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Inhibition of Fatty Acid Amide Hydrolase (FAAH) by Macamides

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Abstract

The pentane extract of the Peruvian plant, *Lepidium meyenii* (Maca), has been demonstrated to possess neuroprotective activity in previous in vitro and in vivo studies (Pino-Figueroa et al. in Ann N Y Acad Sci 1199:77–85, 2010; Pino-Figueroa et al. in Am J Neuroprot Neuroregener 3:87–92, 2011). This extract contains a number of macamides that may act on the endocannabinoid system (Pino-Figueroa et al. in Ann N Y Acad Sci 1199:77–85, 2010; Pino-Figueroa et al., 2011; Dini et al. in Food Chem 49:347–349, 1994). The aim of this study was to characterize the inhibitory activity of four of these maccamides (*N*-benzylstearamide, *N*-benzyloleamide, *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, and *N*-benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide) on fatty acid amide hydrolase (FAAH), an enzyme that is responsible for endocannabinoid degradation in the nervous system (Kumar et al. in Anaesthesia 56:1059–1068, 2001). The four compounds were tested at concentrations between 1 and 100 μM, utilizing an FAAH inhibitor screening assay. The results demonstrated concentration-dependent FAAH inhibitory activities for the four macamides tested. *N*-Benzyloctadeca-9*Z*,12*Z*-dienamide demonstrated the highest FAAH inhibitory activity whereas *N*-benzylstearamide had the lowest inhibitory activity. In addition, *N*-benzylstearamide, *N*-benzyloleamide, and *N*-benzyloctadeca-9*Z*,12*Z*-dienamide demonstrated time-dependent inhibition when tested after a pre-incubation period, indicating that the mechanism of inhibition for these compounds most likely is irreversible. Of interest, unsaturation in the fatty acid moiety resulted in greater FAAH inhibitory activity. LC/MS/MS analysis demonstrated that FAAH was able to hydrolyze *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, suggesting that *N*-benzyloctadeca-9*Z*,12*Z*-dienamide is also a slow substrate for FAAH. These results provide useful information about the mechanism of action of *Lepidium meyenii* and may help with the development of new compounds with FAAH inhibitory or modulatory activity.

Keywords *Lepidium meyenii* (maca) · FAAH · *N*-benzylstearamide · *N*-benzyloleamide · *N*-benzyloctadeca-9*Z*,12*Z*-dienamide · *N*-benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide

Introduction

Investigations of cannabinoids from marijuana resulted in the discovery of the endocannabinoid system [1–4]. This system contains the cannabinoid receptors CB1 and CB2, as well as their endogenous ligands [3, 5]. Both receptors belong to the G protein-coupled receptor (GPCR) family, and both are coupled to G_i proteins. The activation of cannabinoid receptors by cannabinoid agonists inhibits adenylyl cyclase, which leads to decreased intracellular production of cyclic AMP and modulation of ion channel activities [1, 5, 6].

The discovery of cannabinoid receptors has stimulated the study of endogenous ligands or endocannabinoids such as anandamide, arachidonyl ethanolamine (AEA), 2-arachidonoylglycerol (2-AG), and palmitoylethanolamide (PEA) [1, 3, 4]. These endocannabinoid compounds are synthesized and released on demand from postsynaptic neurons in response to increased intracellular calcium [4, 5]. The effects of endocannabinoids are terminated by hydrolase enzymes [1, 7, 8]. AEA is degraded to arachidonic acid and ethanolamine by fatty acid amide hydrolase (FAAH) [9, 10], whereas 2-AG is hydrolyzed to arachidonic acid (AA) and glycerol by monoacylglycerol lipase (MAGL) [4, 11].

Several studies have been conducted to demonstrate that the endocannabinoid system is involved in neuroprotection [8, 9, 11–14], anti-inflammatory activity [1, 15, 16], and analgesic activity [5, 17, 18]. A recent study demonstrated that selective FAAH and MAGL inhibitors had anti-nociceptive effects in acute, inflammatory and neuropathic pain models

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[19–21]. An additional in vivo study of the effect of exogenous AEA on acutely damaged brains demonstrated that exogenous administration of anandamide reduced neuron damage and protected the brain from acute injury by promoting an enhanced pro-inflammatory glial response in the brain [22].

Since the endocannabinoids might have important effects useful in the treatment of several pathologies, the pharmacological blockade of the FAAH enzyme has emerged as a potentially attractive strategy to increase endocannabinoid-mediated activities. FAAH inhibition might avoid the undesirable side effects of direct CB1 agonists, such as weight gain and the impairment of cognition and motor control [2, 23]. This idea has led to the discovery of several FAAH inhibitors which are currently being investigated.

A pentane extract of the Peruvian plant, *Lepidium meyenii* (Maca), has been shown to possess neuroprotective activity in previous in vitro and in vivo studies [24, 25]. This plant, traditionally used as a food and as a folk medicine contains numerous macamides that might act on the endocannabinoid system [26–30]. To investigate this, eleven different macamides were synthesized and preliminarily screened for their FAAH inhibitory activities [31, 32].

The purpose of this study was to further characterize the FAAH inhibitory activity of the four most promising macamides: *N*-benzylstearamide, *N*-benzyloleamide, *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, and *N*-benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide, to determine the importance of unsaturation on the strength of FAAH inhibition (Fig. 1).

Materials

The macamides *N*-benzylstearamide, *N*-benzyloleamide, *N*-benzyloctadeca-9*Z*,12*Z*-dienamide and *N*-benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide and a standard reversible FAAH inhibitor, 1-oxo1-[5-(2-pyridyl)-2-yl]-7-phenylheptane (OL-135) were synthesized in the organic chemistry laboratory at MCPHS University [31]. *N*-Phenyl-4-(quinolin-2-ylmethyl) piperidine-1-carboxamide (PF-750), a standard irreversible FAAH inhibitor, was purchased from Tocris Bioscience (Minneapolis, MN). Benzylamine was from Sigma-Aldrich (St. Louis, MO). Human recombinant FAAH and 7-amino-4-methylcoumarin-arachidonamide (AMC-AA), a substrate for FAAH, were obtained from Cayman Chemical (Ann Arbor, MI).

