CHEMBIOCHEM

DOI: 10.1002/cbic.200800428

CAY10499, a Novel Monoglyceride Lipase Inhibitor Evidenced by an Expeditious MGL Assay

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Monoglyceride lipase (MGL) plays a major role in the metabolism of the lipid transmitter 2-arachidonoylglycerol (2-AG). This endocannabinoid is known to mediate a large number of physiological processes, and its regulation is thought to be of great therapeutic potential. However, the number of available monoglyceride lipase inhibitors is limited, mostly due to the lack of rapid and accurate pharmacological assays for the enzyme. We have developed a 96-well-format assay for MGL using a nonradiolabeled substrate, 4-nitrophenylacetate. The IC_{50} values that were obtained for known inhibitors of MGL using 4-nitrophenylacetate were similar to those reported by using the radiolabeled form of an endogenous substrate, 2-oleoylglycerol. In a first small-scale screening, we identified CAY10499 as a novel monoglyceride lipase inhibitor. Thus, we report here the characterization of this submicromolar inhibitor, which acts on MGL through an unprecedented mechanism for inhibitors of this enzyme.

Introduction

2-Arachidonoyglycerol (2-AG) is a lipid messenger that primarily acts by binding to the cannabinoid G protein-coupled receptors CB_1 and CB_2. Unlike classical neurotransmitters, 2-AG is not stored in vesicles prior to its release; instead, it is produced on demand from membrane lipid precursors.[1,2] After its action, 2-AG is rapidly taken up by cells and hydrolyzed rather than stored in vesicles. Such a mode of action requires tight control of 2-AG synthesis and hydrolysis. 2-AG synthesis is thought to occur through the sequential action of phospholipase C (β- and δ-PLCs) and diacylglycerol lipases (α- and β-DAGs), or through phospholipase A_1 (PLA_1) and lysophospholipase C (lysoPLC) activities.[3–5] On the other hand, the α/β-hydrolase monoglyceride lipase (MGL, E.C. 3.1.1.23) is responsible for the bulk part of 2-AG hydrolysis.[6–8] It was recently suggested that MGL was responsible for roughly 85% of the 2-AG hydrolase activity that is present in the mouse brain membrane.[8] Beside MGL, other 2-AG hydrolase activities have been described at the pharmacological level,[9] whereas a proteomic approach allowed for the recent identification of two additional α/β-hydrolase enzymes that hydrolyze 2-AG, named α/β-hydrolase domain-containing proteins 6 and 12 (ABHD6 and ABHD12).[8] In addition, experiments that used fatty acid amide hydrolase (FAAH) inhibitors suggested that under specific circumstances, 2-AG could also be hydrolyzed by FAAH.[10,11] Thus, 2-AG is hydrolyzed through several pathways, and the predominant one, at least in mouse brain, is by MGL.

2-AG possesses a wide range of pharmacological properties from the modulation of neurotransmitter release to control of neuroinflammation, and from regulation of cancer cell growth to stress-induced analgesia. It is therefore of great interest to have access to a large spectrum of pharmacological tools that would enable us to regulate 2-AG levels.[12] However, despite MGL cloning more than a decade ago, only a limited number of inhibitors have been described so far.[13] These include the potent but unselective methylarachidonoylfluorophosphonate (MAFP),[6] the less-potent carbamate derivative URB602,[14] and the thiuram derivative disulfiram.[15] This disappointing situation might have arisen from the quite expensive and painstaking assay procedures that are most commonly used nowadays to screen for and to characterize MGL inhibitors. One of these assays quantifies the arachidonic acid released upon 2-AG hydrolysis by using HPLC–UV spectroscopy,[16] while the other common assay quantifies the hydrolysis products of radiolabeled MGL substrates (2-AG or 2-oleoylglycerol, 2-OG).[6,15] An interesting alternative to these assays would be the use of a chromogenic substrate that, upon hydrolysis by MGL, would release a chromophore, and thus allow for quantification of MGL activity. Note that a similar approach was successfully used for FAAH by using 4-nitroanilide derivatives as FAAH substrates.[17]

Thus, in the present paper we describe the characterization of a novel, 96-well format assay for purified recombinant human MGL by using 4-nitrophenylacetate (4-NPA) as a substrate. Using this assay, we have identified in a preliminary screening CAY10499 as a MGL inhibitor. Thus, we also report here the biochemical characterization of this novel MGL inhibitor, which is suggested to inhibit MGL through an unprecedented mechanism of action.

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Results and Discussion

Because 4-NPA is a substrate of numerous esterases, we investigated whether recombinant human MGL was able to release the chromogenic moiety, 4-nitrophenol, upon incubation with 4-NPA. As might have been expected, the rapid increase of absorbance, which was measured at 405 nm (the λ_{max} of 4-nitrophenol) confirmed the hydrolysis of 4-NPA by MGL (Figure 1).

