89 ± 0.06	1.24
anandamide (**2**)	2.78 ± 0.51	1.40 ± 0.06	0.50
(*R*)-α-methanandamide (**5**)	33.0 ± 4.3	0.37 ± 0.03	0.011
(*S*)-α-methanandamide (**6**)	7.94 ± 0.93	0.39 ± 0.012	0.049
o-OH-ph-arachidonamide (**15**)	7.31 ± 0.69	3.17 ± 0.11	0.43

a high-affinity anandamide transport system has been shown to be present in neurons and astrocytes.[21] Presumably this reuptake protein is involved in the transport of extracellular anandamide across the plasma membrane which may be subsequently hydrolyzed by the amidohydrolase.

We have been interested in developing metabolically stable anandamide analogues which also possess high cannabinoid receptor affinity. Earlier, we reported[22,23,24] the synthesis and cannabinoid receptor binding affinities of several novel analogues of anandamide with modifications in the ethanolamido headgroup as well as in the arachidonic acid tail portion. Metabolic stability of these analogues was assessed by comparing their receptor affinities in the presence and absence of PMSF. Thus, very little or no effect of PMSF on receptor affinity was taken as an indication of metabolic stability. We have also reported two simple, efficient nonradioactive assays to measure AAH activity in rat brain microsomes that employ reverse-phase high-performance liquid chromatography (RP-HPLC). One of these is used for the quantitation of arachidonic acid[25] produced upon enzymatic hydrolysis, while the second assay measures the amine component of the enzymatic hydrolysis.[26] In this article, we have used these methods to study the hydrolysis of anandamide and its congeners by AAH as well as the inhibition of AAH hydrolysis of anandamide by other cannabimimetic ligands. Such a study will serve to delineate the structural features required for AAH active site recognition and hydrolytic action in enzyme substrates and inhibitors. It also allows us to initiate strategies for the rational development of high-affinity metabolically stable anandamide analogues of potential therapeutic value.