Methods

FAAH Activity Assay

A fluorescence-based assay was used to assess FAAH activity in vitro. This assay utilizes the measurement of fluorescence produced by the hydrolysis of a substrate, 7-amino-4-methylcoumarin-arachidonamide (AMC-AA) by the FAAH enzyme, to the 7-amino-4-methylcoumarin (AMC) product. FAAH inhibitors decrease the fluorescence of the final solution by preventing hydrolysis. FAAH inhibitory activity was assessed as follows:

Various concentrations of the test compounds and standard positive controls were dissolved in DMSO. A buffer of 125 mM Tris-HCl containing 1 mM EDTA at pH 9.0 was used in this assay. Solutions of 78.75 mU/μL FAAH and 66.7 μM AMC substrate were prepared. Blank wells were loaded with 120 μL assay buffer and 20 μL DMSO. One hundred percent (100%) activity wells were loaded with 90 μL assay buffer, 20 μL DMSO, and 30 μL of 78.75 mU/μL FAAH solution (final FAAH concentration 11.8 mU/μL). Test wells were loaded with 90 μL assay buffer, 20 μL inhibitor solution, and 30 μL of 78.75 mU/μL FAAH solution (final FAAH concentration 11.8 mU/μL). The reaction was initiated by adding 60 μL of 66.6 μM AMC substrate solution (final concentration 20 μM in all wells). Fluorescence was measured 60 min after substrate addition at room temperature with a BioTek Synergy HT microplate reader (BioTek, Winooski, VT), with excitation and emission wavelengths of 360 and 460 nm, respectively.

Pre-Incubation Assay

To study the effect of time on enzyme inhibiton, the previous FAAH inhibitory activity assay was repeated using different pre-incubation times (60 min and 120 min) before substrate addition at 37 °C. Inhibition was determined 60 min after substrate addition.

LC/MS/MS Analysis

To determine whether the most active macamide, *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, acted as a substrate or simply as an inhibitor of FAAH we used LC/MS/MS. In a glass vial 90 μL of assay buffer, 20 μL of 1000 μM *N*-benzyloctadeca-9*Z*,12*Z*-dienamide (final concentration of 143 μM), and 30 μL of 78.75 mU/μL FAAH solution (final concentration 11.8 mU/μL) were mixed. In another glass vial, 120 μL of assay buffer was mixed with 20 μL of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide solution to produce 140 μL total volume, and this vial was treated as a control. Both vials were incubated for 60 min at 37 °C, after which the FAAH enzyme was inactivated by adding 280 μL acetonitrile. Samples from both incubated mixtures were analyzed to determine concentrations of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide and benzylamine resulting from the possible degradation of this compound by FAAH. An LC/MS/MS system was utilized with a Kinetex C18 column (100 × 3.00 mm,

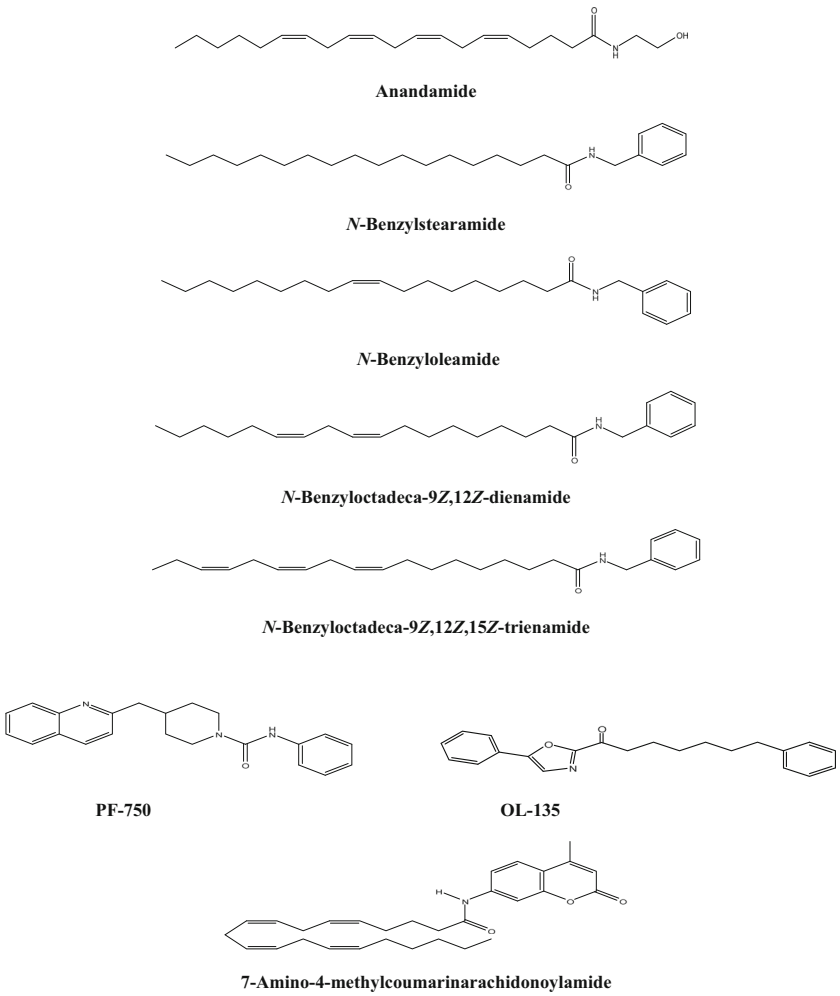

Fig. 1 Chemical structures of macamides and the standard FAAH inhibitors used in the study

2.6 μm particles, 100 Å pores, Phenomenex, Torrance, CA), in an Agilent 1100 HPLC system (Agilent, Santa Clara, CA) coupled to an AB SCIEX API 3000 triple-quadrupole mass spectrometer (AB SCIEX, Framingham, MA). The LC/MS/MS system was controlled by Analyst 1.5 software (AB SCIEX), and data were analyzed. Mobile phase A consisted of nanopure water (18-MΩ resistance, NANOpure, Thermo Fisher, Waltham, MA), filtered through a Sep-Pak C18 cartridge (Waters, Milford, MA), then filtered through a 0.2-μm filter (Millipore, Billerica, MA), with 0.1% formic acid added (Sigma-Aldrich, St. Louis, MO). Mobile phase B was HPLC-grade acetonitrile (EMD, Gibbstown, NJ) with 0.1% formic acid. The flow rate was 0.250 mL/min. For analysis of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, a gradient elution was used as follows: 90% B to 100% B at 4 min, held at 100% B until 10 min, to 90% B at 10.1 min and equilibrated at 90% B until 17 min, followed by the next injection. *N*-Benzyloctadeca-9*Z*,12*Z*-dienamide had a retention time of 5.4 min and (*Z*)-1-(octadec-9-en-1-yl)-3-benzylurea, utilized as an internal standard, eluted in 7.3 min. For analysis of benzylamine, an isocratic elution was used with 10% B, resulting in a retention time of 1.8 min for this compound. The API 3000 was operated in positive ion mode with turbo ion spray as the ion source operating with a voltage of + 3000 V and the temperature of nitrogen gas in the ion source at 325 °C. The transitions were determined and monitored in multireaction monitoring (MRM) mode [107.9/91.1 for benzylamine, 370.2/90.9 for *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, and 401.5/91.2 for (*Z*)-1-(octadec-9-en-1-yl)-3-benzylurea], and mass analyzer potentials were optimized manually with standards of each compound in methanol at 10 μg/mL. Calibrations were made by adding standards to the sample matrix without enzyme and diluting in sample matrix to appropriate concentrations.