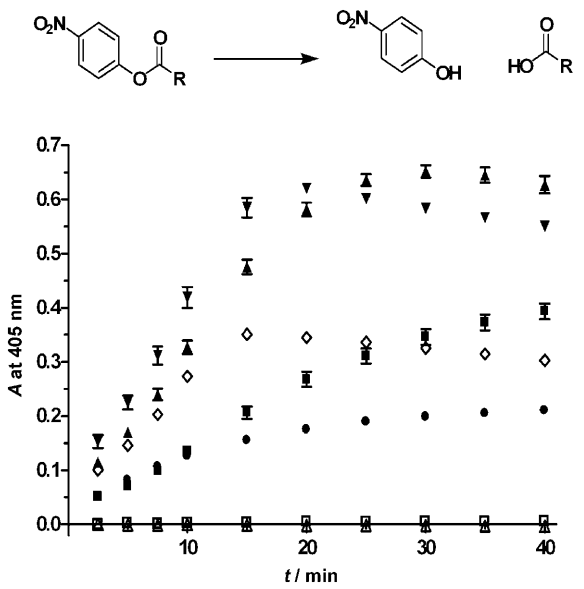


Figure 1. 4-Nitrophenyl alkyl ester derivatives are MGL substrates. The absorbance of 4-nitrophenol released by the hydrolysis of 4-nitrophenyl alkyl ester derivatives (0.25 mM) was monitored at 405 nm over 40 min. The following putative substrates were assayed: 4-nitrophenylacetate (■), 4-nitrophenylpropionate (▲), 4-nitrophenylbutyrate (▼), 4-nitrophenylvalerate (◇), 4-nitrophenylcaproate (●), 4-nitrophenyllaurate (□). The amide 4-nitrophenylacetamide (△) is not hydrolyzed by MGL.

To develop a colorimetric assay for MGL based on such a reaction, we further investigated the hydrolysis of 4-nitrophenyl alkyl ester derivatives by MGL to find the best-suited substrate. Thus, six 4-nitrophenyl alkyl esters with varying alkyl chain lengths ranging from C_2 to C_{12} were incubated with MGL, and the absorbance was measured over a 40 min period (Figure 1). Except for 4-nitrophenyllaurate (4-NPL, C_{12}), all tested compounds are MGL substrates, as evidenced by the increase in absorbance that was observed over time. However, due to the poor solubility of 4-NPL, we cannot rule out that this compound is a MGL substrate. Note that we also tested the well-known amidase substrate, 4-nitrophenylacetamide, but its incubation with MGL did not result in increased absorbance (λ_{max} for 4-nitroaniline is 382 nm); this is in line with the reported lack of hydrolase activity of MGL towards anandamide.[6,15] In light of 4-NPAs good behavior as substrate combined with its higher solubility, we selected it as a substrate for our following experiments.

We next characterized the kinetic parameters of 4-NPA hydrolysis by MGL (Figure 2). 4-NPA is hydrolyzed in a protein- and time-dependent manner; this further confirms that it is acting as a substrate. When measuring MGL activity as a function of substrate concentration, a Michaelis–Menten-type curve is obtained, which results in K_m and V_{max} values of 0.20 ± 0.03 mM and 52.2 ± 2.3 μmol min^{-1} mg^{-1}, respectively (Figure 2C). Based on these results, we selected the conditions—16 ng of MGL, 15 min incubation, and 250 μM 4-NPA—for a MGL activity assay that would allow for the characterization of potential inhibitors.

