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Fatty Acid Amide Hydrolase (FAAH) Inhibitors: Discovery in *Lepidium meyenii* (Maca) Extracts.

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Abstract

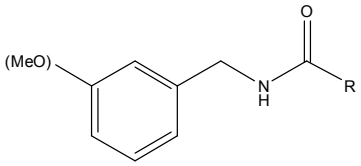
The pentane extract of the Peruvian plant *Lepidium meyenii* (Maca) has been shown to possess neuroprotective activity *in vitro* and *in vivo*. The involvement of the endocannabinoid system has been shown to be a possible mechanism of action. Some of the lipophilic constituents of Maca such as the macamides have been reported to possess significant pharmacological properties as shown in cell culture. However, their fatty acid amide hydrolase (FAAH) inhibitory activity was not determined. The aim of this study was to determine the FAAH inhibitory activity of the pentane extract from Maca, macamides and synthetic derivatives in an *in vitro* model using human recombinant FAAH. The pentane fraction from Maca and some of the macamides demonstrated a dose-dependent FAAH inhibitory activity. LC-MS/MS analysis demonstrated the presence of the macamides in the pentane extract. These results suggest a potential application of macamides from Maca or new synthesized derivatives (alkamides) as FAAH inhibitors to be used as analgesics, antidepressants, or antianxiety agents.

Keywords: Fatty acid amide hydrolase FAAH inhibitor(s), *Lepidium meyenii* (maca), macamides.

1. Introduction

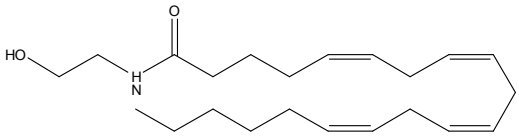
Lepidium meyenii (Maca) belongs to the Cruciferare (Brassicaceae) family. It is cultivated in the central Andes of Peru [19]. Maca has been used as an important medicinal crop in Peru for thousands of years [23]. The medicinal properties attributed to Maca include an aphrodisiac effect, regulation of hormonal levels, regulation of metabolism, immunostimulation, improvement of memory and anti-depressant activity among others [1, 5, 11, 19]. However, the biological mechanism of action is unclear.

The components of the dry Maca hypocotyls include several secondary metabolites such as alkaloids, glucosinolates and macamides [7, 10, 17, 24]. Macamides are claimed to be responsible for many of the pharmacological effects of Maca[24]. Macamides have a general chemical structure formed by a phenylamine linked to a fatty acid by an amide bond. The fatty acid can have 12 to 24 carbons and double bonds with cis or trans configurations. Some macamides also possess a methoxy group on the benzyl ring at the meta position. The general structure of macamides is shown in Figure 1.



Firgure 1. The general structure of macamides. The R group represents the fatty acid alkyl chain which can have 12 to 24 carbons and double bonds with cis or trans configurations.

The endocannabinoid system is a lipid signaling system which regulates neurotransmission, hormone release, gastrointestinal tract function and the immune and reproductive systems [9]. More specifically, endocannabinoids are involved in physiologic processes such as pain processing, inflammation, sleep, feeding, blood pressure and immunity[13]. The endocannabinoids act as agonists on the cannabinoid (CB) receptors which control or modulate the function of other neurotransmitters. They are metabolites of arachidonic acid [9, 16]. Some of the endocannabinoids include anandamide, 2-arachidonyglycerol, virodhamine, noladin ether, and oleamide. They have been found in the central nervous and peripheral tissues in several animal species including human beings [3, 6, 9, 21]. Thus, the endocannabinoid system is an important target in analgesic, antidepressant and antianxiety therapy. The structure of anandamide, the main endocannabinoid, is shown in Figure 2.



Firgure 2. Structure of anandamide

The action of endocannabinoids is terminated by reuptake or hydrolysis. Fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase (MAGL) are the main hydrolytic terminators of endocannabinoid signaling [9]. FAAH hydrolyzes lipid and ethanol amides including anandamide, oleamide, 2-arachidonoylglycerol, N-acylethanol amides and N-acyl amides [15, 20, 22, 23]. When FAAH is inhibited, the amount of endocannabinoids, mainly anandamide, increases in the cannabinergic synapses, manifesting an elevated activity of the endocannabinoid system. FAAH inhibitors have been screened regarding possible new treatments for anxiety, depression and chronic pain. CB1 receptors are abundant in the brain regions which regulate pain and behavior [6, 12, 16]. Inhibition of FAAH is a new strategy replacing the use of CB1 receptor agonists which produce many side and adverse reactions including psychotropic effects, addiction, dizziness and coordination-cognition impairment [2, 4, 15]. Animal studies with FAAH inhibitors did not show behavioral changes or avert toxicity as shown by CB receptor agonists [20]. The finding of new FAAH inhibitors has recently become crucial and promising for the treatment of neurological and neurodegenerative diseases, pain, epilepsy, anxiety, depression and others.