Data Analysis

All experiments were performed in triplicate and repeated three times for each concentration of test compound. Data are presented as mean ± SEM. Data were subjected to statistical analysis via one-way ANOVA with minimum significance set at $p < 0.05$. If the p-value in the ANOVA test was significant, Tukey's test was then performed. Dose-response non-linear regression fit was used to calculate IC_{50} values. All of the analyses were performed with SigmaPlot 11.0.

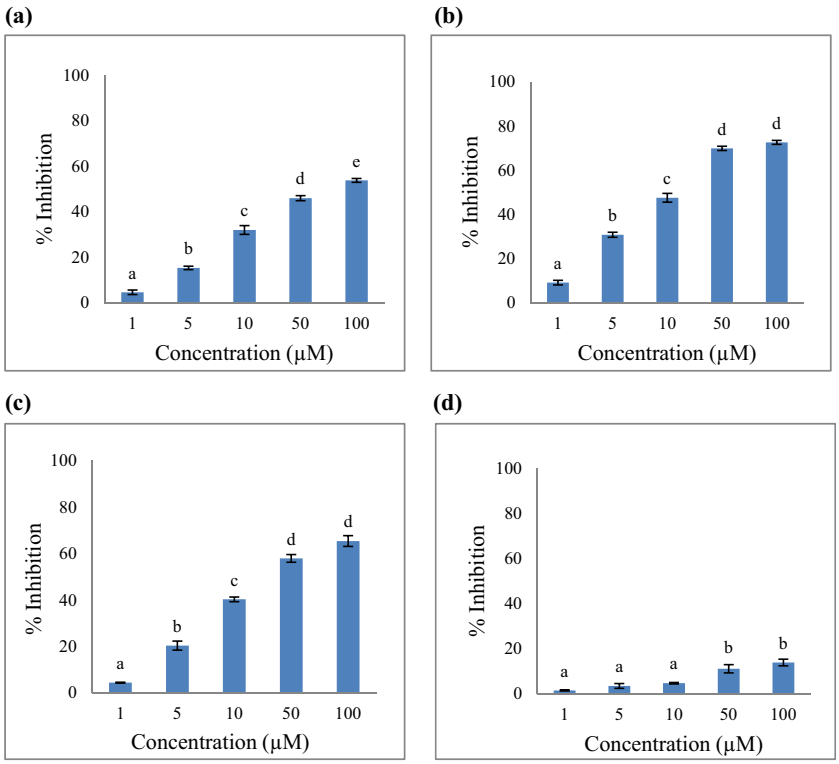

Fig. 2 Concentration-dependent inhibition of FAAH by (**a**) *N*-benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide, (**b**) *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, (**c**) *N*-benzyloleamide and (**d**) *N*-benzylstearamide without pre-incubation. FAAH activity was measured 60 min after initiation of the reaction. Values are expressed as mean ± SEM from three independent experiments. Bars with different superscript letters are significantly different ($p < 0.05$)

Results

Concentration-Dependent FAAH Inhibition

All four macamides tested, in the range of 1-100 μM, demonstrated concentration-dependent inhibitory activity on FAAH; however, the activities of the four compounds were significantly different from each other (Fig. 2). The stearic acid macamide was only slightly active in inhibiting FAAH (maximum 16% at the highest concentration tested). Figure 3 illustrates the inhibitory effects of 1 μM OL-135 and PF-750, standard reversible and irreversible FAAH inhibitors, respectively. Percent FAAH inhibition was determined without pre-incubation 60 min after the start of the reaction.

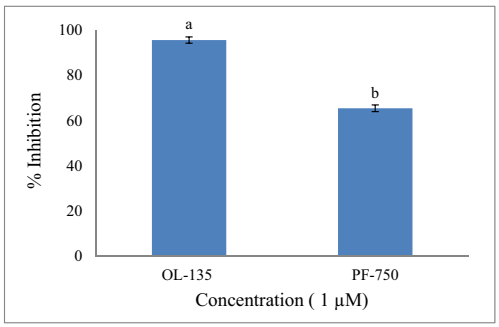

Fig. 3 FAAH inhibitory effect of OL-135 and PF-750 at 1 μM without pre-incubation. FAAH activity is measured 60 min after initiation of the reaction. Values are expressed as mean ± SEM from three independent experiments. Bars with different superscript letters are significantly different ($p < 0.001$)

Pre-Incubation Studies

The effects of pre-incubation times on the inhibiton of FAAH by *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, *N*-benzyloleamide, and *N*-benzylstearamide at concentrations from 1 to 100 μM are illustrated in Figs. 4, 5, and 6, respectively. In these figures, the three macamides demonstrated time-dependent inhibition of FAAH activity after 60 and 120 min pre-incubations. In Fig. 4c, percent inhibition of FAAH by *N*-benzyloctadeca-9*Z*,12*Z*-dienamide was significantly different between a 120 min pre-incubation and both a 60 min pre-incubation and no pre-incubation at concentrations of 10, 50, and 100 μM, whereas the inhibition by 5 μM of this compound was significantly different only between the 120 min pre-incubation and no pre-incubation. In addition, there was no significant difference in FAAH inhibition by this compound when it was pre-incubated at 1 μM. Time-dependent inhibition of FAAH by *N*-benzyloleamide is shown in Fig. 5c; the percent FAAH inhibition was significantly different among no pre-incubation, 60 and 120 min pre-incubations at concentrations of 1, 5, and 50 μM. At 10 μM and 100 μM there was a significant difference only between no pre-incubation and a 120 min pre-incubation. Figure 6c shows the significant difference between a 120 min pre-incubation of *N*-benzylstearamide and both no pre-incubation and a 60

