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A critical cysteine residue in monoacylglycerol lipase is targeted by a new class of isothiazolinone-based enzyme inhibitors

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Background and purpose: Monoacylglycerol lipase (MGL) is a presynaptic serine hydrolase that inactivates the endocannabinoid neurotransmitter, 2-arachidonoyl-sn-glycerol. Recent studies suggest that cysteine residues proximal to the enzyme active site are important for MGL function. In the present study, we characterize the role of cysteines in MGL function and identify a series of cysteine-reactive agents that inhibit MGL activity with nanomolar potencies by interacting with cysteine residue 208.

Experimental approach: A series of cysteine traps were screened for the ability to inhibit MGL in vitro. Rapid dilution assays were performed to determine reversibility of inhibition. Molecular modelling and site-directed mutagenesis were utilized to identify cysteine residues targeted by the inhibitors.

Key results: The screening revealed that 2-octyl-4-isothiazolin-3-one (octhilinone) inhibited purified rat recombinant MGL (IC₅₀ = 88 ± 12 nM) through a partially reversible mechanism. Initial structure–activity relationship studies showed that substitution of the n-octyl group of octhilinone with a more lipophilic oleoyl group increased inhibitor potency (IC₅₀ = 43 ± 8 nM), while substitution with a methyl group produced the opposite effect (IC₅₀ = 239 ± 68 nM). The inhibitory potency of octhilinone was selectively decreased by mutating cysteine 208 in MGL to glycine (IC₅₀; wild-type, 151 ± 17 nM; C208G, 722 ± 74 nM), but not by mutation of other cysteine residues (C32, C55, C201, C208 and C242).

Conclusions and implications: The results indicated that cysteine 208 plays an important role in MGL function and identified a novel class of isothiazolinone-based MGL inhibitors with nanomolar potency in vitro.

Keywords: monoacylglycerol lipase; 2-arachidonoylglycerol; endocannabinoids

Abbreviations: 1-HG, 1(3)heptadecanoylglycerol; 2-AG, 2-arachidonoyl-sn-glycerol; 2-OG, 2-oleoylglycerol; DMF, dimethylformamide; DTT, dithiothreitol; MGL, monoacylglycerol lipase; MAFP, methyl arachidonylfluorophosphonate; NAM, N-arachidonoylmaileimide

Introduction

Monoacylglycerol lipase (MGL) is a cytosolic serine hydrolase that cleaves monoacylglycerols into fatty acid and glycerol through a catalytic mechanism that involves a classical serine-aspartate-histidine triad (Karlsson et al., 1997). MGL is a member of the α/β-hydrolase family of enzymes (Ollis et al., 1992) and is distantly related to microbial lysophospholipases and haloperoxidases (Karlsson et al., 1997). Because of its abundant expression in mammals in lipid-metabolizing tissues, such as white fat and liver, MGL is thought to catalyse the final step of the lipolytic cascade that releases fatty acids from triacylglycerol stores (Chon et al., 2007). In the brain, however, a primary role of MGL may be to carry out the hydrolysis and inactivation of the endocannabinoid neurotransmitter, 2-arachidonoyl-sn-glycerol (2-AG) (Dinh et al., 2002).

Several findings implicate MGL in the termination of neuronal 2-AG signalling. First, virally induced MGL overexpression attenuates receptor-dependent accumulation of 2-AG in primary cultures of rat cortical neurons (Dinh et al., 2002) and, conversely, RNAi-mediated silencing of MGL increases 2-AG levels in HeLa cells (Dinh et al., 2004). Second, electron microscopy studies have shown that MGL is localized to presynaptic nerve terminals of glutamatergic projection neurons and γ-aminobutyric acid (GABA)-ergic interneurons.
where cannabinoid type-1 (CB1) receptors targeted by 2-AG are also found (Gulyas et al., 2004). Third, blockade of MGL activity by pharmacological inhibitors, such as biphenyl-3-ylicarbamic acid cyclohexyl ester (URB602), elevates 2-AG levels in rat brain (Hohmann et al., 2005; Makara et al., 2005; King et al., 2007) and enhances 2-AG-mediated signalling in both acutely dissected hippocampal slices (Makara et al., 2005; Hashimotodani et al., 2007) and midbrain periaqueductal grey substance in vivo (Hohmann et al., 2005).