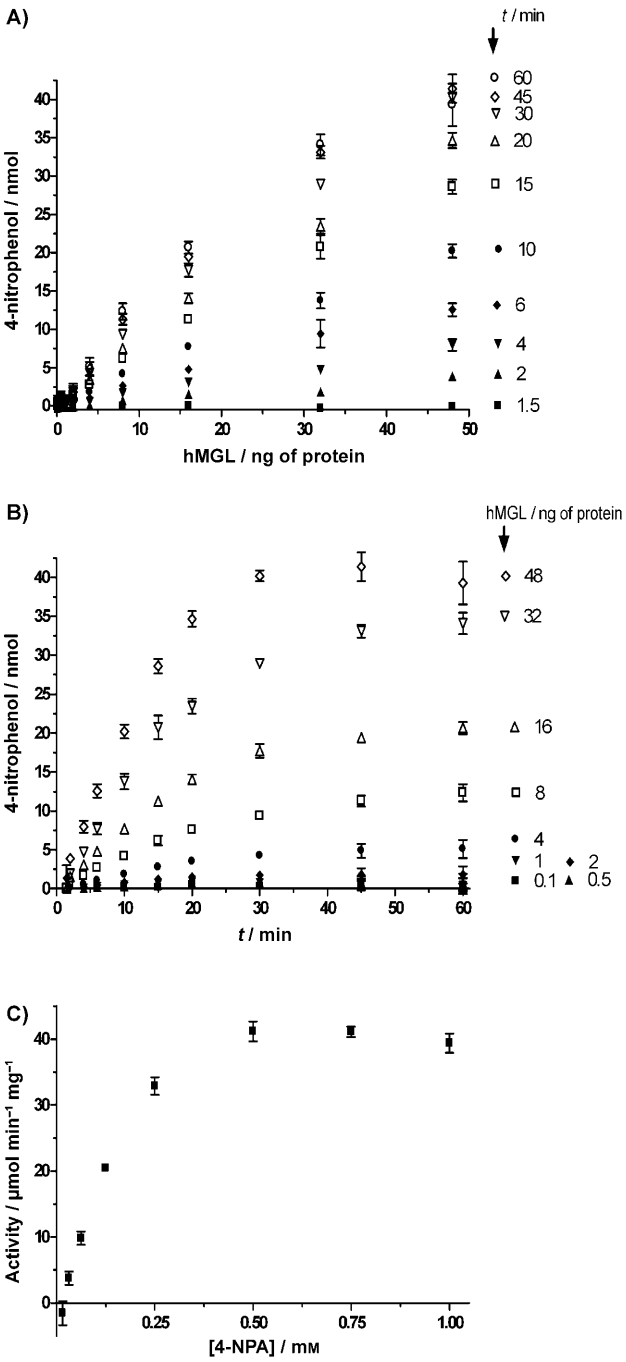


Figure 2. 4-NPA hydrolysis by human MGL. 4-NPA hydrolysis by MGL is A) protein concentration dependent, and B) time dependent. C) Varying the substrate concentration leads to a Michaelis–Menten-type curve (K_m = 0.20 ± 0.03 mM and V_{max} = 52.2 ± 2.3 μmol min^{-1} mg^{-1}).

To confirm the validity of our approach, we assayed known lipases and MGL inhibitors. As previously reported, PMSF inhibited MGL only at very high concentrations (Figure 3A). Similarly, the lipase inhibitor RHC80267 also required a relatively high concentration to inhibit MGL activity. Interestingly, we obtained a complete sigmoidal curve ($IC_{50} = 0.46 \pm 0.09$ μM) with the lipase inhibitor tetrahydrolipstatin (THL). However, this inhibitor failed to fully inhibit the enzyme, suggesting that it might not completely block the active site.

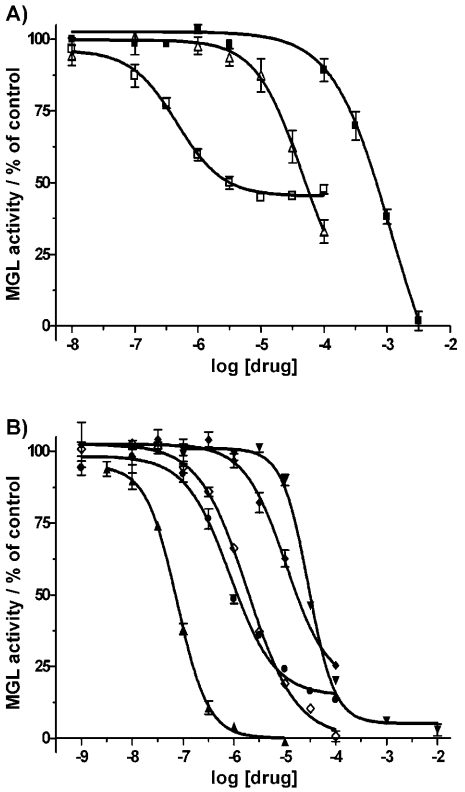


Figure 3. Characterization of MGL inhibition. A) MGL was incubated in the presence of increasing concentrations of PMSF (■), THL (□), RHC80267 (△) and the activity was measured by using 4-NPA hydrolysis. B) The MGL inhibitors MAFP (▲), NEM (▼), NAM (◆), ATFMK (◇), and disulfiram (●) all dose-dependently inhibited 4-NPA hydrolysis by MGL.

The fluorophosphonate inhibitor MAFP ($IC_{50} = 0.076 \pm 0.004$ μM; Figure 3B), the maleimide derivative *N*-ethylmaleimide (NEM, $IC_{50} = 28 \pm 1.7$ μM), the trifluoromethylketone arachidonoyltrifluoromethylketone (ATFMK, $IC_{50} = 1.84 \pm 0.14$ μM), and the thiuram derivative disulfiram ($IC_{50} = 0.8 \pm 0.09$ μM) all inhibited MGL with IC_{50} values that are similar to those we previously reported with human purified MGL and radiolabeled 2-OG as a substrate.[15] *N*-arachidonylmaleimide (NAM) inhibited 4-NPA hydrolysis with an IC_{50} value of 10.5 ± 3.8 μM (compared to 4.9 ± 0.9 μM with [^{3}H]-2-OG as a substrate). Although this is lower than the value that was reported by Saario et al.,[18] the IC_{50s} that are reported here for NAM are consistent with what was reported by Blankman et al.,[8] and they are also consistent with what we observed when using rat brain homogenates (3.2 ± 0.5 μM). Taken together, these data confirm that 4-NPA can be used as chromogenic substrate for characterizing MGL inhibitors.

