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New metabolically stable fatty acid amide ligands of cannabinoid receptors: Synthesis and receptor affinity studies

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Abstract—We investigated the structure–activity relationships for the interactions of fatty acid amide analogs of the endocannabinoid anandamide with human recombinant cannabinoid receptors. Thirty-five novel fatty acid amides were synthesized using five different types of acyl chains and 11 different aromatic amine 'heads.' Although none of the new compounds was a more potent ligand than anandamide, we identified three amine groups capable of improving the metabolic stability of arachidonoylamides and their CB_1/CB_2 selectivity ratio to over 20-fold, and several aromatic amines capable of improving the affinity of short chain or monosaturated fatty acids for cannabinoid CB_1 receptors. For the first time a tertiary amide of arachidonic acid was found to possess moderate affinity (K_i = 300 nM) for cannabinoid CB_1, but not CB_2, receptors.

Anandamide (*N*-arachidonoyl-ethanolamine) is one of the two best studied endogenous ligands of cannabinoid receptors. This compound binds both the CB_1 and CB_2 subtypes of cannabinoid receptors with sub-micromolar affinity (K_i ranging between 40 and 250 nM depending on the assay conditions),[1,2] although it behaves as a partial agonist at CB_1 receptors and is almost functionally inactive at CB_2 receptors.[3] The structure–activity relationships for the interactions of anandamide with both cannabinoid receptors have been widely investigated,[2] and have led, among other things, to the only selective CB_1 receptor ligands identified to date,[4] *N*-arachidonyl-2-chloroethylamide and *N*-arachidonyl-cyclopropylamide. However, these compounds lack metabolic stability.[4] With the present study we aimed at: (1) expanding our knowledge of the structure–activity relationships for anandamide interactions with human CB_1 and CB_2 receptors, and (2) developing CB_1-selective ligands with higher metabolic stability, by studying the affinity of 35 novel *N*-acyl-amides obtained from the condensation of 5 different fatty acids with 6 or, as in the case of arachidonic acid, 11 different aromatic amine 'heads.' We report for the first time the development of weak non-arachidonoyl-containing CB_1 ligands with some selectivity over CB_2, and of metabolically stable and moderate affinity, CB_1-selective, arachidonic acid amide ligands.

As shown in Scheme 1, the synthesis of the amides was accomplished by direct condensation between *trans*-2-undecenoic acid (tail 1), 10-undecenoic acid (tail 2), erucic acid (tail 3), oleic acid (tail 4), or arachidonic acid (tail 5), and several amines, that is, 4-methoxyaniline (head A), 4-methoxybenzylamine (head B), 4-methoxy-phenethylamine (head C), 3,5-di-methoxybenzylamine (head D), 2,4-di-methoxybenzylamine (head E), 4-(4-morpholino)aniline (head F), using 1-propylphosphonic acid cyclic anhydride (PPAA) as catalyst, with 65–75% yields[5] (see Supplementary materials). For the arachidonoyl tail, we utilized five more amines, that is, 2,5-dimethoxybenzylamine (UP61), 2,4-di-methoxy-benzylamine (UP63), 1-(3,4-dimethylphenyl) piperazine (UP67), 1-(4-chlorophenyl)piperazine (UP69), and 1-(4-fluoro-phenyl)piperazine (UP70). The affinity for cannabinoid receptors of the synthesized amides was assessed by binding assays,[6] and the results obtained are reported in Table 1.

Data obtained from the binding assays allow us to make the following considerations regarding the importance

Keywords: Cannabinoid; Anandamide; CB_1; CB_2; Receptor.
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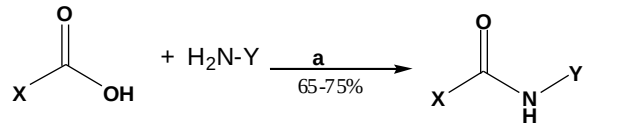

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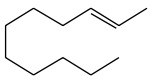
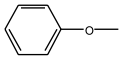
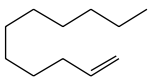
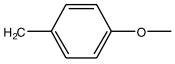
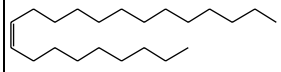
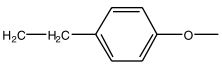
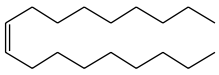
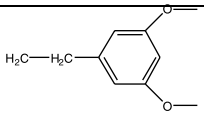
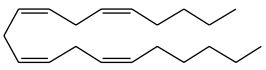
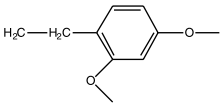
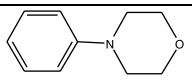
1		A	
2		B	
3		C	
4		D	
5		E	
	a= PPAA, Et_3N, CH_2Cl_2	F	

Scheme 1. General scheme for the synthesis of the new compounds.