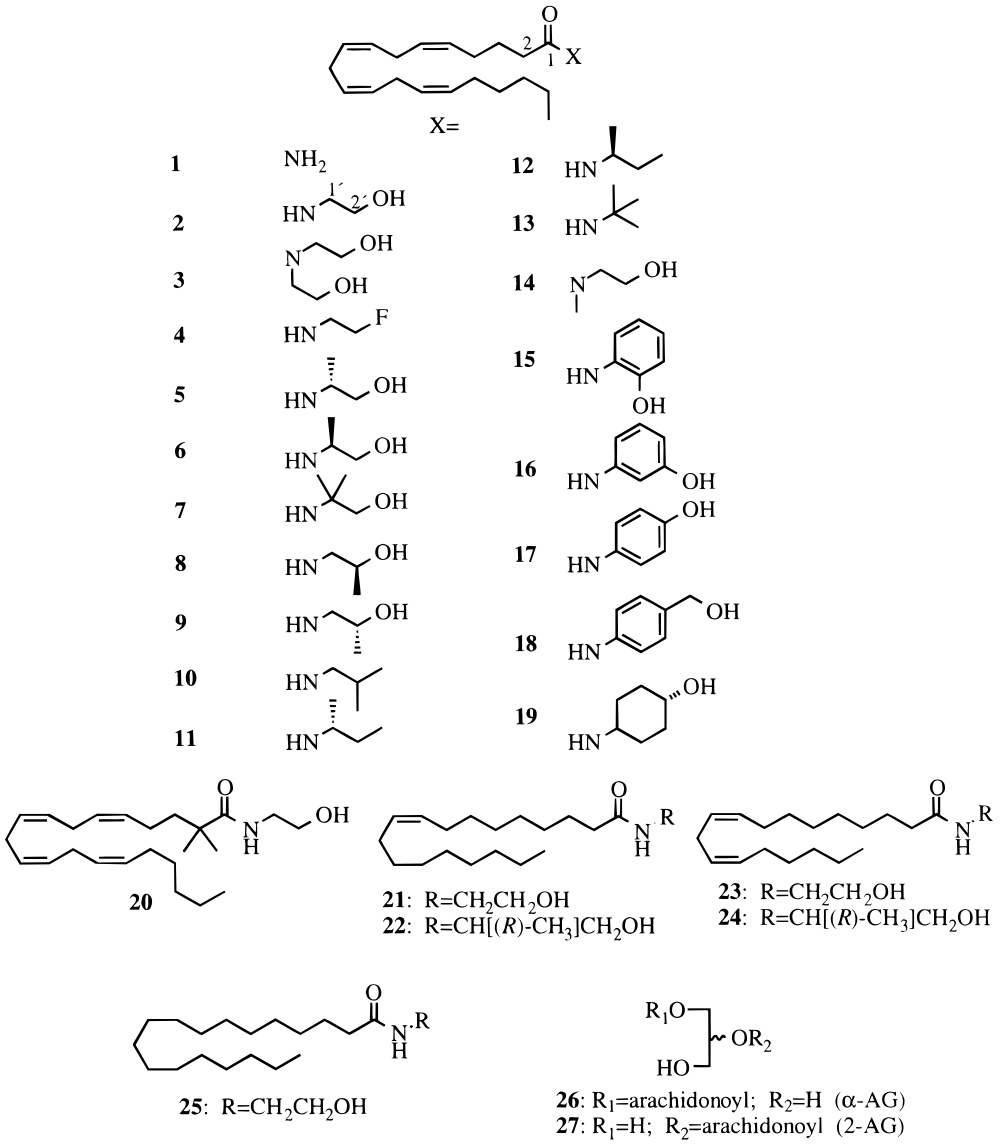
Results and Discussion

Kinetic Parameters of Representative Anandamide Analogues. Five representative anandamide congeners: arachidonamide (**1**), anandamide (**2**), (*R*)-α-methanandamide (**5**), (*S*)-α-methanandamide (**6**), and *N*-(*o*-hydroxyphenyl)arachidonamide (**15**), were chosen for a kinetic study aimed at elucidating binding affinities and turnover rates for the amidohydrolase (Table 1). Different amounts of the substrate were incubated with 0.3 mg/mL microsomal protein at 37 °C and pH 7.4 and sampled at least four times in the linear range of the initial time progress curve using bovine serum albumin (BSA) as a carrier protein. Kinetic analysis was carried out by plotting initial velocity of arachidonic acid (AA) formation versus substrate concentration and by curve-fitting the data to the Michaelis−Menten equation. In this regard, Tawakuwa et al.[27] have recently demonstrated that the kinetics of a substrate and an enzyme both bound to the same membrane can be analyzed using the Michaelis−Menten equation. Table 1 lists the kinetic parameters of the five substrates for their hydrolysis by microsomal AAH. Arachidonamide (**1**) was found to be the optimal substrate with an apparent K_m of 2.34 μM and a V_{max} of 2.89 nmol/min/mg of protein, while anandamide had a K_m of 2.78 μM, very similar to one recently reported,[28] but exhibited a V_{max} only one-half that of arachidonamide. Substrate specificity (V_{max}/K_m) decreased in the rank order of arachidonamide > anandamide > *N*-(*o*-hydroxyphenyl)-arachidonamide > (*S*)-α-methanandamide > (*R*)-α-methanandamide. For (*R*)-α-methanandamide, the ratio of V_{max}/K_m was 45-fold less than that of anandamide and about 4-fold less than that of (*S*)-α-methanandamide. The lower K_m value for (*S*)-α-methanandamide suggests that the enzyme active site can tolerate an (*S*)-α-methyl group better than an (*R*)-α-methyl group. The fast turnover for **15**, approximately equal to that of **1**, can be attributed to anchimeric assistance by the phenolic OH group during the hydrolytic step.

Hydrolysis of Anandamide Analogues by AAH. For a rapid comparative study of AAH substrate specificity, we elected to use a substrate concentration of 100 μM. Such a high concentration ensures V_{max} conditions thus allowing direct comparison of substrate rates of hydrolysis under steady-state conditions with one exception of (*R*)-α-methanandamide where V_{max} is not achieved because of its high K_m value (Table 1). For each analogue, the absolute rate and rate of hydrolysis relative to anandamide (rate = 100%) are reported.