We also questioned whether the use of pure MGL would constitute a prerequisite for the present assay or if tissue homogenates could also be used. Several authors described strong MGL activity in rat brain homogenates,[16,19] and we therefore thought they might be suitable for our assay. Thus, 4-NPA was incubated in the presence of increasing amounts of rat cerebellum membranes. In this way, progress curves were generated that were similar to those obtained with pure MGL (See Figures 2 and 4).

Three inhibitors, MAFP, disulfiram, and NEM were also tested and found to inhibit 4-NPA hydrolysis to various extents. Note that the inhibition curves that were obtained with rat cerebellar membranes (Figure 4C) differ from those that were obtained by using pure recombinant human MGL (Figure 3B). For instance, MAFP and NEM completely inhibited 4-NPA hydrolysis by pure MGL, whereas only 72 and 33% of the hydrolysis by rat cerebellum membranes was inhibited. These data can be explained by the large number of esterases that are present in tissue homogenates, and suggest that the present assay might not be suitable for tissues homogenates when the aim is the characterization of MGL inhibitors.

To further test this assay, we screened (at 10 and 100 μM) a set of compounds that have been reported to interact with endocannabinoid-hydrolyzing enzymes. These include nonsteroidal anti-inflammatory drugs,[20] trifluromethylketone derivatives,[21] carbamates,[22–24] hydantoins,[25] and substrate analogues. With the exception of the hormone-sensitive lipase (HSL) carbamate inhibitor CAY10499,[26] and the benzoylthiocarbamate SC17, all the compounds were devoid of an inhibitory effect on 4-NPA hydrolysis by MGL (Table 1, and Table S1 in the Supporting Information). Because both CAY10499 and SC17 behaved as good inhibitors in this screening, we determined their IC_{50} values. MGL-mediated 4-NPA hydrolysis was inhibited with IC_{50} values of 5 ± 0.8 μM and 0.5 ± 0.03 μM for SC17 and CAY10499, respectively. When assayed by using 2-OG as a substrate, IC_{50} values of the same magnitude were observed (20 ± 3.15 μM and 0.4 ± 0.04 μM, respectively).

Because there are only a limited number of available submicromolar MGL inhibitors (for a review see ref. [13]), we decided to further characterize MGL inhibition by CAY10499. The inhibition of MGL by CAY10499 cannot be overcome by increasing the substrate concentration (Figure 5A). Actually, K_m values are increased in the presence of increasing concentrations of inhibitor (0, 0.1, 0.3, 1, and 10 μM of CAY10499) whereas the V_{max} values are decreased by the inhibitor. Interestingly, the IC_{50} values that were measured after preincubation of the inhibitor with the enzyme showed a strong increase in the inhibition potential, with IC_{50} values reaching a plateau of around 20 nM after 15 min of preincubation in the absence of any substrate (Figure 5B).

These data show that CAY10499 is also able to interact with MGL in the absence of substrate. The reversibility of the inhibition was also investigated by performing a high dilution (400×) of the enzyme–inhibitor solution prior to the substrate addition. The inhibitor concentration was selected to fully in-