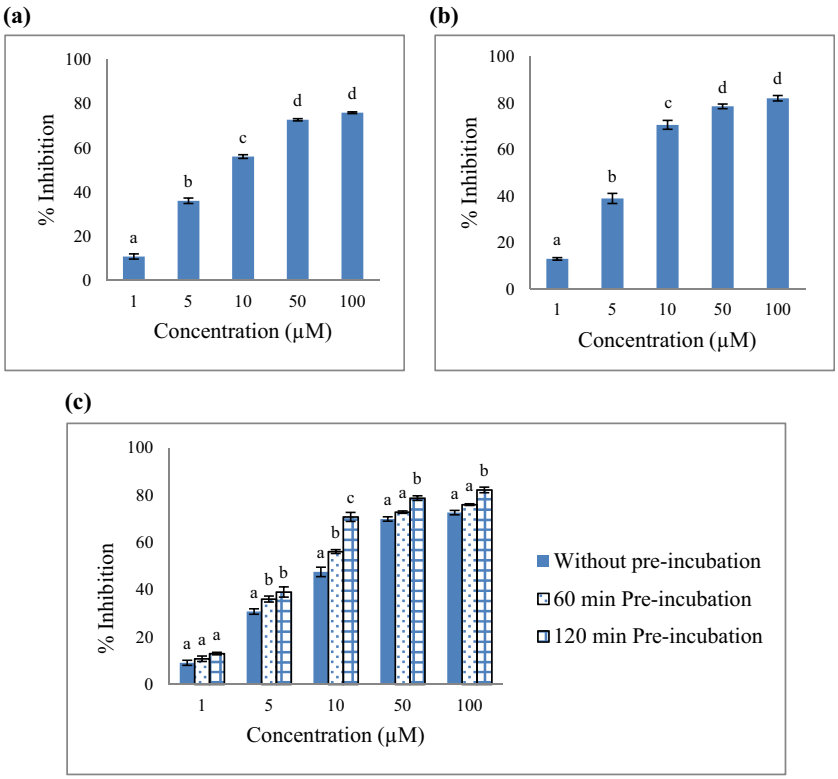

Fig. 4 Pre-incubation studies of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide. Percent FAAH inhibition was determined with (**a**) 60 min and (**b**) 120 min pre-incubation. Difference in percent inhibition without, and with 60 and 120 min pre-incubations are expressed in (**c**). Values are expressed as mean ± SEM from three independent experiments. Bars with different superscript letters are significantly different ($p < 0.05$)

min pre-incubation at concentrations from 1 to 100 μM. In contrast, with this compound there was no significant difference between a 60 min pre-incubation and no pre-incubation. The pre-incubation effects of the standard reversible and irreversible inhibitors OL-135 and PF-750 at 1 μM are shown in Fig. 7. Percent FAAH inhibition was determined without, and

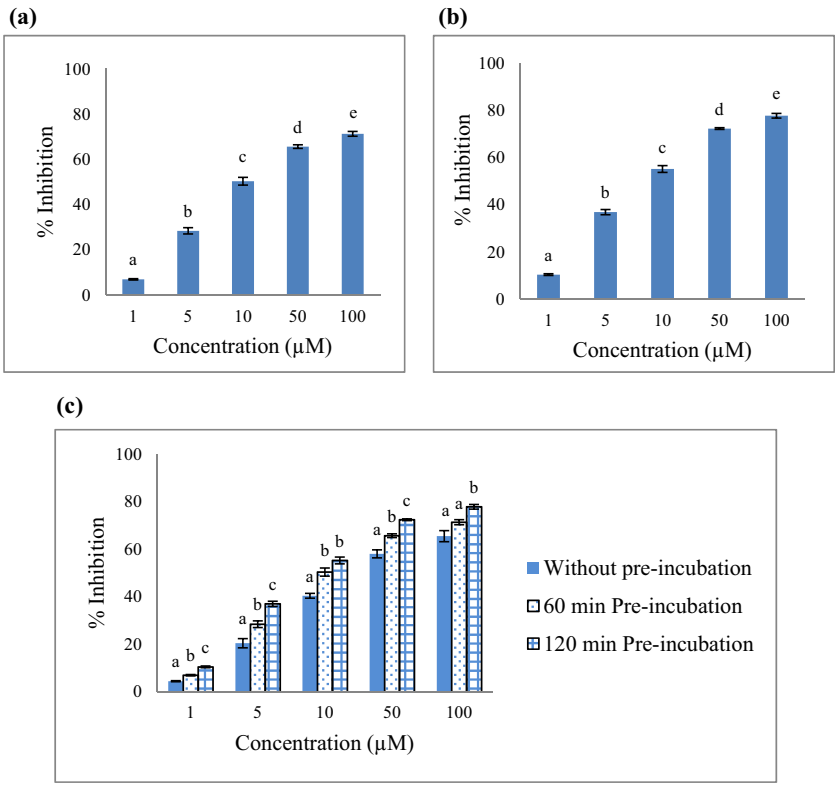

Fig. 5 Pre-incubation studies of *N*-benzyloleamide. Percent FAAH inhibition was determined with (**a**) 60 min and (**b**) 120 min pre-incubation. Difference in percent inhibition without, and with 60 and 120 min pre-incubations are expressed in (**c**). Values are expressed as mean ± SEM from three independent experiments. In **a** and **b**, bars with different superscript letters are significantly different ($p < 0.001$). In **c**, bars with different superscript letters are significantly different ($p < 0.05$)

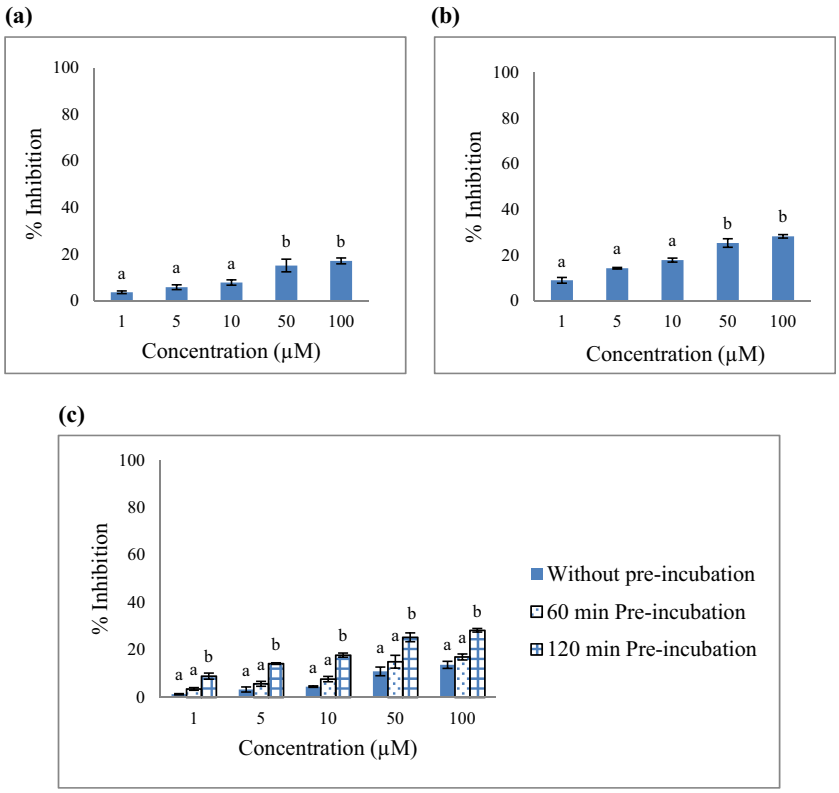

Fig. 6 Pre-incubation studies of *N*-benzylstearamide. Percent FAAH inhibition was determined with (**a**) 60 min and (**b**) 120 min pre-incubations. A difference in percent inhibition without, and with 60 and 120 min pre-incubations are expressed in (**c**). Values are expressed as mean ± SEM from three independent experiments. Bars with different superscript letters are significantly different ($p < 0.05$)

with 60 min and 120 min pre-incubations. Pre-incubation time had a significant effect on the FAAH inhibitory activity of PF-750, while no effect was observed with OL-135.