The metabolic stability of several headgroup analogues of anandamide (Chart 1) was determined using an HPLC assay developed in our laboratory.[25] From the data presented in Table 2, it can be seen that headgroup analogues possessing a methyl group at the α-position (C1′) exhibit remarkable resistance to enzymatic hydrolysis. Thus, both (*R*)-α-methanandamide (**5**) and (*S*)-α-methanandamide (**6**) were hydrolyzed at slower rates than anandamide. The enzyme also exhibited high substrate stereoselectivity. (*R*)-α-Methanandamide (2.4%) was found to be about 10 times metabolically more stable than (*S*)-α-methanandamide (23%). Indeed, the V_{max}/K_m ratio, a measure of substrate specificity, for (*S*)-α-methanandamide is about 4 times higher than that of (*R*)-α-methanandamide (Table 1) suggesting that the former is a better enzyme substrate. Interestingly, a reverse trend in stereoselectivity was observed for β-substituted methanandamides. Thus, the relative rate of hydrolysis for (*S*)-β-methanandamide (**8**) was only 21%, while (*R*)-β-methanandamide (**9**) underwent facile hydrolysis with a relative rate of 121%, higher even than that of anandamide. Metabolic stability of α,α-dimethylanandamide (**7**) was found to be comparable to that of (*R*)-α-methanandamide. These data show that metabolism of anandamide analogues can be reduced significantly by the introduction of a methyl group at the headgroup and practically blocked in the (*R*)-α-methyl analogue. Moreover, this latter substitution is

Chart 1. Structures of Anandamide Analogues



also known to increase the CB1 receptor affinity by severalfold.[22]

Replacement of the ethanolamido hydroxyl group of anandamide with fluorine (**4**) increases the rate of hydrolysis by 27%. On the other hand, analogues devoid of a hydroxyl group, e.g., **10**−**13**, were found to be metabolically very stable and underwent little or no hydrolysis. Of these, **10**, **11,** and **13** have CB1 receptor affinities approximately equal to that of anandamide. It can thus be argued that the enzyme favors anandamide congeners with H-bonding and/or electron-withdrawing substituents such as hydroxyl and fluoro groups at the β-headgroup position. The enzyme also requires the presence of at least one NH amide nitrogen for activity, while tertiary amides such as **3** and **14** are not susceptible to enzymatic activity. Interestingly, such analogues also exhibit poor CB1 receptor affinity.[23]

Three phenolic analogues were tested as substrates for AAH. Of these, only the 4-hydroxyphenyl analogue AM404 (**17**) was found to be resistant to enzymatic hydrolysis. The metabolic stability of this analogue, which was recently shown[21] to be an inhibitor of anandamide transport, is probably due to the electron-donating properties of a *p*-OH phenyl substituent. This leads to reduced susceptibility of the carbonyl group to a nucleophilic attack at the enzyme catalytic site. Such a mesomeric effect is absent from the corresponding *meta* analogue **16** in which the hydroxyl group now acts as an electron-withdrawing substituent and enhances the electrophilic character of the carbonyl carbon leading to its increased reactivity with nucleophilic amino acid residues at the enzyme active site. Indeed, **16** was found to be a good substrate for AAH. According to the above argument, analogue **15** with an electron-donating *ortho* phenolic hydroxyl was expected to be resistant to enzymatic hydrolysis. However, **15** was shown to be an excellent substrate for AAH with a relative rate of hydrolysis almost equal to that of arachidonamide (**1**). This high reactivity of **15** can be attributed to anchimeric assistance of the *ortho* phenolic OH at the carbonyl group during enzymatic hydrolysis. This effect helps to overcome the mesomeric deactivating effect of the *o*-hydroxy group. It should be pointed out that these results are not in agreement with conclusions drawn by Edgemond et al.[29] based on CB1 receptor binding assays for *o*- and *p*-hydroxyphenyl analogues where