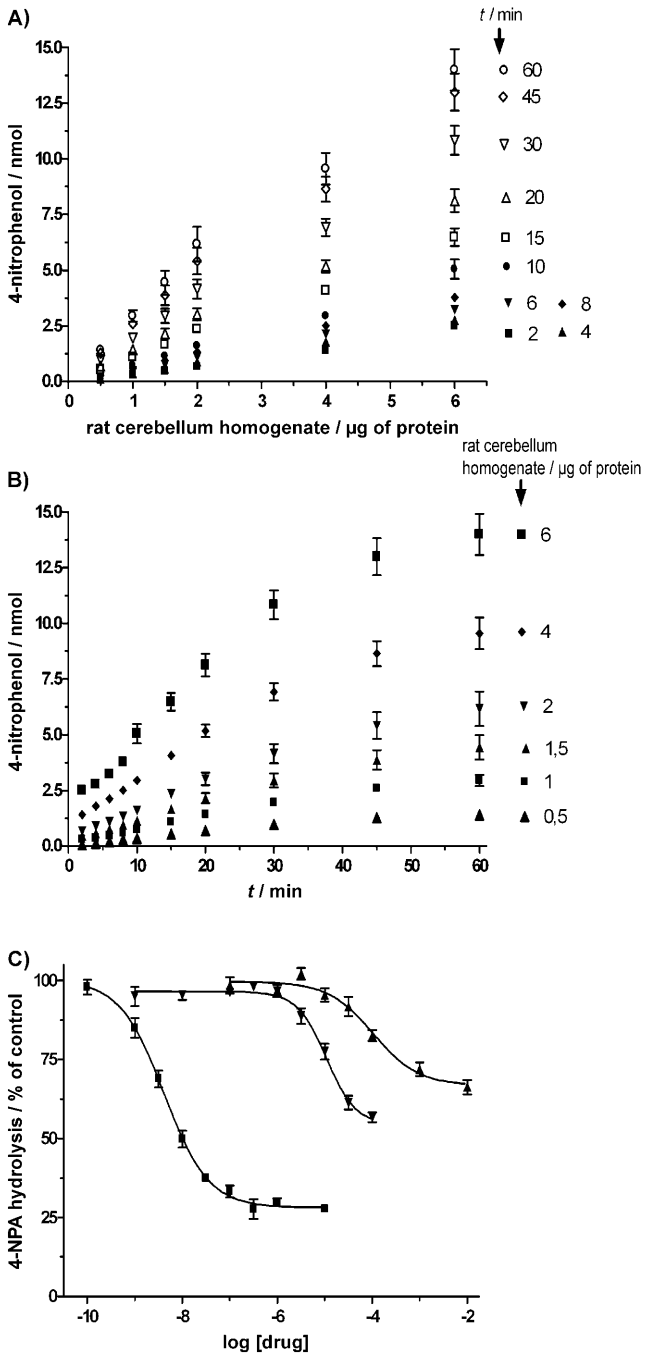


Figure 4. 4-NPA hydrolysis by rat cerebellar membranes. 4-NPA hydrolysis by rat cerebellar membranes is A) protein concentration dependent and B) time dependent. C) The hydrolysis of 4-NPA by rat cerebellar membrane was partially inhibited by MAFP (■), disulfiram (●), and NEM (▼).

Table 1. Screening of a small set of compounds. Representative compounds of classes that are known to interact with endocannabinoid metabolism (that is, NSAIDs, carbamates, acylesters, ureas, trifluoromethylketones) were screened against MGL activity.

Compound	MGL activity [% of control]		Compound class
	100 µM	10 µM	
SC1	51	82	NSAIDs
SC2	89	80	NSAIDs
SC3	55	67	NSAIDs
SC4	84	79	NSAIDs
SC5	68	64	NSAIDs
SC6	70	84	NSAIDs
SC7	103	100	NSAIDs
SC8	90	102	acylester
SC9	98	97	benzylglycerol
SC10	101	108	dibenzylcarbonate
SC11	88	90	carbamate
SC12	92	88	carbamate
CAY10499	5	5	carbamate
CAY10433	101	98	carbamate
WWL70	89	99	carbamate
URB602	69	84	carbamate
SC13	82	93	carbamate
SC14	80	84	trifluoromethylketone
SC15	92	111	trifluoromethylketone
SC16	100	101	trifluoromethylketone
SC17	12	36	benzoylthiocarbamate
SC18	63	80	benzoylcarbamate
SC19	79	83	benzoylcarbamate
SC20	104	105	benzhydryl
SC21	109	110	benzhydryl
SC22	102	98	hydantoin

hibit the enzyme during the 1 h preincubation.[27] Thus, after 1 h of incubation of MGL with vehicle, and following a 400× dilution prior to substrate addition, MGL still time-dependently hydrolyzed 4-NPA; this shows that the 1 h preincubation per se did not affect MGL activity (Figure 5C). Adding ATFMK as an inhibitor during the preincubation had no effect on the MGL activity after the 400× dilution, as was expected for this reversible electrophilic carbonyl-type inhibitor.[28,29] On the other hand, the active-site-directed covalent inhibitor MAFP fully and irreversibly inhibited MGL. No MGL activity was detected even after a 1 h incubation with the substrate (Figure 5C).

A similar profile is apparent after MGL incubation with CAY10499; this suggests that the inhibition is irreversible, at least over the time course of the assay. Note that we tested the same inhibitors in parallel at their 400× diluted concentration, and neither MAFP nor CAY10499 inhibited MGL (data not shown). This demonstrates that the inhibitor that is bound to the enzyme is indeed responsible for the MGL inhibition that was observed after the dilution.

We further characterized CAY10499 by assessing its effect on FAAH activity as well as its affinity for the cannabinoid receptors. CAY10499 was able to fully inhibit [^{3}H]-AEA hydrolysis by human recombinant FAAH with an IC_{50} of 76 nM. On the other hand, CAY10499 displaced only 25% and 20% of [^{3}H]-CP-55940-specific binding to the CB_1 and CB_2 receptors, respectively, and thus is devoid of significant affinity for the cannabinoid receptors.