Concentration-Response Curve

The 50% inhibitory concentrations (IC_{50s}), defined as the conconcentrations of inhibitor that reduced the FAAH enzyme activity to 50% of its maximum effect, are shown in Figs. 8, 9, 10 and 11. The IC_{50} of *N*-benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide without pre-incubation was 8.5 μM (Fig. 8). The IC_{50} values for *N*-benzyloctadeca-9*Z*,12*Z*-dienamide without, and with 60 and 120 min pre-incubations were 7.2, 6.0 and 5.7 μM, respectively (Fig. 9). The IC_{50} values for *N*-benzyloleamide without, and with 60 and 120 min pre-incubations were 7.9, 6.6 and 5.9 μM, respectively (Fig. 10). The

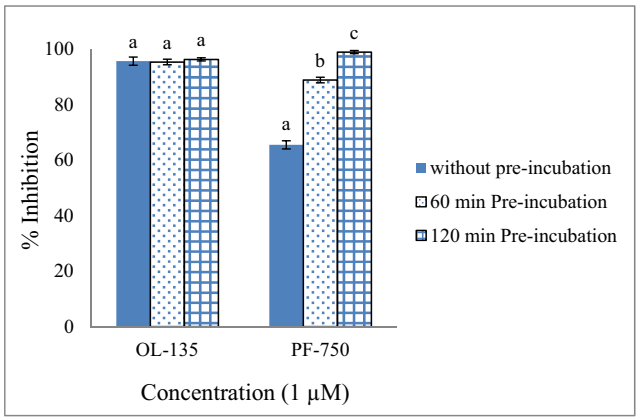

Fig. 7 Pre-incubation studies of OL-135 and PF-750 at 1 μM. Percent FAAH inhibition was determined without, and with 60 min and 120 min pre-incubation. Values are expressed as mean ± SEM from three independent experiments. Bars with different superscript letters are significantly different ($p < 0.001$)

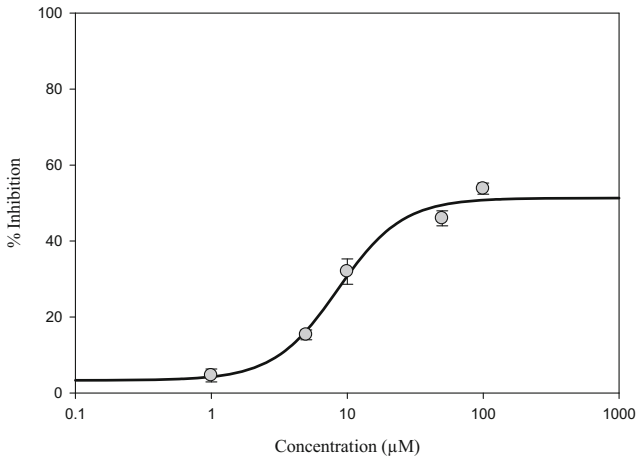

Fig. 8 Concentration-response curve for the inhibition of human recombinant FAAH by *N*-benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide without pre-incubation ($IC_{50} = 8.5 \pm 0.8$ μM). Values are expressed as mean ± SEM from three independent experiments

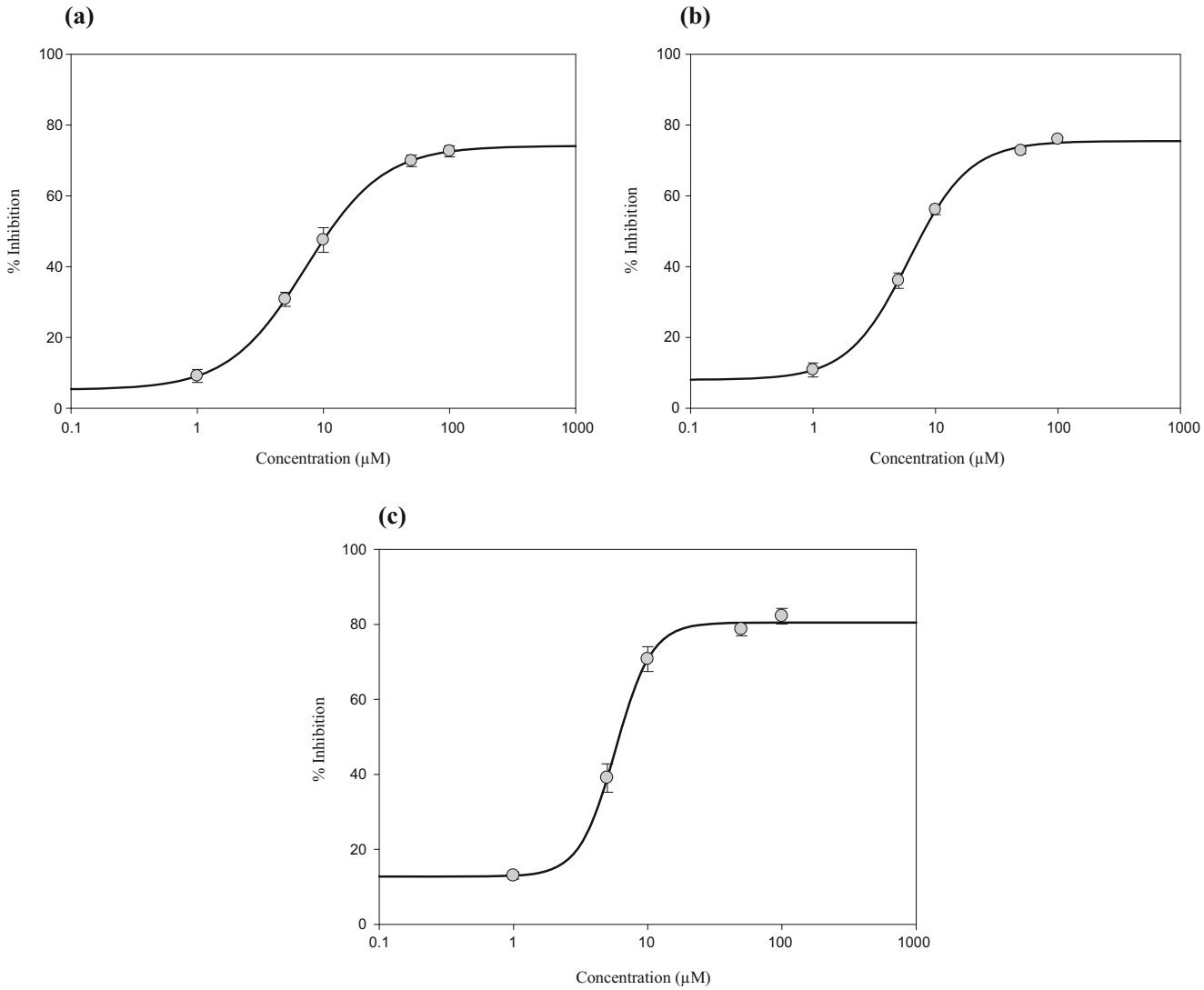

Fig. 9 Concentration-response curve for the inhibition of human recombinant FAAH by *N*-benzyloctadeca-9*Z*,12*Z*-dienamide. Percent FAAH inhibition was determined (**a**) without pre-incubation and (**b**) with 60 min and (**c**) with 120 min pre-incubation. IC_{50} values were 7.2 ± 0.4, 6.0 ± 0.2 and 5.7 ± 0.2 μM, respectively. Values are expressed as mean ± SEM from three independent experiments