Table 2. Rates of AAH Hydrolysis and CB1 Receptor Binding Affinities of Anandamide Analogues

substrate no.	rate of hydrolysis (nmol/min/mg)	relative rate of hydrolysis (%)	CB1 receptor binding affinity K_i (nM)
1	2.85 ± 0.105	219 ± 8.1	9600 ± 100[a]
2	1.30 ± 0.025	100 ± 1.9	78 ± 2[b]
3	0 ± 0.025	0 ± 2.0	174 ± 8[c]
4	1.65 ± 0.026	127 ± 2.0	8.6 ± 1.1[d]
5	0.030 ± 0.016[g]	2.4 ± 1.2	20 ± 1.6[b]
6	0.299 ± 0.022	23 ± 1.7	173 ± 26[b]
7	0.056 ± 0.016	4.3 ± 1.2	151 ± 12
8	0.273 ± 0.023	21 ± 1.8	26 ± 5[b]
9	1.58 ± 0.035	121 ± 2.7	119 ± 5[b]
10	0.025 ± 0.025	2.0 ± 2.0	90.2
11	0 ± 0.029	0 ± 2.3	73.6
12	0.082 ± 0.021	6.3 ± 1.6	287.2
13	0.022 ± 0.015	1.8 ± 1.1	51.2
14	0 ± 0.012	0 + 0.9	4980 ± 380[e]
15	2.51 + 0.143	193 ± 11	1660 + 110[c]
16	1.98 ± 0.048	152 ± 3.7	1560 ± 120[c]
17	0.208 ± 0.035	16 ± 2.7	1760 ± 140[c]
18	0 ± 0.016	0 ± 1.2	>3000[c]
19	0.299 ± 0.033	2 ± 2.5	217 ± 3[c]
20	0.0094 ± 0.002	0.7 ± 0.2	72.2[f]
21	0.79 ± 0.034	61.1 ± 2.6	>10000[g]
22	0.47 ± 0.034	3.6 ± 1.9	>10000[g]
23	0.98 ± 0.055	75 ± 4.2	>10000[g]
24	0.27 ± 0.068	20.5 ± 5.2	>10000[g]
25	0.20 ± 0.016	15.0 ± 1.2	217[g]
26 (α-AG)	3.43 ± 0.159	264.2 ± 12.2	
27 (2-AG)	3.27 ± 0.072	251.3 ± 5.6	

[a] Data from Felder et al.[3] [b] Data from Abadji et al.[22] [c] Data from Khanolkar et al.[23] [d] Data from Adams et al.[10a] [e] Data from Adams et al.[10b] [f] Unpublished results. [g] Data from Lin et al.[24] [h] Data not obtained under V_{max} conditions.

PMSF was reported to have no effect on the receptor binding of either of these analogues. Our results also show that benzyl (**18**) and cyclohexyl (**19**) headgroup analogues are poor enzyme substrates.

In addition to the headgroup analogues, we also studied the hydrolysis of 2-methylanandamide analogues, other fatty acid amides, and arachidonylglycerols (Chart 1) using an HPLC-based assay developed in our laboratory for determining the concentrations of hydrolytic products resulting from the amino headgroup via *o*-phthaldialdehyde (OPA) precolumn derivatization.[26] Our results indicate that introduction of *gem*-dimethyl groups in the 2-position of arachidonic acid as in **20** imparts considerable metabolic stability. This compound also exhibits respectable CB1 receptor affinity approximately equal to that of anandamide. The present study also confirmed[13,15] that the ethanolamides of oleic, linoleic, and stearic acids are AAH substrates although not as good as the parent compound. Thus, compared to anandamide (rate = 100%), analogues **21**, **23**, and **25** were hydrolyzed to an extent of about 61, 75, and 15%, respectively. This can be attributed to conformational differences between the different fatty acid amides which may affect their respective abilities to interact with the enzyme active site. As with the arachidonic acid series, (*R*)-methanandamide analogues of oleic and linoleic acids **22** and **24** showed much slower turnover rates by the enzyme.