Potent and selective MGL inhibitors are not currently available. Nevertheless, such inhibitors would be useful to uncover physiological functions of 2-AG (see Freund et al., 2003; Piomelli, 2003) and validate MGL as a target for therapeutically important drugs (for review, see Piomelli, 2005). Three classes of chemical compounds that interact with MGL have been described thus far. The compound URB602, mentioned above, inhibits MGL activity through a non-competitive, partially reversible mechanism (Hohmann et al., 2005; Makara et al., 2005; King et al., 2007). By contrast, lipophilic serine-reactive agents, such as methyl arachidonylfluorophosphonate (MAFP), irreversibly block MGL, presumably by forming a covalent bond with the catalytic nucleophile, serine 122 (Goparaju et al., 1999). An irreversible mechanism has also been postulated to explain the ability of cysteine-reactive agents, such as N-arachidonylmaleimide (NAM) (Saario et al., 2005) and tetraethylthiuram disulfide (disulfiram) (Labar et al., 2007), to inhibit MGL with nanomolar potencies. In particular, computational and mass spectrometry studies have shown that NAM forms a Michael addition product with cysteine 242 in MGL, which is located in close proximity of the catalytic serine 122 (Saario et al., 2005; Zvonok et al., 2008).

In the present study, we combined structure–activity relationship (SAR) and computational strategies to further characterize the role of cysteine residues in MGL function and identify a new family of isothiazolinone-based MGL inhibitors. Kinetic and site-directed mutagenesis studies showed that the prototype in this class of compounds, ochtolinone, inhibits MGL through a partially reversible mechanism that involves a specific interaction with cysteine 208.

### Methods

**MGL expression and purification**

Purified MGL was prepared as previously described (King et al., 2007). Briefly, a full-length rat MGL cDNA was subcloned into the pET15b vector (Novagen, La Jolly, CA, USA) containing a N-terminal histidine tag and transformed in DH5α Escherichia coli cells. Positive clones were expressed in Rosetta 2(De3)pLysS E. coli cells (Novagen) using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were lysed by using a French press, and membrane fractions were collected and resuspended in 50 mM HEPES pH 7.4, 300 mM NaCl, 3 mM β-mercaptoethanol, 1% Triton X-100. Solubilized supernatant was loaded onto a TALON column (Clontech, Mountain View, CA, USA), and MGL was eluted from the column by using a stepwise gradient of imidazole (10 to 200 mM) at a concentration around 75 mM.

**MGL assay**

Monoacylglycerol lipase activity was measured as previously described (King et al., 2007). Briefly, either 10 ng of purified MGL or 2.5–50 μg of protein from MGL-transfected HeLa cell lysates were preincubated with inhibitors for 10 min at 37°C in assay buffer (50 mM Tris-HCL, pH 8.0, 0.5 mg·mL⁻¹ bovine serum albumin, fatty acid-free). Following preincubation, 2-oleoylglycerol (2-OG) substrate (10 μM final) was added, and samples were incubated for an additional 10 min at 37°C. Reactions were stopped with chloroform : methanol (2:1, vol : vol), containing heptadecanoic acid (5 nmol) as an internal standard. In some experiments with cell lysates, 1(3)heptadecanoylglycerol (1-HG) and heptadecanolic acid were used as substrate and internal standard respectively. Samples were subjected to centrifugation at 2000×g at 4°C for 10 min, and the organic layers were collected and dried under a stream of N₂. The residues were suspended in chloroform : methanol (1:3, vol : vol) and analysed by liquid chromatography/mass spectrometry (LC/MS).