IC_{50} values for *N*-benzylstearamide without, and with 60 and 120 min pre-incubations were 43.7, 21.5 and 16.1 μM, respectively (Fig. 11).

LC/MS/MS Analysis

To evaluate whether macamides are inhibitors or substrates of FAAH, LC/MS/MS analyses were performed on reaction mixtures. Standards of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide and benzylamine were used to prepare calibration curves. The concentration of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide in the sample that was incubated for 60 min with FAAH enzyme was 76.1 μM, while in the sample incubated without FAAH enzyme it was 113 μM. Also, the concentrations of benzylamine in the samples that were incubated with and without FAAH enzyme were 40.8 and 0.95 μM, respectively (Table 1), indicating metabolism of the macamide by FAAH.

Discussion

The medicinal properties of cannabinoids have been recognized for centuries and can be largely attributed to CB1 receptor activation in the central nervous system. However, the beneficial effects of cannabinoids, which include neuroprotection, anti-inflammatory and antidepressant activates, as well as relief of pain and spasticity, are counterbalanced by adverse effects such as cognitive and motor dysfunction [1, 3, 4, 33]. The discovery of the endocannabinoid anandamide, and the FAAH enzyme that terminates anandamide signaling, has

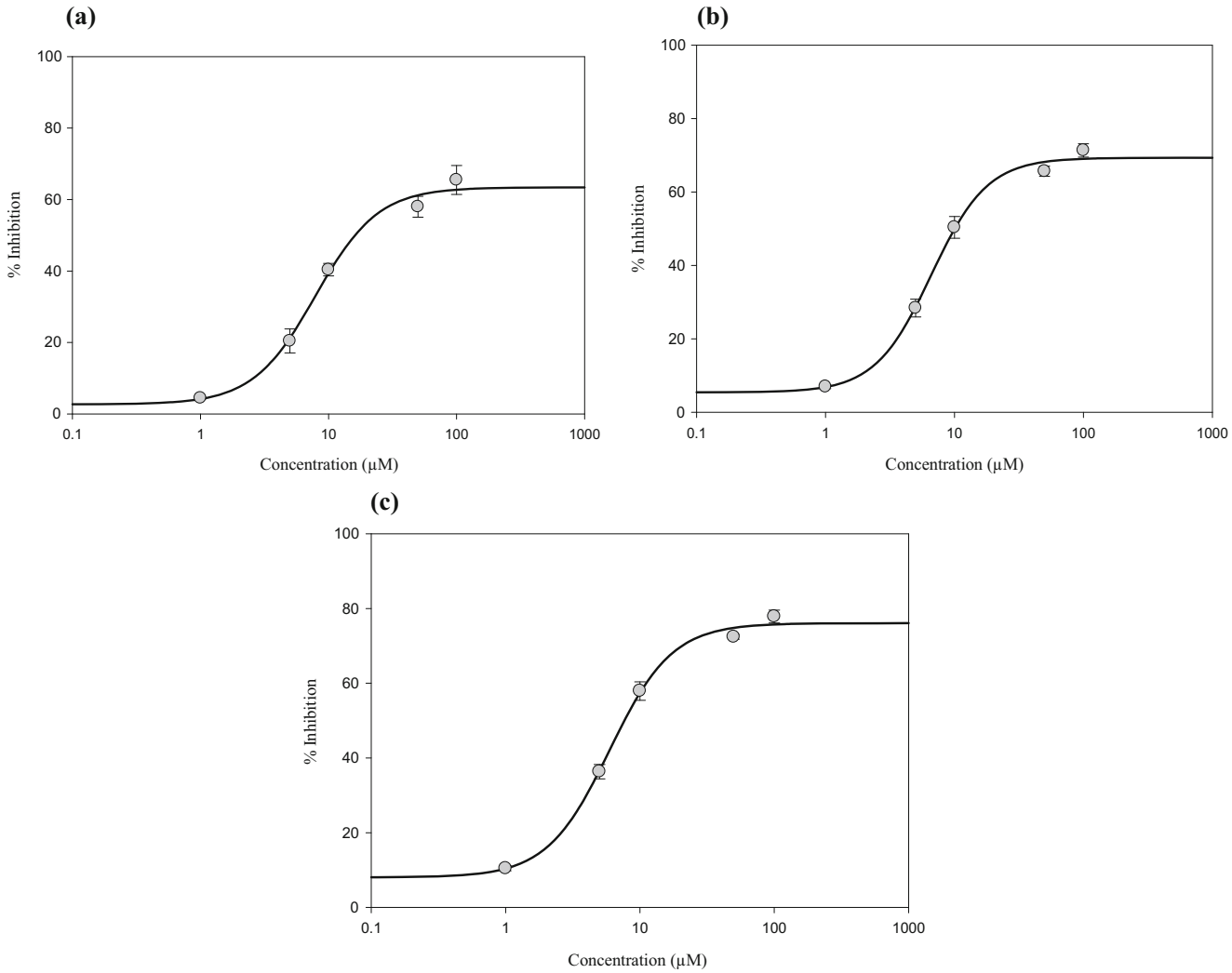

Fig. 10 Concentration-response curve for the inhibition of human recombinant FAAH by *N*-benzyloleamide. Percent FAAH inhibition was determined (**a**) without pre-incubation (**b**) with 60 min and (**c**) 120 min pre-incubation. IC_{50} values were 7.9 ± 0.6, 6.6 ± 0.4 and 5.9 ± 0.3 μM, respectively. Values are expressed as mean ± SEM from three independent experiments

inspired the search for pharmacological strategies to augment endogenous cannabinoid activity with FAAH inhibitors. Such inhibitors might exhibit superior selectivity in their elicited effects compared to direct CB1 agonists [34, 35]. Several FAAH inhibitors have been tested in vitro and in vivo but until now there is no FAAH inhibitor that has been approved by the FDA for clinical use [36–38]. However, very potent and active FAAH inhibitors are not always a good alternative since they can be toxic and can cause brain damage [39]. Therefore, a search for natural compounds with structural similarities to endocannabinoids, which could produce a partial inhibition of FAAH with a lower risk to develop side or adverse effects, is desirable.