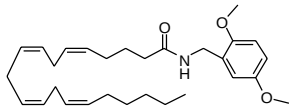
of the alkyl chain in fatty acid amide ligands of cannabinoid receptors:

(1) In the *trans*-2-undecenoic acid series, some notable affinity for CB_1 receptors was observed and the most active compounds were UP3-238 and UP4-14, which, however, exhibited only a ~2-fold selectivity over CB_2 receptors. UP1-13 showed a slightly higher affinity for CB_2. Interestingly, the affinity for both CB_1 and CB_2 receptors was lost if the double bond in the acyl chain was moved to Δ^{10}. This indicates that a certain rigidity near the amide group is necessary for these 'short' fatty acid amides to interact with the two receptors.

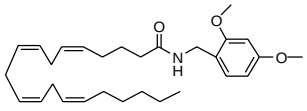
(2) When long-chain mono-unsaturated acyl chains were used, notable affinity for CB_1 receptors was observed mostly for oleic acid derivatives, which were almost all more potent than the corresponding *trans*-2-undecenoic acid derivatives. In particular, we observed the following rank of activity: UP28 > UP27-18 > UP30 > UP29 > UP26-7 > UP31-8. A higher than 20-fold selectivity over CB_2 receptors was also found, for example, with UP28 and UP27-18. Interestingly, the same order of activity was not observed for those erucic acid derivatives that exhibited some affinity for cannabinoid receptors.

(3) The derivatives of arachidonic acid yielded, as expected, the most potent ligands. They exhibited not only the highest affinity for CB_1 receptors, but also selectivity for this receptor type over CB_2 receptors. For this reason we tested five more arachidonate derivates (UP61, 63, 67, 69 and 70), which also showed quite good affinity and selectivity for CB_1 receptors.

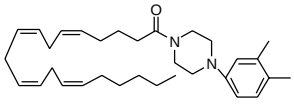
These findings are in full agreement with previous findings on the role of the alkyl chain in fatty acid amide interactions with the cannabinoid receptors.[2,8] However, we have also shown here that the affinity of alkyl chains (i.e., *trans*-2-undecenoic, oleic and erucic) that, when amidated with ethanolamine, were previously found to be inactive at these receptors (K_i > 5 μM, V. Di Marzo's unpublished observations) can be significantly improved when using certain aromatic amines.



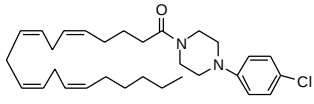
UP61 (CB_1 K_i = 0.5 μM; CB_2 K_i = 4.1 μM)



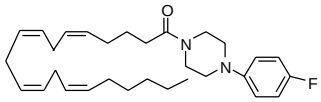
UP63 (CB_1 K_i = 0.2 μM; CB_2 K_i = 4.0 μM)



UP67 (CB_1 K_i = 0.4 μM; K_i = 4.2 μM)



UP69 (CB_1 K_i = 0.6 μM; CB_2 CB_2 K_i > 10 μM)



UP70 (CB_1 K_i = 0.3 μM; CB_2 K_i = 5.1 μM)

We also identified new structural requirements for the design of fatty acid amides not only with improved affinity with cannabinoid receptors, but also with higher selectivity over CB_2 than anandamide. In particular:

(1) The addition of a methylene group between the amide and the *para*-methoxy-phenyl group increases affinity for CB_1 receptors in the oleic acid series, decreases affinity for both CB_1 and CB_2 in the *trans*-2-undecenoic acid series and, unlike the *para*-hydroxy-phenyl 'head',[7] has no effect in the case of the arachidonoyl derivative.

(2) The addition of a second methylene group between the amide and the *para*-methoxy-phenyl group increases affinity for both CB_1 and CB_2 receptors of the *trans*-2-undecenoic acid derivative, and worsens it in the case of arachidonate derivative, with no effect on the oleic acid derivative.