**LC/MS analysis**

We used a reversed-phase Eclipse C18 column (30 × 2.1 mm i.d., 1.8 μM, Agilent Technologies, Wilmington, DE, USA) eluted with 95% of solvent A and 5% solvent B for 0.6 min at a flow rate of 0.6 mL·min⁻¹ with column temperature set at 50°C. Solvent A consisted of methanol containing 0.25% acetic acid and 5 mM ammonium acetate. Solvent B consisted of water containing 0.25% acetic acid and 5 mM ammonium acetate. Under these conditions, analytes eluted from the column at the following retention times: oleic acid, 0.34 min; heptadecanoic acid, 0.37 min; heptadecanolic acid, 0.29 min. Electrospray ionization was in the negative mode, capillary voltage was set at 4 Kv, and fragmentor voltage was 100 V. N₂ was used as drying gas at a flow rate of 13 L·min⁻¹ and a temperature of 350°C. Nebulizer pressure was set at 60 psi. For quantification purposes, we monitored the [M-H]⁻ ions of m/z = 281.3 for oleic acid, m/z = 269 for heptadecanoic acid, and m/z = 267 for heptadecanolic acid.

**Rapid dilution assay**

Rapid dilution assays were performed as previously described (Copeland, 2005). Briefly, samples containing of purified MGL (100-fold concentrated compared with standard assays) were preincubated with 10-fold the IC₅₀-equivalent concentration of ochtolinone, MAFP or vehicle (dimethylsulphoxide, DMSO, final concentration 2%) for 20 min at 37°C. Samples were then diluted 100-fold with assay buffer containing substrate to initiate reactions, and the time course of product formation was measured by LC/MS.

**Molecular modelling**

The crystal structure of chloroperoxidase I (EC 1.11.1.10) from Streptomyces lividans (PDB code 1A88) was used as a template (Hofmann et al., 1998; Saario et al., 2005) to produce a first-generation model of the P20-W289 MGL segment, based on the 1A88-MGL alignment previously reported (Saario et al., 2005). MODELLER 7.0 software was used for 3D
model building, and standard loop modelling settings were applied (Sali and Blundell, 1993). A second-generation alignment was created, moving a small gap from one loop (between S185 and R186, in the loop connecting helices α5 and α6) to another (between A203 and G204, in the loop connecting helices α6 and α7) within the lid domain. This modification resulted in C201 being buried within the active site rather than being exposed to the solvent, as in the first-generation alignment, while retaining the same identity, similarity and gap scores, and maintaining comparable matches in the secondary structure between chloroperoxidase 1 and rMGL as suggested by Saario (Saario et al., 2005) (Figure S1). Five second-generation 3D models were produced. PROCHECK (Laskowski et al., 1993) was used to assess the overall geometric quality of the structures, and the best model (G-factor of −0.23, 86% of allowed residues and 0.8% of disallowed residues) was selected and utilized for modelling purposes. Hydrogen atoms were added by the Biopolymer module of Sybyl (Version 7.2, Tripos Inc., St. Louis, MO, USA), choosing the tautomeric states of histidines that maximized the number of hydrogen bonds within the protein; then the geometry was relaxed by energy minimization. The 2-AG substrate was interactively docked into the rMGL-binding site by using the Dock_minimize module of Sybyl. The docking procedure was followed by an energy minimization of the complex and by 20 cycles of simulated annealing (heating phases of 1000 fs at 700 K followed by cooling phases of 1000 fs at 200 K). The annealed rMGL/2-AG complex was finally submitted to 500 ps of molecular dynamics at 310 K followed by energy minimization. Octhilinone was docked in the active site by using the Dock_minimize procedure. Starting from the resulting complex, a covalent adduct was built, modifying the topology of the inhibitor and C208. The resulting adduct was submitted to a final energy minimization. In all the energy minimizations, simulated annealing and molecular dynamics calculations, the backbone atoms of the protein were kept frozen to preserve the rMGL tertiary structure. MMFF94s (Halgren, 1999) and MMFF94 (Halgren, 1996) force fields were employed, with the dielectric constant set to 1. Energy minimizations were performed to a gradient of 0.1 kcal-(mol·Å)⁻¹.