Both β-arachidonylglycerol (2-AG), a *meso* ester shown to be an endogenous CB2 ligand,[30] and α-arachidonylglycerol, its racemic isomer, underwent facile hydrolysis by the amidase in agreement with a recent report[31] according to which AAH hydrolyzes 2-AG 4 times faster than anandamide. The amidase used in this study was only partially purified; therefore, hydrolysis of these esters may not be exclusively due to AAH. However, this is an important result as it indicates that the amidase may also be involved in the inactivation of 2-AG.

Table 3. Relative Rates of Hydrolysis of Anandamide (2) by AAH in the Presence of Arachidonic Acid Congeners

compound	concn (μM)	relative rate of hydrolysis (%)
none	-	100
arachidonyl alcohol	30	102.1 ± 1.0
	100	85.2 ± 6.4
arachidonyl aldehyde	30	33.5 ± 2.9
	100	21.1 ± 1.0
arachidonic acid	30	58.8 ± 2.2
	100	40.9 ± 3.8
anandamine	30	98.1 ± 2.7
	100	43.8 ± 1.1

AAH Inhibition by Arachidonic Acid Congeners and Cannabimimetic Ligands. In an effort to identify the structural features required for enzyme inhibition, we measured the rate of AAH-induced anandamide hydrolysis in the presence of a carefully selected group of nonhydrolyzable compounds. Our study included arachidonic acid congeners as well as representative ligands from the different cannabimimetic classes. The results, as represented in Table 3, show that arachidonyl alcohol and anandamine exhibit no significant AAH inhibition at a 30 μM ligand concentration and about 15 and 56% inhibition, respectively, at 100 μM. Conversely, arachidonyl aldehyde was more effective exhibiting 66 and 79% enzyme inhibition at 30 and 100 μM concentrations, respectively. This inhibitory activity may be attributed to a covalent attachment of the aldehyde group at the enzyme active site through the formation of a tetrahedral intermediate. We also confirmed the inhibitory effect of arachidonic acid on anandamide hydrolysis by AAH as reported earlier.[26,28] Indeed, our data show a 30% inhibition when equimolar quantities (3 μM) of arachidonic acid and anandamide are used. Interestingly, this concentration is very similar to anandamide's K_m value for AAH.

The representative group of cannabimimetic ligands which were tested as potential AAH inhibitors included cannabinoid receptor agonists from the classical and nonclassical cannabinoids and aminoalkylindole classes (Chart 2). It also included SR141716A, a biarylpyrazole CB1 receptor antagonist. The data (Table 4) indicate that the classical cannabinoid, (−)-Δ^8-THC, exhibits a moderate ability to inhibit AAH. The results are congruent with a recent report[32] indicating that (−)-Δ^8-THC as well as cannabidiol and cannabinol inhibited anandamide hydrolysis by the enzyme. Our data also indicate that this inhibitory ability is reduced with cannabinoids carrying additional OH groups and longer side chains such as the classical (−)-11-OH-DMH-Δ^8-THC and the nonclassical cannabinoids CP55,940 and CP55,244. Furthermore, no enzyme inhibition was observed with a cannabinoid ligand in which the phenolic hydrogen was substituted with a methyl group as in OMe-Δ^8-THC. Of the remaining cannabimimetic structural prototypes, SR141716A exhibited AAH inhibitory properties while the cannabimimetic aminoalkylindole WIN55212-2 did not show significant inhibition.