(3) The addition of an *ortho*-methoxy group to the *para*-methoxy-phenylethyl derivatives decreases affinity for CB_1 receptors of the *trans*-2-undecenoic and oleic acid derivatives and also for CB_2 receptors in the case of *trans*-2-undecenoic acid; it improves affinity for both receptors in the case of the arachidonic acid derivative; and it improves affinity only for CB_1 receptors for the erucic acid derivative. Addition of a *ortho*-methoxy-group (UP63) to the *para*-methoxy-benzyl derivative of arachidonic acid (UP58) also slightly improves affinity for CB_1. Methylation of the *para*-phenyl group results

Table 1. Affinity constants for human recombinant cannabinoid CB_1 and CB_2 receptors as calculated in binding assays

Compounds	X	Y	Human CB_1 K_i (μM)	Human CB_2 K_i (μM)
UP1-13	1	A	4.9	2.5
UP2	1	B	>10	>10
UP3-238	1	C	2.4	5.1
UP4-14	1	D	2.8	5.2
UP5	1	E	>10	>10
UP7-229	1	F	>10	>10
UP10	2	A	>10	>10
UP11-5	2	B	>10	>10
UP12	2	C	>10	>10
UP13	2	D	>10	>10
UP14	2	E	>10	>10
UP16-28	2	F	>10	>10
UP19	3	A	>10	>10
UP20-2	3	B	>10	>10
UP21	3	C	>10	>10
UP22-3	3	D	5.4	>10
UP23	3	E	3.7	>10
UP24-11	3	F	6.4	>10
UP26-7	4	A	1.6	>10
UP27-18	4	B	0.5	>10
UP28	4	C	0.4	>10
UP29	4	D	1.3	>10
UP30	4	E	0.8	>10
UP31-8	4	F	>10	>10
UP57	5	A	0.5	4.0
UP58	5	B	0.6	4.5
UP59	5	C	2.4	>10
UP62	5	D	>10	>10
UP60	5	E	0.5	4.0
UP66	5	F	0.15	4.3
Anandamide			0.072	0.18
WIN55,212			0.021	0.002
CP55,940			0.013	0.0007
AM404			1.76[7]	—

K_i values of reference compounds tested here are also shown. Data are means of three measurements. SD values were never higher than 10%.

in a 3-fold higher affinity for CB_1 receptors (compare UP57 and AM404).

(4) The shift of the two methoxy groups from *ortho–para* to *meta–meta* in di-methoxyphenylethyl derivatives improves affinity for both CB_1 and CB_2 receptors with *trans*-2-undecenoic acid, decreases affinity for both receptors in the case of arachidonic acid, and decreases affinity for CB_1 receptors in the case of the oleoyl and erucoyl derivatives. In the case of the dimethoxybenzyl derivatives of arachidonic acid, also the shift of just one methoxy group from *para* to *meta* causes a slight decrease of affinity for CB_1 receptors (compare UP63 and UP61).

(5) The amidation of arachidonic acid, but not of the other fatty acids, with 2,5-dimethoxybenzylamine (UP61), 2,4-dimethoxybenzylamine (UP63), 1-(3,4-dimethylphenyl)piperazine (UP67), 1-(4-chloro-phenyl)piperazine (UP69), 1-(4-fluoro-phenyl)piperazine (UP70) and, particularly, 4-(4-morpholino)aniline (UP 66) was shown here for the first time to result in moderate to high affinity ligands for CB_1 but not CB_2 receptors.

Table 2. Affinity constants of some of the most potent ligands developed in this study as calculated in binding assays carried out in the absence or presence of PMSF (200 μM)

Compounds	Human CB_1		FAAH IC_{50} (μM)
	K_i (μM) without PMSF	K_i (μM) with PMSF	
UP26-7	1.6	1.5	>25
UP27-18	0.5	0.6	>25
UP28	0.4	0.3	>25
UP29	1.3	1.0	>25
UP30	0.8	0.6	>25
UP63	0.2	0.2	>25
UP66	0.15	0.15	>25
UP70	0.3	0.3	>25

Data are means of three measurements. SD values were ⩽10%.