The aim of this research is focused on the structural similarities between macamides (Figure 1) and anandamide (Figure 2) and considering the medicinal claims and pharmacological properties of the Maca plant. It is hypothesized that the Maca plant, by way of its macamides, could be directly or indirectly acting on the endocannabinoid system. The investigation of the macamides' FAAH inhibitory potential is the first step to demonstrate their possible mechanism of action on the endocannabinoid system. In this research, several macamides present in the Maca plant were screened for their inhibitory capacity on the human FAAH.

2. Materials and Methods

FAAH inhibitory activity assay: The FAAH inhibitor screening assay kit (Cayman) is a fluorescent-based method where the product of the hydrolysis of the substrate (AMC-arachidonyl amide) by FAAH is the fluorescent substance, 7-amino-4-methyl coumarin (AMC). FAAH inhibitors prevent hydrolysis, thus decreasing fluorescence in the final solution. Maca pentane extract, macamides and the control FAAH inhibitor *N*-benzyl-4-(quinolin-3-

ylmethyl) piperidine-1-carboxamide (PF-750) were dissolved in DMSO to prepare a number of test solutions. In a 96-black well plate human recombinant FAAH and test solutions were mixed into the wells. After a 20 minute pre-incubation period at room temperature the substrate was added into each well. The plate was incubated at 37°C for 30 minutes. Fluorescence was then measured with the Synergy TM HT Multi-Detection Microplate Reader with an excitation wavelength of 340-360 nm and an emission wavelength of 450-465 nm. Fluorescence data were used to calculate the % FAAH inhibitory activity of test compounds [21].

Maca pentane extract: Dried hypocotyls of *Lepidium meyenii*, obtained from Arequipa, Peru, were botanically identified at the faculty of Pharmacy and Biochemistry of the Catholic University of Arequipa, and were then chemically characterized at the Massachusetts College of Pharmacy and Health Sciences (MCPHS) and the University of Mississippi. The dried hypocotyls (750 g) were ground into a powder and extracted with 3 L of methanol (HPLC grade, Pharmco-AAPER, Brookfield, CT) for 48 hours. The methanol extract was then filtered, concentrated in a rotary evaporator to 1 L and mixed with an equal volume of distilled water, followed by a continuous liquid-liquid re-extraction with 98% *n*-pentane (Sigma-Aldrich), for 24 hours. After this the *n*-pentane fraction was dried in a rotary evaporator and the residue was refrigerated.

Pure natural macamides and derivatives: All tested compounds were synthesized by Hui Wu and Charles Kelley (Organic Chemistry Laboratory at MCPHS). All macamides and derivatives were stored at room temperature and dissolved in DMSO. All tested compounds are listed in Table 1.