The hypocotyls of the maca plant, originally cultivated in the Andean mountains of Peru, have been used as food and for many medical purposes [28, 29, 40]. Macamides are secondary metabolites isolated from the hypocotyls, and they have shown analgesic, antidepressant, memory enhancing and neuroprotective effects [24, 25, 41–43]. Macamides' neuroprotective effects have been clearly demonstrated in our lab with studies of maca extracts in vitro on rat neuroblastoma cells as well as in vivo in a rat focal ischemic stroke model [24, 25]. These effects could be due to the similarities between the macamides' chemical structures and those of the endogenous cannabinoids, e.g., anandamide, which possess a long chain fatty acid and an amide group. Since macamide structures mimic the structure of anandamide, they could bind to the FAAH enzyme and protect anandamide from metabolism by the enzyme [Fig. 1; 27, 44]. In previous studies in our lab, eleven macamides have been synthesized and analyzed for FAAH inhibitory activity. Some of these macamides have demonstrated varying inhibitory effects on the FAAH enzyme [31, 32]. One of these studies investigated *N*-3-methoxybenzyllinoleamide, which has a methoxy group

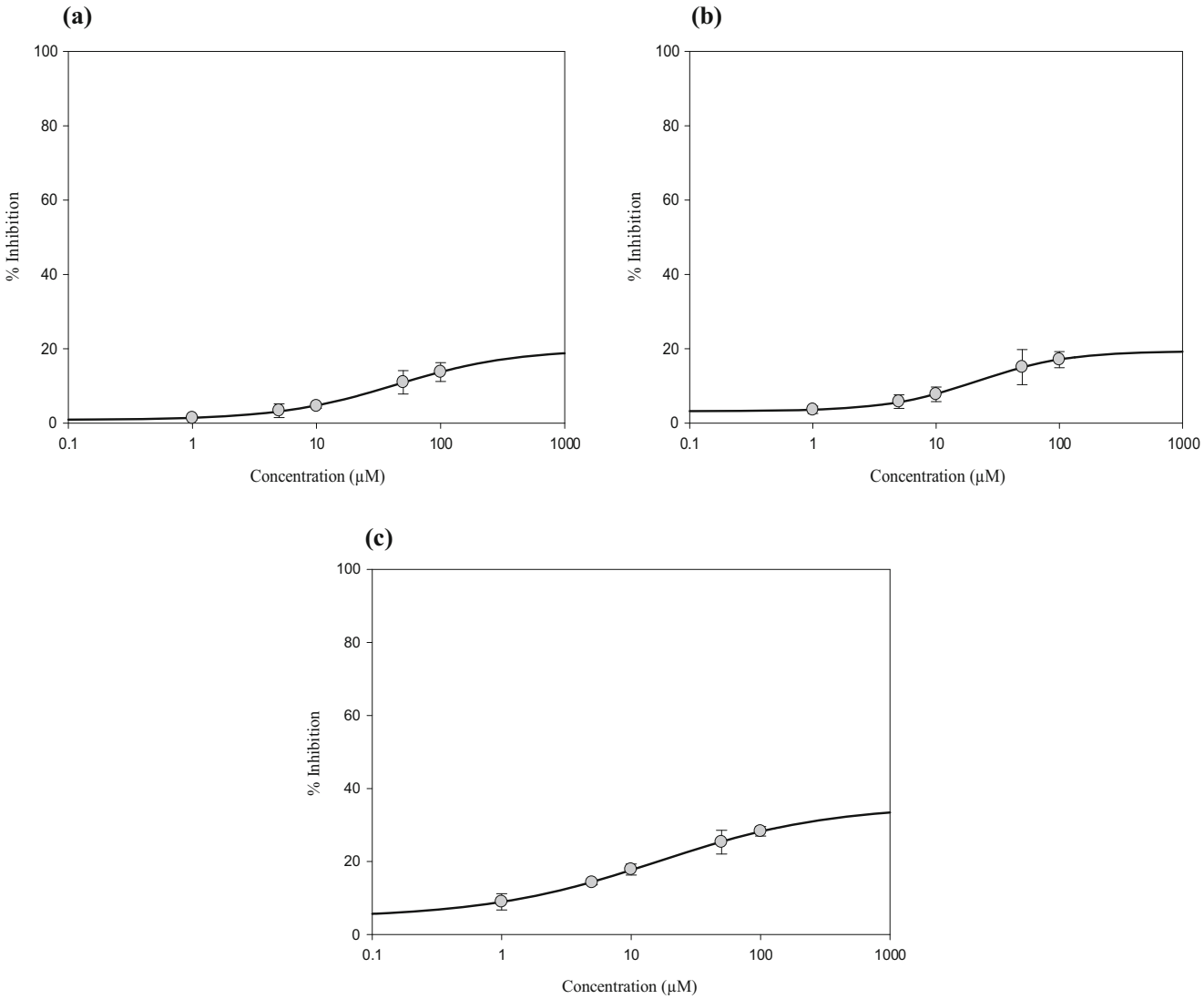

Fig. 11 Concentration-response curve for the inhibition of human recombinant FAAH by *N*-benzylstearamide. Percent FAAH inhibition was determined (**a**) without pre-incubation (**b**) with 60 min and (**c**) 120 min pre-incubation. IC_{50} values were 43.7 ± 37.3, 21.5 ± 19.2 and 16.1 ± 17.1 μM, respectively. Values are expressed as mean ± SEM from three independent experiments

in its structure, and which demonstrated a significant inhibitory effect on FAAH [32]. Since macamides that were previously tested in our lab demonstrated inhibition of FAAH activity, this research was undertaken to study how effective these four tested compounds would be at inhibiting the FAAH enzyme. This study has demonstrated the effect of unsaturation on FAAH inhibitory activity of the natural macamides; *N*-benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide, *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, *N*-benzyloleamide, and *N*-benzylstearamide. The fatty acids moieties of these macamides have the same number of carbons (18), but they differ in the presence and number of double bonds (Fig. 1).