(6) Again for the first time, tertiary amides of arachidonic acid were found to significantly bind to CB_1 but not CB_2 receptors; the affinity seems to improve in the presence of not too hindering *para* electronegative groups in the aromatic moiety.

The stability of these compounds to enzymatic hydrolysis was assessed in two ways: (1) by testing the effect of the thirty-five compounds on [^{14}C]anandamide hydrolysis by rat brain membranes, where a very abundant fatty acid amide hydrolase (FAAH) activity is present;[9] (2) by assaying some of the highest affinity ligands in binding assays carried out in the presence of the non-selective hydrolase inhibitor PMSF (200 μM). The fact that none of the new compounds was capable of inhibiting anandamide hydrolysis up to a 25 μM concentration (Table 2 and data not shown) indicates that they are not substrates for FAAH, and hence not hydrolysed by this enzyme. The fact that the affinity of some of the most potent compounds did not improve in the presence of PMSF (Table 2) indicates that, unlike the other previously identified CB_1-selective fatty acid amides,[4] they were not easily hydrolysed during the binding assay conditions.

In conclusion, we have identified here: (1) at least three novel (UP63, UP66 and UP70) moderate affinity (K_i = 150–300 nM) ligands for cannabinoid CB_1 receptors with 17- to 28-fold selectivity over CB_2 receptors and metabolic stability to enzymatic hydrolysis; additionally, one of these compounds, UP70, by containing a tertiary amine, might yield an ammonium salt under acidic conditions, which might result in more water-soluble CB_1 ligand than those currently available; and (2) new chemical features necessary to fatty acid amides to interact with cannabinoid CB_2 and, particularly, CB_1 receptors; in particular, three amine groups, when amidated to oleic acid, which is less expensive and less sensitive to oxidation than arachidonic acid, yield fatty acid amides (UP27-18, UP28 and UP30) that exhibit metabolic stability, sub-micromolar affinity for CB_1 receptors (K_i = 400–800 nM) and 10- to >20-fold selectivity over CB_2 receptors. To the best of our knowledge these three compounds are the first ligands of cannabinoid receptors to be developed from oleic acid and might therefore be suitable for in vivo use. The agonist or antagonist activity of these

new compounds will have to be tested next, using the appropriate functional assays.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.09.023.

References and notes

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5. To a solution of fatty acid in DCM, TEA (4.0 equiv) and PPAA (1.2 equiv) were added with stirring. After 15 min at room temperature, the appropriate amine (1.2 equiv) was added and the mixture was stirred at room temperature for 2 h. The organic phase was evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using hexane/AcOEt as eluants. Melting points were determined with a Gallenkamp hot stage apparatus. Infrared spectra were recorded with a Shimadzu 800 spectrophotometer. ^{1}H NMR spectra (see Supplementary materials) were measured with a Bruker 300 MHz spectrometer using $CDCl_3$ as solvent. Column chromatography was carried out using Merck silica gel 60 (230–400 mesh).
6. For CB_1 and CB_2 receptor binding assays, the new compounds were tested by using P_2 membranes from HEK cells transfected with either the human CB_1 or CB_2 receptor and [^{3}H]-(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethyl-heptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol ([^{3}H]CP-55,940) (K_d = 0.27 nM) as the high affinity ligand, as described by the manufacturer (Perkin-Elmer, Italia). In some experiments, membranes were pre-incubated for 10 min with PMSF (200 μM), which was also left during the incubation. Displacement curves were generated by incubating drugs with 0.27 nM [^{3}H]CP-55,940. In all cases, K_i values were calculated by applying the Cheng–Prusoff equation to the IC_{50} values (obtained by GraphPad) for the displacement of the bound radioligand by increasing concentrations of the test compounds.
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9. The effect of increasing concentrations of the synthetic compounds on the enzymatic hydrolysis of anandamide was studied as described previously[10] by using membranes prepared from rat brain. Membranes were incubated with increasing concentrations (1–5–10–25–50 μM) of the test compounds and [^{14}C]anandamide, 4 μM (10,000 cpm) in 50 mM Tris–HCl, pH 9, for 30 min at 37 °C. [^{14}C]Ethanolamine produced from [^{14}C]anandamide hydrolysis was used to calculate FAAH activity and was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3$/MeOH 1:1 (by vol.).
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