Chart 2. Structures of Cannabimimetic Ligands Tested as Enzyme Inhibitors

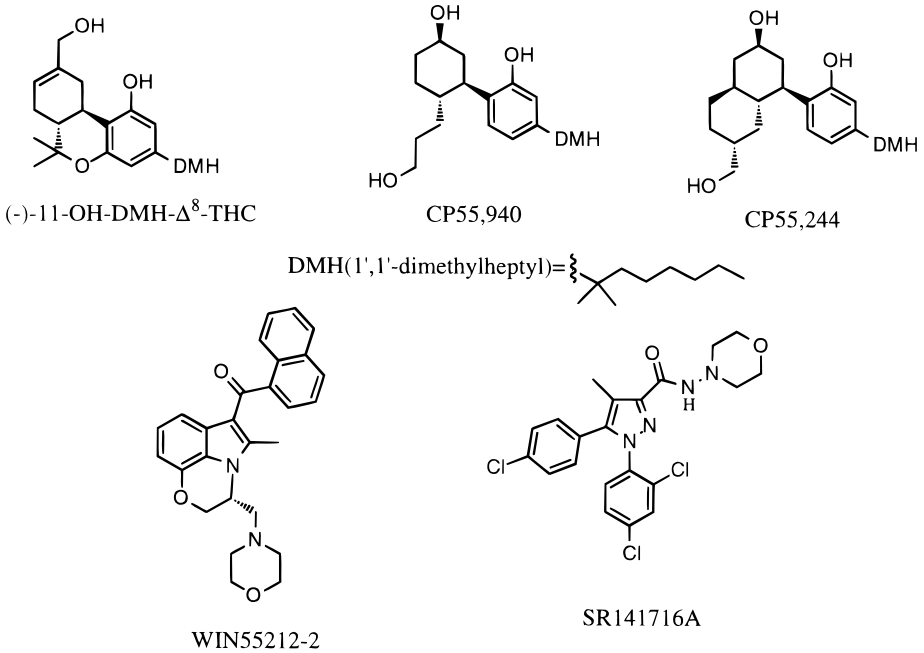


Table 4. Relative Rates of Hydrolysis of Anandamide (2) by AAH in the Presence of Cannabimimetic Ligands

compound	concn (μM)	relative rate of hydrolysis (%)
anandamide	30	100
(−)-Δ^8-THC	30	76.8 ± 7.2
(−)-11-OH− DMH-Δ^8-THC	30	87.7 ± 2.5
OMe-Δ^8-THC	30	106.4 ± 6.7
CP55,940	30	97.4 ± 4.1
CP55,244	30	87.9 ± 4.8
WIN55212−2	30	91.1 ± 2.9
SR141716A	30	70.0 ± 1.6

Comparison of Enzymatic Hydrolysis and Receptor Binding Affinity. The endogenous cannabinoid receptor ligand, anandamide, has a moderate affinity for the CB1 receptor (K_i 78 nM) but is also highly susceptible to amidohydrolase activity. Introduction of a bulky headgroup such as a *tert*-butyl group or elimination of the hydroxyl group leads to increased resistance to enzymatic hydrolysis. However, such modifications do not result in increased receptor affinity as is observed with **10**−**13**. On the other hand replacement of the OH group with a fluoro group (**4**) leads to a 10-fold increased receptor affinity but reduced metabolic stability. Methyl substitution in the headgroup produced analogues which were less susceptible to AAH hydrolysis and also maintained considerable affinity for the CB1 receptor. Among the four stereoisomeric methanandamides, (*R*)-α-methanandamide (**5**) and (*S*)-*β*-methanandamide (**8**) were metabolically most stable and also exhibited 4- and 3-fold higher affinities for CB1, respectively. In this regard, it is noteworthy that the enzyme−substrate stereoselectivity is opposite to that observed with the CB1 receptor so that the enantiomer with the higher receptor affinity is also found to exhibit lower susceptibility to AAH activity. This is true for all three enantiomeric pairs of analogues tested, e.g., **5**/**6**, **8**/**9**, and **11**/**12**. Thus, for example, (*R*)-α-methanandamide shows about 8 times higher affinity for CB1 than (*S*)-α-methanandamide but it is hydrolyzed by AAH 10 times slower than the latter.

Conclusions

The present study has served to explore the molecular features involved in the hydrolysis of anandamide by AAH and to identify within AAH ligands those structural features required for active site recognition and catalytic hydrolysis. Our results can be summarized as follows:

(1) Arachidonamide (**1**) and *N*-(*o*-hydroxyphenyl)-arachidonamide (**15**) are found to be the best enzyme substrates among the analogues tested with relative rates of hydrolysis approximately twice that of anandamide. Enzymatic hydrolysis of **15** is facile presumably due to anchimeric assistance by the phenolic hydroxyl group.