Table 1 LC/MS/MS analysis of hydrolysis of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide and formation of benzylamine

Sample	Concentration (μM)
N-Benzyloctadeca-9*Z*,12*Z*-dienamide (μM)	
Incubated with FAAH enzyme	76.1
Incubated without FAAH enzyme	113
Benzylamine (μM)	
Incubated with FAAH enzyme	40.8
Incubated without FAAH enzyme	0.95

Results presented in Fig. 2 demonstrate that the presence of double bonds has an important role in FAAH inhibitory activity. *N*-Benzyloctadeca-9*Z*,12*Z*,15*Z*-trienamide, with three double bonds, inhibited FAAH with a maximum inhibition of 54% at 100 μM. The percent inhibition increased to 73% with *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, which has two double bonds in its

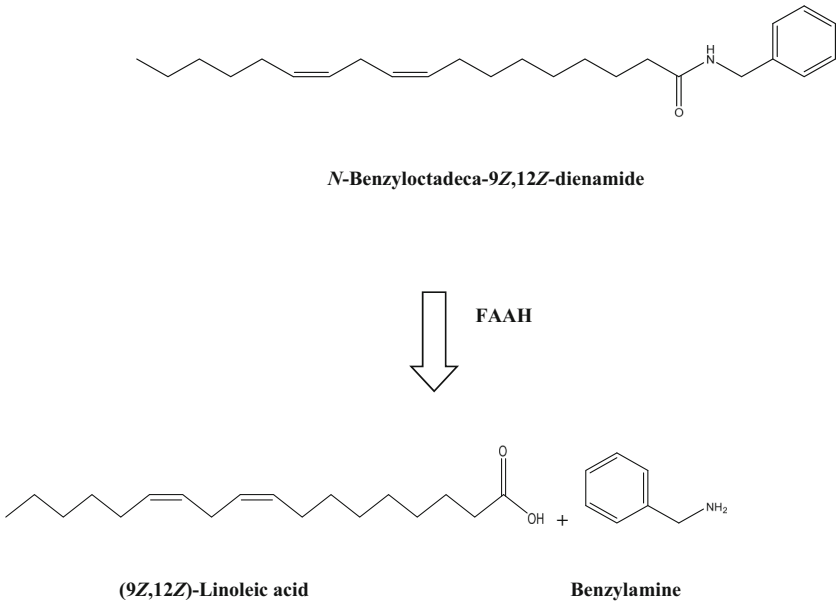

Fig. 12 Proposed hydrolysis of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide by FAAH

chemical structure. *N*-Benzyloleamide, with one double bond produced 64% inhibition at 100 μM. *N*-Benzylstearamide, which has no double bonds, produced only 13% inhibition of FAAH at the same concentration, the lowest inhibitory activity among the group of compounds tested. These results demonstrate that two double bonds in the fatty acid moiety are optimal for FAAH inhibition.

The pre-incubation study was performed to investigate the mechanism of inhibition by these compounds. The goal was to determine whether there were differences in FAAH inhibition between no pre-incubation and several different pre-incubation times, which could determine whether the enzyme inhibition is reversible or irreversible. When a pre-incubation exposure increases the activity of an inhibitor, this mechanism of time-dependent inhibition is characteristic of irreversible inhibitors [45]. In this study, the inhibitory activity assays with *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, *N*-benzyloleamide, and *N*-benzylstearamide at concentrations between 1 and 100 μM were repeated with 60 and 120 min pre-incubation times before substrate addition. The results in Figs. 4c, 5c, and 6c demonstrate that increasing the concentrations of these compounds as well as prolonging the pre-incubation time to 120 min resulted in significant differences in FAAH inhibitory activity. Figures 4c and 5c illustrate high FAAH inhibitory activities of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide and *N*-benzyloleamide, respectively; these are compounds which have double bonds in the fatty acid moieties compared to *N*-benzylstearamide, which has no double bonds in its structure (Fig. 6c). These three compounds demonstrate decreased IC_{50} values with increased FAAH inhibitory activity, in response to increased pre-incubation times. This is characteristic of time-dependent inhibitors or irreversible inhibitors, which might have a different pharmacological profile than classical reversible inhibitors.

The FAAH enzyme has a primary role in hydrolyzing many fatty acid amides [36]. As a consequence, in this study we hypothesized that *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, as it demonstrated the highest FAAH inhibitory activity (lowest IC_{50}) among the compounds tested, would also be degraded by FAAH to benzylamine and linoleic acid (Fig. 12). LC/MS/MS was utilized to determine whether the degradation of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide by the FAAH enzyme had occurred, by detecting the amount of benzylamine produced and by measuring the concentration of the macamide remaining in the sample. Table 1 shows that the concentration of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide remaining in the sample incubated with FAAH enzyme was less than that remaining in the sample incubated without enzyme by a ratio of 1:1.5. Analysis of the sample incubated with FAAH enzyme showed a significant accumulation of benzylamine, one product of the degradation reaction, compared to a similar sample incubated without FAAH enzyme, by a ratio of 43:1. The percent degradation of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide without incubation with FAAH enzyme was 0.8% while the percent degradation of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide after incubation with FAAH enzyme for 60 min was 35%. This result indicates that *N*-benzyloctadeca-9*Z*,12*Z*-dienamide is degraded by the FAAH enzyme, acting as a slow substrate by binding to the enzyme active site. The substrate and inhibitor nature of this compound might compete for access to the enzyme's active site so that the inhibitor is

slowly hydrolyzed, or it could also be an allosteric modulator which binds both to allosteric and active sites of the enzyme, explaining the irreversible inhibitory activity. Its binding to the allosteric site would make conformational changes to the active site of the enzyme, leading to slow degradation of the substrate. The benzylamine or fatty acid products of the reaction could also be possible inhibitors of the FAAH enzyme. Further investigations, such as kinetic studies, will clarify the mechanism of inhibition of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide. Testing the possible inhibitory activity of benzylamine or a fatty acid might help us to a better understanding this result.

Conclusions

Based on the collective data, the four macamides studied demonstrated concentration-dependent FAAH inhibitory activity. Double bonds appeared to have an important impact on FAAH inhibitory activity. The macamides containing oleic, linoleic, and linolenic acids produced FAAH inhibition of 64, 73, and 54%, respectively, while the macamide containing stearic acid and lacking a double bond displayed only 13% percent inhibition at 100 μM. *N*-Benzyloctadeca-9*Z*,12*Z*-dienamide (two double bonds) demonstrated the greatest inhibitory activity (lowest IC_{50}), indicating that two double bonds significantly influence the inhibition of FAAH by macamides. Pre-incubation studies showed significant differences in the FAAH inhibitory activity of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide, *N*-benzyloleamide, and *N*-benzylstearamide, indicating that the inhibitory mechanism of these compounds is time-dependent and could be irreversible. Incubation of *N*-benzyloctadeca-9*Z*,12*Z*-dienamide with FAAH enzyme for 60 min resulted in a 44% degradation, when analyzed by LC/MS/MS. This result indicates that *N*-benzyloctadeca-9*Z*,12*Z*-dienamide likely acts as a slow substrate for the FAAH enzyme. Based on this results, the presence of double bonds in macamides significantly increases FAAH inhibition, which could improve the potential neuroprotective and analgesic activities. These inhibitors' activities need further pharmacological study to investigate both in vitro and in vivo effects.

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