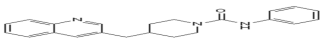
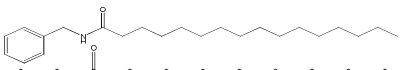
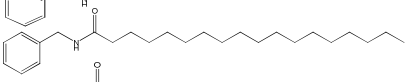
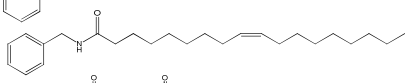
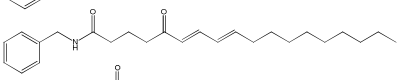
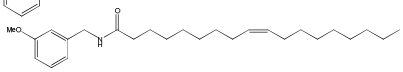
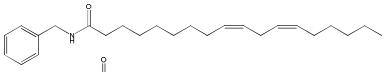
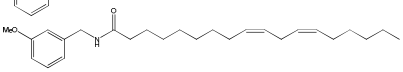
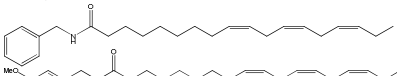
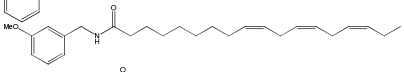
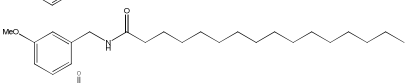
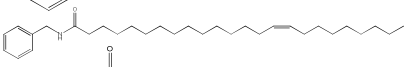
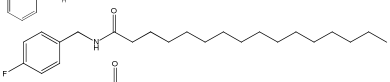
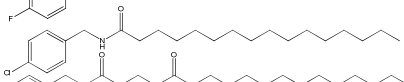
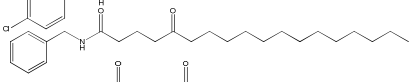
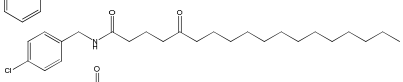
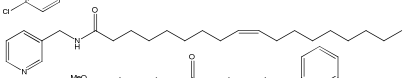
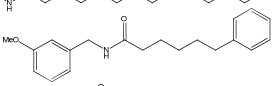
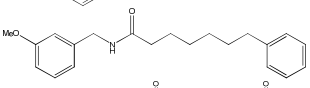
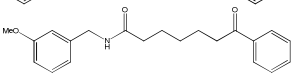
LC-MS/MS analysis: The pentane extract and macamide standard solutions were analyzed with the API 3000 LC-MS/MS system (AB/SCIEX). The compounds were separated on a Kinetex RP-C_{18} column (150 × 3 mm, 2.6 µm particles, 100 Å pores, Phenomenex). The mobile phase consisted of HPLC grade water (A) and acetonitrile (B), both containing 0.1% formic acid (Sigma-Aldrich) with a linear gradient elution from 45% A/55% B to 0% A/100% B over a 14 minute period. The flow rate was 125 µL/min and 10 µL aliquots of samples and standards were injected. In the triple quadrupole MS, multiple reaction monitoring experiments were used to examine unique transitions for each of the ten compounds. Each molecular ion was filtered in Q1 and the molecular ions were fragmented with specific collision energies for each molecular ion in Q2. Unique product ions for each compound were filtered in Q3 and the amplified signals of the product ions were detected and the data were recorded and processed with Analyst 1.4.2 software (AB/SCIEX). Peaks were assigned by comparing the retention times and MRM transitions with those of the standard compounds. Mixtures of all ten standards were analyzed at different concentrations so that any ion suppression phenomena caused by the coelution of similar compounds would be consistent in both mixtures of standards and in the pentane extract.

Statistical analysis of results: The averages of the percent inhibition were calculated for each test compound concentration. Preliminary compound screening with two concentrations (500 µM and 10 µM) was performed to identify inhibitory FAAH activity. Concentration-dependent inhibitory effect was determined in active compounds at concentrations from 0.3 µM to 500 µM to determine the inhibitory concentration 50% (IC_{50}). The statistical analysis included the sigmoidal correlation, t-test, one-way analysis of variance (ANOVA), and *post hoc* Dunn or Bonferroni tests.

3. Results

A preliminary screening allowed the selection of the significant inhibitory compounds. The FAAH inhibitory concentration-response curves of selected compounds were analyzed in order to calculate the IC_{50} and the maximal inhibitory efficacy. Figure 3 illustrates the concentration-response curve of the pentane extract with an IC_{50} value of 7.5 µg/ml and a calculated maximal inhibitory effect of 95.8%. Table 2 illustrates the IC_{50} and inhibitory efficacy of all tested compounds.

Table 1. Chemical information on tested compounds

CODE	NAME	MOLECULAR WEIGHT (g/mol)	STRUCTURES
PF 750	N-Benzyl-4-(3-quinolinylmethyl)-1-piperidinecarboxamide	345.44	
11	N-benzylhexadecanamide	345	
12	N-benzyloctadecanamide	373	
13	N-benzyl-9Z-octadecenamide	371	
56	N-benzyl-5-oxo-6E,8E-octadecadienamide	383	
70	N-(3-methoxybenzyl) -9Z-octadecanamide	401	
75	N-benzy-(9Z,12Z)-octadecadienamide	369	
76	N-(3-methozybenzyl) -(9Z,12Z)-octadecadienamide	399	
77	N-benzy-(9Z,12Z,15Z)-octadecatrienamide	367	
78	N-(3-methozybenzyl) -(9Z,12Z,15Z)-octadecatrienamide	397	
79	N-(3-methoxybenzyl)-hexadecanamide	375	
80	N-benzyl-(15Z)- tetraisocenamide	455	
14	N-(4-florobenzyl)-hexadecanamide	363	
15	N-(4-chlorobenzyl)-hexadecanamide	380	
32	N-benzyl-5-oxoctadecanamide	422	
33	N-(4-chlorobenzyl)-5-oxoctadecanamide	422	
71	N-pyridine-9Z-octadecenamide	372	
72	N-(3-methoxybenzyl)-6-phenylhexanamide	311	
73	N-(3-methoxybenzyl)-6-phenylheptanamide	325	
74	N-(3-methoxybenzyl)-7-oxo-7-phenylheptanamide	339	

The presence of the studied natural compounds in the Maca pentane extract was confirmed by LC-MS/MS analysis. All of the studied natural macamides were present in the pentane extract at the concentrations illustrated in table 3. Other constituents represent 93.74% of the extract which could be different macamides or other primary and secondary metabolites.