Carbamate moieties are known to interact with numerous enzymes, including several enzymes of the endocannabinoid system. Indeed, FAAH is inhibited by such derivatives as URB597[22] and JP-83,[24] whereas MGL is inhibited, albeit to a much lesser extent, by the carbamate URB602.[14] Finally, the 2-arachidonoylglycerol hydrolase ABDH6 was recently shown to be inhibited by WWL70.[8,30] Thus, it is interesting to compare the potent inhibition that we obtained with the carbamate de-

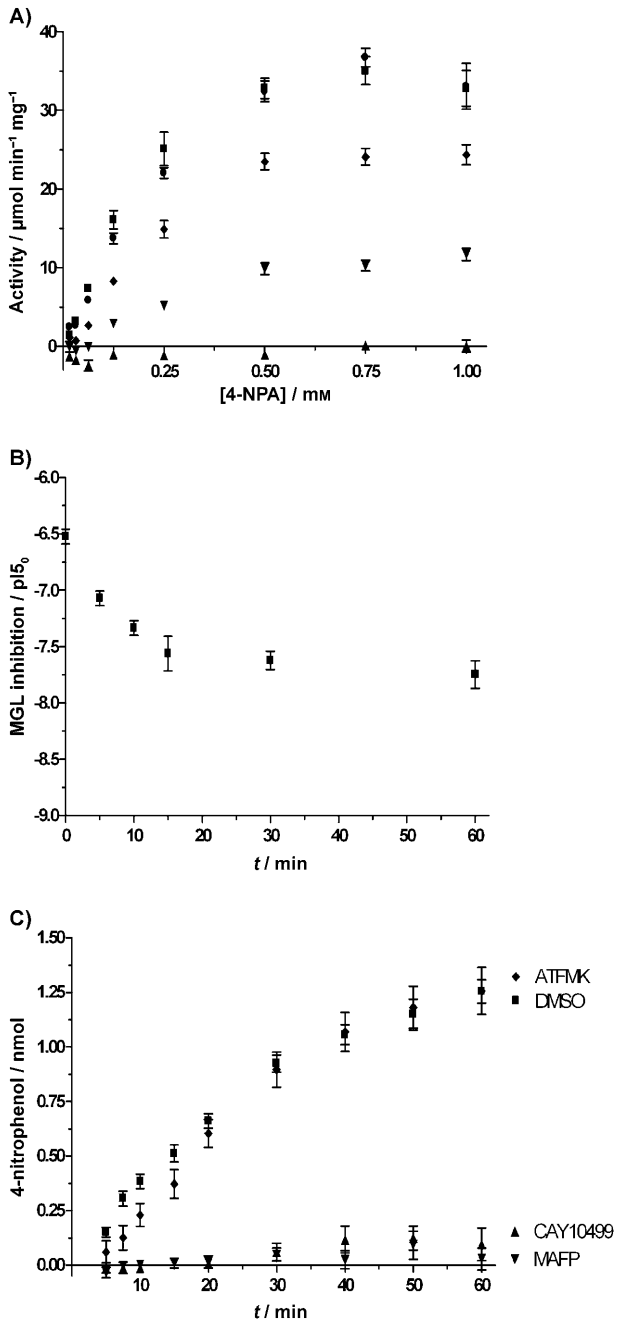
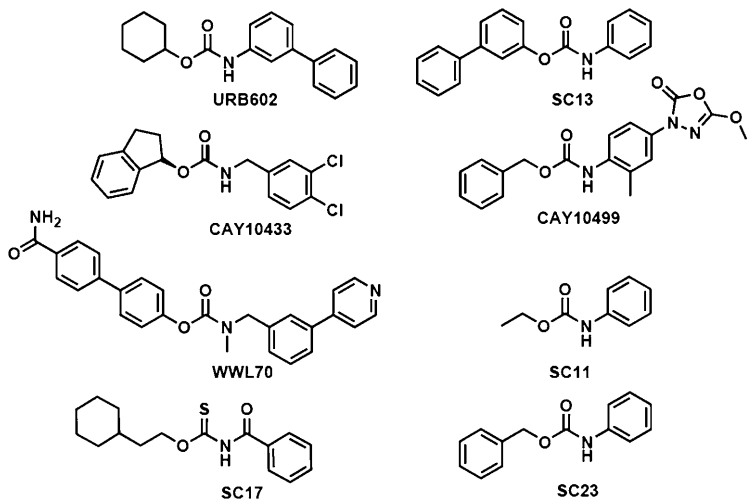


Figure 5. Characterization of CAY10499 as an MGL inhibitor. A) In the presence of CAY10499 (■ 0 µM; ● 0.1; ◆ 0.3; ▼ 1 µM; ▲ 10 µM), K_m values are increased while V_{max} values are decreased. B) Preincubation of CAY10499 with MGL in the absence of substrate results in increased inhibition (that is, decreased $-\log IC_{50}$ values). C) Following 1 h preincubation of an enzyme–inhibitor mixture and 400× dilution, MGL time-dependently hydrolyzes 4-NPA (■ DMSO, control) as does the MGL that had been preincubated with ATFMK (◆). Conversely, MGL that was preincubated with MAFP (▼) and CAY10499 (▲) was still inhibited; this demonstrates that the inhibition is irreversible.

rivative CAY10499 with the lack of inhibition that was shown by all the other carbamate inhibitors that were tested here (Table 1 and Scheme 1).