(2) Introduction of a methyl group in either the C2, C1′, or C2′ position of anandamide leads to metabolically more stable analogues with, in some cases, concomitant increase in the CB1 receptor affinity. The resistance to hydrolysis among these analogues can be attributed to increased steric hinderence around the carbonyl group. Thus, introduction of a single (*R*)-1′-methyl and (*S*)-2′-methyl or *gem*-dimethyl groups at the C2 or C1′position leads to metabolically stable analogues.

(3) AAH exhibits considerable stereoselectivity. Interestingly, this stereoselectivity with methylanandamide substrates is opposite to that observed for the CB1 receptor; i.e., the enantiomer with the lower enzyme turnover shows higher receptor affinity.

(4) The enzyme appears to favor anandamide substrates in which the nitrogen of the ethanolamido headgroup is either primary or secondary. Analogues containing a tertiary amide nitrogen are poor enzyme substrates and, interestingly, also have poor CB1 receptor affinity.

(5) Anandamide analogues with H-bonding and/or electronegative headgroup substituents, such as hydroxyl or fluoro, are more prone to hydrolysis by the amidase than their counterparts that are devoid of such groups.

(6) Although all C-18 and C-20 ethanolamide analogues tested were found to be AAH substrates, these fatty acid analogues possessing only one or two *cis* double bonds demonstrated lower susceptibility to hydrolysis than their *cis*-tetraene congener, while the total absence of double bonds diminished this activity very considerably. This observation may be attributed to the requirement of a hairpin conformation[33] for substrate recognition at the AAH active site. This low-energy conformation for anandamide involves a folding in the middle of the fatty acid chain so that its distal part is in close proximity with the headgroup. Arguably this hairpin conformation is best accommodated by the presence of four *cis* nonconjugated double bonds as in anandamide. Such a conformation is thermodynamically less favored in the *cis*-diene linolenyl and *cis*-ene oleyl analogues and much less so in the fully saturated palmitoyl analogue, thus decreasing the ability of these substrates to recognize the AAH catalytic site.

(7) Among the different classes of cannabimimetic ligands, the classical cannabinoids are capable of recognizing the AAH catalytic site. This ligand−enzyme interaction requires the presence of a free phenolic OH group in the cannabinoid structure but is reduced by the presence of additional hydroxyl groups and/or longer side chains. The biarylpyrazole CB1 antagonist SR141716A was also shown to interact with the enzyme, while the aminoalkylindole cannabimimetic ligand failed to do so.

A general conclusion to be drawn from the results described in this article is that arachidonic acid congeners and cannabimimetic ligands have specific structural requirements for interaction with the rat brain AAH enzyme. According to the above data, some of the substrate and ligand requirements are similar to those involved in the recognition and binding to the CB1 receptor, a functional protein with which the enzyme shares a common endogenous ligand, anandamide. However, our results also indicate significant differences between the requirements for ligand interaction with these two cannabimimetic sites. This is highlighted by the opposite requirements in absolute configuration for the methylanandamide ligands and by the inability of cannabimimetic aminoalkylindoles to interact with the AAH site. These differences can, thus, serve as the basis for the development of selective AAH ligands and/or inhibitors which produce cannabimimetic effects without directly interacting with cannabinoid receptors. Such inhibitors may prove to have unique pharmacological profiles of potential therapeutic value.

Experimental Section

Materials. Arachidonic acid, phenylmethanesulfonyl fluoride, and essentially fatty acid-free BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Arachidonyl alcohol and arachidonamide were purchased from Aldrich Chemical Co. and Biomol Reseach Labs, Inc. (Plymouth Meeting, PA), respectively. Anandamide and its analogues used in this study were synthesized as described previously.[22−24] Ethanolamides of oleic and linoleic acids were prepared from the corresponding fatty acid chloride and appropriate ethanolamine. Arachidonyl aldehyde was prepared from arachidonyl alcohol by oxidation with pyridinium dichromate. (−)-Δ^8-THC was obtained from the National Institute on Drug Abuse.