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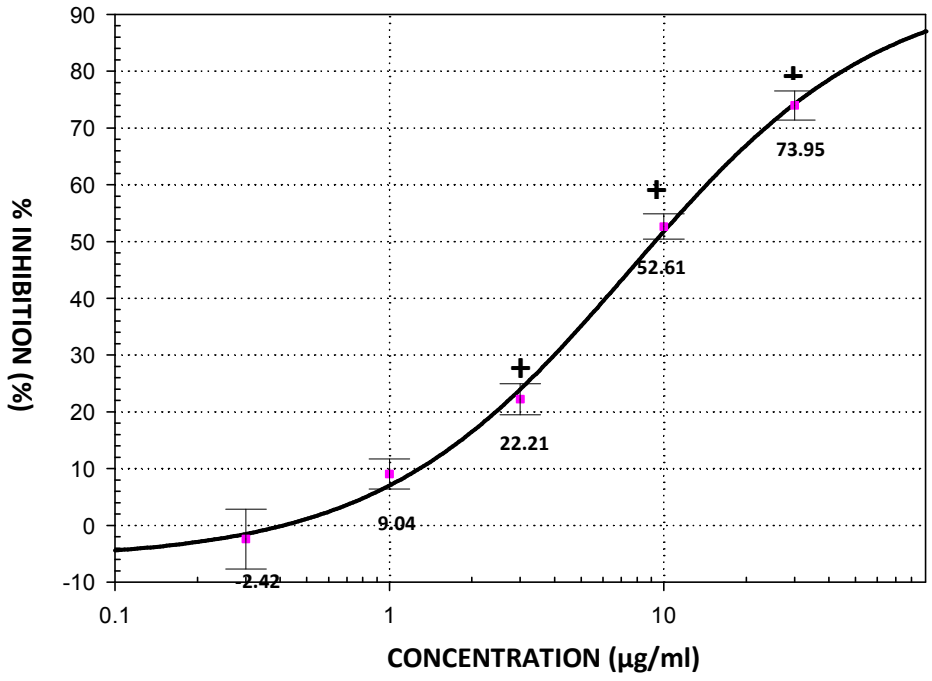

Figure 3 Concentration-response curve for the inhibition of human FAAH by Maca pentane extract in the range from 0.3 to 30 µg/mL (n=5). IC_{50} = 7.5±3.1 µg/mL. Statistical ($P<0.05$) sigmoidal correlation (r^2=0.9978). Values represent the mean ± S.E.M.

Table 2. FAAH IC_{50} and maximal inhibitory activity of selected inhibitory compounds

Compounds	IC_{50}	Maximum FAAH inhibitory activity (%)
PF 750	1.40 ± 0.55 µM	100.00
Pentane Extract	7.51 ± 3.12 µg/ ml	95.77 ± 20.00
13	14.18 ± 3.51 µM	76.13 ± 3.55
70	7.84 ± 1.64 µM	76.39 ± 1.42
75	6.91 ± 0.82 µM	83.20 ± 1.74
76	7.24 ± 0.91 µM	87.50 ± 2.00
77	26.94 ± 1.51 µM	79.72 ± 1.51
78	11.76 ± 1.56 µM	82.78 ± 2.26
71	6.74 ± 0.95 µM	90.10 ± 4.91

Table 3. Studied macamides and their percentages in the pentane extract

Compounds	Percentage in the Pentane Extract
Other Compounds	93.76%
Compound 11	1.75%
Compound 12	0.16%
Compound 13	0.34%
Compound 70	0.14%
Compound 75	1.26%
Compound 76	0.10%
Compound 77	2.13%
Compound 78	0.26%
Compound 79	0.08%
Compound 80	0.02%

4. Discussion

Traditional and popular claims as well as research studies suggest that the Maca plant may have many health promoting properties such as aphrodisiac effect, regulation of hormonal levels, regulation of metabolism, immunostimulation, improvement of memory and anti-depressant activity [1, 5, 11, 19]. According to the present results, the macamides have demonstrated FAAH inhibitory activity. The chemical structure of macamides partially resembling the structure of endocannabinoids may explain this ability to bind to FAAH and inhibit its activity. The benzyl group in the macamide structure may produce some steric hindrance, however, this does not determine whether a substance is a FAAH inhibitor or not [23]. Regarding the structure of anandamide, the main substrate of FAAH, the hydroxyl group is reported not to play an important role in interacting with the FAAH enzyme [23]. The key determining affinity for FAAH is actually the electrophilic carbonyl in the amide group [3].