This difference in activity incited us to ask whether the carbamate moiety or the 5-methoxy-3-(3-phenoxyphenyl)-1,3,4-oxadiazol-2(3*H*)-one moiety is responsible for MGL inhibition. Indeed, the later moiety was involved in the inhibition of HSL by 3-phenyl-5-methoxy-1,3,4-oxadiazolon-2-one (compound 7600), a close analogue of CAY10499.[31] To answer this question, the most straightforward approach was to synthesize a derivative lacking one of the two moieties. We therefore synthesized and tested benzyl phenylcarbamate (SC23), which lacks the 5-methoxy-3-(3-phenoxyphenyl)-1,3,4-oxadiazol-2(3*H*)-one moiety. SC23 was devoid of any activity against MGL (data not shown). Because CAY10499 activity is increased by preincubation with the enzyme, we also preincubated SC23 for 20 min before adding the substrate, and again found no activity for this carbamate. Together, these data suggest that the active moiety of CAY10499 is not the carbamate but more likely the 5-methoxy-1,3,4-oxadiazol-2(3*H*)-one moiety.



Scheme 1. Carbamates used in this study.

Conclusion

We report here the characterization of a fast, inexpensive, and non-radiolabeled enzymatic assay for assessing MGL activity by using 4-NPA as chromogenic substrate. The assay was validated by using known MGL inhibitors, which all showed inhibition values that were similar to those that we reported earlier using [^{3}H]-2-OG as a substrate. The use of this assay in a small-scale screening allowed us to detect, for the first time, the inhibitory activity of CAY10499 against MGL. Upon further study, CAY10499 appears to be an irreversible nanomolar inhibitor that interacts with MGL through an original mechanism for this enzyme. This novel mechanism combined with its good inhibition of MGL should warrant further development of this class of compounds as inhibitors of this crucial enzyme in the endocannabinoid 2-AG metabolism.

Experimental Section

Materials: 4-NPA, 4-nitrophenylbutyrate, 4-nitrophenylcaproate, 4-NPL, 4-nitrophenylvalerate, *N*-(4-nitrophenyl)acetamide, disulfiram, PMSF, NEM, THL, RHC80267, and 2-OG were purchased from

Sigma. 4-Nitrophenyl propionate was obtained from MP Biomedicals (Illkirch, France). CAY10499, CAY10433, WWL70, MAFP, NAM, were purchased from Cayman Chemicals (Ann Arbor, MI, USA). [^{3}H]-2-OG and [^{3}H]-AEA were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [^{3}H]-CP-55 940 (101 Ci mol^{-1}) was purchased from PerkinElmer. Pure human MGL was obtained as previously described.[15] Enzyme amounts are given in mg of protein (determined by Bradford's method). Incubation and absorbance measurement were performed at 37 °C by using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Measurement of MGL activity using 4-NPA hydrolysis: Assays were carried out in a 96-well microtiter plate (200 μL total volume). Pure human MGL (16 ng per well) in Tris–HCl (150 μL, 100 mM, pH 7.4, 0.1% *w/v* fatty-acid-free BSA [final]) was added to each well, which also contained drug in DMSO (10 μL) or DMSO alone as a control. Hydrolysis was initiated by rapidly adding a solution of 4-NPA in Tris–HCl (1.25 mM, 40 μL; 250 μM final concentration) to each well. The 96-well plate was incubated at 37 °C for 15 min prior to absorbance measurement at 405 nm. Wells that contained buffer only were used as controls for chemical hydrolysis (blank) and this value was systematically subtracted. For the characterization of the method, assays were performed by using different protein amounts ranging from 0.1 to 48 ng, or different incubation periods (from 2 min to 40 min). A calibration curve for 4-nitrophenol was built-up under the same conditions and used to convert absorbance values into amounts of product (data not shown).

Measurement of MGL activity using [^{3}H]-2-OG hydrolysis: The assay was performed as previously reported.[15] Briefly, pure human MGL (5 ng) in Tris–HCl (165 μL, 100 mM, pH 7.4, 0.1% *w/v* fatty acid-free BSA [final]) was added to glass tubes that contained either 10 μL of drug in DMSO or DMSO alone (control). Hydrolysis was initiated by adding a solution of [^{3}H]-2-OG in Tris–HCl (80 μM, 25 μL, 50 000 dpm; 10 μM final concentration). The tubes were incubated in a shaking water bath at 37 °C for 10 min. Tubes containing buffer only were used as controls for chemical hydrolysis (blank) and this value was systematically subtracted. Reactions were stopped by adding ice-cold MeOH/$CHCl_3$ (1:1, 400 μL), and the radiolabeled glycerol was extracted by mixing and subsequent centrifugation at 1700 *g* (5 min). The upper layer (200 μL) was recovered and the radioactivity was measured by liquid scintillation.