Preparation of Rat Brain Microsomal Fraction. Rat brain microsomes were prepared as previously described.[25] Briefly, adult Sprague−Dawley rat brains were homogenized with a Potter-Elvehjem tissue grinder in five volumes of ice-cold buffer containing 20 mM Tris·HCl and 1 mM EGTA (pH 7.4). The homogenate was centrifuged at 2000*g* for 10 min, and the supernatant was further centrifuged at 10700*g* for 30 min and 105000*g* for 60 min at 4 °C, sequentially. The pellet from the last centrifugation step (microsomal fraction) was resuspended in 40 mL of Tris buffer. Aliquots (1.5 mL) of the suspension were stored at −80 °C until use. Protein concentration was determined using a modification of Lowry's assay.[34]

HPLC Analysis. HPLC was performed using a Beckman System Gold system (Fullerton, CA) consisting of a 128 pump, a 166 UV detector, and a Rheodyne 7725i injector with a 20-μL loop. An IBM 466DX2/Si computer was interfaced to the system, and Beckman System Gold software was used for system control and data processing. Separations were carried out on a Beckman Ultrasphere ODS guard column (5 μm, 45 × 4.6-mm i.d.). Aqueous 8.5% phosphoric acid−acetonitrile (10:90 v/v) was used as the mobile phase at a flow rate of 1 mL/min. Peak areas at 204-nm wavelength were used for quantitation of arachidonic acid. A standard calibration curve was used to calculate the amount of arachidonic acid as described.[25]

For ethanolamides of fatty acids other than arachidonic acid, amidase activity was measured by *o*-phthaldialdehyde (OPA) precolumn derivatization of the ethanolamine moiety followed by UV detection at 230 nm as we have described elsewhere.[26]

Enzyme Assay. For the substrate hydrolysis assay, 50 nmol of the appropriate anandamide analogue was incubated with 150 μg of rat brain microsomal protein in a final volume of 500 μL (100 μM) of buffer containing 50 mM Tris·HCl, 1 mM EDTA, and 0.1% BSA (pH 7.4) at 37 °C for 15 min with shaking. The reaction was terminated by pipetting 200 μL of the suspension into a microcentrifuge tube containing 800 μL of acetonitrile. The tube was vortexed and then centrifuged at room temperature for 4 min to remove the proteins; 20 μL of the supernatant was then injected into the HPLC system for quantitation. An identical incubation was carried out in the absence of substrate to measure the amount of arachidonic acid existing naturally in the brain microsomal preparation. The rate of hydrolysis was calculated from the average of four experiments with results determined in duplicate. The amount of arachidonic acid (AA) in the blank was subtracted from that in the sample.

The enzyme inhibitory activity of anandamide-like compounds and other cannabimimetics was assessed by preincubating the analogue being tested (15 or 50 nmol) with 150 μg of rat brain microsomal protein for 20 min followed by addition of 15 nmol of anandamide. After further incubation for 20 min, the arachidonic acid produced was quantitated by HPLC analysis as described earlier.[25,26] An identical incubation was carried out in the absence of the test compound as a control. The same amount of protein was also incubated in the absence of both anandamide and the test compound as a blank. The assays were performed in quadruplicate. The percentage of relative rate of hydrolysis was calculated directly as

$$\%\ \text{rate} = \frac{A_s - A_b}{A_c - A_b} \cdot 100$$

where A_s refers to the peak area of arachidonic acid from the sample, A_c is the peak area of arachidonic acid from the control, and A_b is the peak area from the blank experiment.

To confirm that the arachidonic acid produced in the reaction is due to enzymatic and not chemical hydrolysis, the stability of each analogue toward alkaline hydrolysis was tested by incubating 20 mM analogue with 1 N aqueous sodium hydroxide at ambient temperature. No arachidonic acid was detected under these conditions for up to 1 h.

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