An important consideration from the results is the methoxy group at the meta position on the benzyl group, which is found in certain natural macamides. This group seems to improve the compound FAAH inhibitory activity. Compound 13 and 70 contain oleic acid and they are only different by the meta-methoxy group on compound 70 (Table 1). This difference improved the potency of the compound 70 about two-fold without a change in the efficacy. A similar difference can be seen between compounds 75 and 76 which contain linoleic acid. Compound 76 holds the meta-methoxy group on the benzyl group (Table 1). The methoxy group in this case did not improve the potency but the efficacy of the compound is increased. Compounds 77 and 78 contain linolenic acid and the meta-methoxy group is held by compound 78 (Table 1). In this case Compound 78 showed improved potency and efficacy compared to compound 77. The possible explanation for this improvement in the FAAH inhibitory activity by the methoxy group is that this group has the potential to produce a hydrogen bond in the active site of the FAAH enzyme. Based on the X-ray studies of some of the FAAH inhibitors whose structures are similar to the macamides, the oxygen of the methoxy group could increase the affinity of the compounds to the FAAH enzyme and its intermediation [8, 14, 15]. More studies such as X-ray crystallography need to be conducted to confirm this interaction. Compound 71, a synthetic derivative, showed a higher potency and efficacy than the other natural compounds. Compound 71 contains a pyridine ring instead the benzyl group (Table 1). The same explanation applies for why the pyridine ring could improve the FAAH inhibitory activity. Nitrogen is a stronger hydrogen bond acceptor than oxygen, resulting in higher potency and efficacy than all natural tested macamides.

The fatty acid of the natural macamides and derivatives also play a significant role to determine the FAAH inhibitory activity of the compounds. A significant observation is that cis-double bonds in the fatty acid tail produce better inhibition than saturated or trans-double bonds. Compound 12 with stearic acid did not have significant FAAH inhibitory activity as shown by compound 13 with oleic acid. In the same fashion, compound 75 with two double bonds (linoleic acid) and compound 77 with three double bonds (linolenic acid) showed an increased FAAH inhibitory activity than Compound 13. Compounds with cis-double bond(s) are potentially more potent FAAH inhibitors because these compounds resemble the endogenous anandamide with four cis-double bonds (Figure 2). It

seems that cis-double bonds allow the fatty acid to bend and fit into the enzyme pocket. Other studies also confirmed this observation [18].

Compounds 32, 33 and 56 are unique natural macamides found in the Maca plant. They contain a fatty acid showing a carbonyl group in position 5. These compounds did not show significant FAAH inhibitory activity. The carbonyl group at the position 5 of the fatty acid could be the main reason affecting the interaction with the FAAH. The enzyme active site is surrounded by many hydrophobic residues which attract the fatty acid moiety [3, 15]. The carbonyl group with the polar nature of the oxygen will reduce this capacity of interaction. Compound 56 also contains two trans-double bonds which could not provide the required conformation to produce the affinity for the FAAH enzyme site.

The LC-MS/MS chromatography analysis showed the presence of the studied macamides in the pentane extract. The separation of all of the studied macamides was difficult to achieve because of their chemical similarity. The MS/MS detection system, using the specific transitions for every compound, made it possible to detect and quantify all ten natural macamides. Apparently the main FAAH inhibitory activity of the pentane extract is produced by Compounds 75 and 77 which demonstrated the highest concentration in the pentane extract and significant FAAH inhibitory activity as mentioned previously. The pentane extract from Maca showed the highest efficacy of all the studied compounds. This can be explained by the synergistic effect which is currently observed in natural products and extracts.

In conclusion, the plant *Lepidium meyenii* (Maca) and its constituents (macamides) might produce its pharmacological effects by acting through the endocannabinoid system, specifically by inhibiting the FAAH enzyme. For the first time, there is experimental evidence about the mechanism of action of the Maca plant and its macamides. New synthetic FAAH inhibitors with potential in analgesic, anti-anxiety and antidepressant applications can be synthesized based on the structure of the macamides. Further *in vivo* tesing will be performed to confirm these properties.

5. Acknowledgement

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