Measurement of FAAH activity: The assay was performed similarly to the radiolabeled substrate assay for MGL activity by using [^{3}H]-AEA and human recombinant FAAH (5 μg of protein/tube)[32] instead of [^{3}H]-2-OG and human recombinant MGL, respectively.

Cannabinoid receptors competition assay: The assay was performed as previously reported.[25] Briefly, hCB_1-CHO or hCB_2-CHO cell homogenates (40 μg of protein in 450 μL of a buffer containing Tris–HCl (50 mM), EDTA (1 mM), $MgCl_2$ (3 mM), and 1% fatty-acid-free BSA at pH 7.4) were added to glass tubes that contained either 0.5 μL of drug in DMSO or DMSO alone (0.1%, total binding) and [^{3}H]-CP-55,940 (50 μL, 1 nM final). Nonspecific binding was determined in the presence of 10 μM HU-210. The tubes were incubated for 1 h at 30 °C, then cold buffer was rapidly added to the tubes, the solutions were filtered through presoaked glass-fiber filters (Whatman GF/B, Maidstone, UK) by using a Brandell harvester, and the tubes were rinsed twice with cold buffer. Radioactivity on the filter was counted by using scintillation liquid (10 mL).

Inhibition reversibility study: The reversibility of MGL inhibition was assessed essentially as described by Ahn et al.[27] The inhibitors were added to a microvial at a concentration of 20× (MAFP) or 50×(CAY10499, ATFMK) their IC_{50} value. MGL (220 ng) in Tris–HCl (50 μL, 100 mM, pH 7.4, 0.1% *w/v* fatty-acid-free BSA [final]) was also added. After 1 h incubation at room temperature, three aliquots (5 μL) were diluted 300×, and this solution (150 μL) was added to a 96-well plate that contained DMSO (10 μL) and 4-NPA (40 μL, 250 μM final concentration). The hydrolysis was monitored (405 nm) over 60 min.

Preparation of rat cerebellum homogenate and 4-NPA assay: Tissue preparation was conducted essentially as described by Vandevoorde et al.[19] Briefly, rat cerebella were homogenized at 4 °C in sodium phosphate buffer (50 mM, pH 8) containing sucrose (0.3 M). The homogenates were then centrifuged at 100 000 *g* for 50 min at 4 °C, and the pellets were subsequently resuspended in Tris–HCl (100 mM, pH 7.4). The fractions were stored frozen in aliquots at −80 °C until use. Inhibition of 4-NPA hydrolysis assay was conducted essentially as described above, by using rat cerebella homogenates (4 μg per well; 20 min incubation) instead of pure recombinant human MGL.

Synthesis of *N*-benzoylthiocarbamic-cyclohexylethyl ester (SC17): 2-cyclohexylethan-1-ol (4.9 g, 38 mmol) was added dropwise to a mixture of benzoylisothiocyanate (5 g, 30 mmol) and Et_3N (3.1 g, 30 mmol) in dry toluene (60 mL). The resulting solution was refluxed under an inert gas atmosphere for 12 h. The toluene was then removed under reduced pressure, and the resulting solid was recrystallized from EtOH to give a white solid (35%). ^{1}H NMR (400 MHz, [D_6]DMSO): $\delta = 11.91$ (s, 1H), 7.85–7.83 (d, 2H), 7.64–7.60 (t, 2H), 7.52–7.46 (t, 1H), 4.25–4.49 (t, 2H), 1.69–1.55 (m, 2H), 1.42–1.38 (m, 1H), 1.24–1.10 (m, 2H), 0.95–0.86 ppm (m, 2H); ^{13}C NMR (100 MHz, [D_6]DMSO): $\delta = 188.36$, 163.66, 131.83, 131.27, 127.16, 126.89, 68.00, 33.43, 32.12, 31.02, 24.53, 24.15 ppm; IR: $\tilde{\nu} =$ 2925, 1700, 1530 cm^{-1}.

Synthesis of benzylphenylcarbamate (SC23): Benzyl alcohol (7 g, 64 mmol) was added dropwise to a mixture of phenylisocyanate (5 g, 42 mmol) and Et_3N (4,2 g, 42 mmol) in dry toluene (60 mL), and the resulting solution was refluxed under an inert gas atmosphere for 12 h. The toluene was then removed under reduced pressure, and the resulting solid was recrystallized from hexane/acetone to yield a white solid (65%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 7.50–7.34 (m, 10H), 5.26 ppm (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.39, 138.08, 136.29, 129.11, 128.68, 128.39, 128.36, 123.59, 118.91, 67.18 ppm; IR: $\tilde{\nu} = 1691$, 1549 cm^{-1}.

Acknowledgements

G.G.M. is a postdoctoral researcher from the National Fund for Scientific Research (FNRS, Belgium). This work was supported by a FRFC grant (FNRS, grant number 2.4654.06.)

Keywords: enzymes · esterases · high-throughput screening · hydrolase · monoacylglycerol lipase

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Received: June 24, 2008
Published online on October 15, 2008