Site-directed mutagenesis
A full-length rat MGL cDNA was subcloned into the pEF6/V5-His vector (Invitrogen, San Diego, CA, USA). The MGL gene was modified by using the QwikChange II XL Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA, USA) following the manufacturer’s instructions. Primers were commercially synthesized (Invitrogen). Following DNA sequencing, positive clones were identified and transfectioned into HeLa cells by using the Superfect transfectant reagent (Qiagen, Valencia, CA, USA), following standard protocol. Cells were resuspended in ice-cold Tris-HCL (50 mM, pH 8.0) containing 0.32 M sucrose and harvested by using a cell scraper. Lysates were sonicated on ice for 1 min followed by three rounds of freeze thawing. Samples were subjected to centrifugation at 100 000×g at 4°C for 30 min to separate membrane (pellet) and cytosolic (supernatant) fractions.

Protein analysis
Protein concentrations were measured by using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Samples (5 μg) were electrophoresed on a 4–20% SDS-polyacrylamide gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ, USA). Membranes were blocked in 10% milk and incubated in the presence of anti-V5 monoclonal antibody (1:3000, 12 h; Invitrogen). Immuno-reactive bands were visualized by using the ECL-Plus kit (Amersham), exposed to high performance chemiluminescence film (Amersham) and developed with a Mini-Medical film processor (AFP Imaging, Elmsford, NY, USA). As a loading control, membranes were stripped for 15 min at room temperature and probed again by using an anti-actin monoclonal antibody (1:10 000, 12 h; Calbiochem, La Jolla, CA, USA).

Statistical analyses
All results are expressed as mean ± SEM. Non-linear regression analyses were performed by using Prism (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was assessed by the Student’s t-test.

Materials
Methyl arachidonylfluorophosphonate, NAM and NEM were purchased from Cayman Chemical (Ann Arbor, MI, USA). Compounds 1, 2, 6–8 and dithiothreitol (DTT) were from Sigma-Aldrich (St. Louis, MO, USA). Compound 5 was from Acros Organics (Morris Plains, NJ, USA). 2-OG and 1-HG were from Indofine Chemical (Hillsborough, NJ, USA) and Nu-Chek Prep (Elysian, MN, USA) respectively. All drug and molecular target nomenclature follows the Guide to Receptors and Channels (Alexander et al., 2008).

Compound 3 (2-octadec-9-enyl-isothiazol-3-one) was prepared by cyclization of N-oleyl-propiolamide as described below. (i) Preparation of N-octadec-9-enyl-propiolamide: N-oyleyamine (16 mmol) was added under nitrogen atmosphere to a cooled (−40°C) mixture of propiolic acid (16 mmol), triethylamine (16 mmol) and ethylchloroformate (16 mmol), and the resulting mixture was stirred for 90 min at −40°C and then for 16 h at −10°C. Solvent evaporation and purification by flash chromatography (SiO2; DCM : MeOH 98:2) gave N-octadec-9-enyl-propiolamide (yield: 68%) as yellow oil. 1H NMR (300 Mz, CDC13): δ 0.88 (t, 3H, J = 6.39 Hz), 1.28 (m, 22H), 1.52 (m, 2H), 2.01 (m, 4H), 2.77 (s, 1H), 3.29 (q, 2H, J = 6.93), 5.36 (m, 2H), 6.12 (bs, 1H). APCI-MS m/z 320.5 [MH]+.

(ii) Preparation of 2-octadec-9-enyl-isothiazol-3-one: a mixture of N-octadec-9-enyl-propiolamide (1.5 mmol) and ammonium thiosulphate (1.5 mmol) in water-dimethylformamide (DMF) was stirred at 0°C for 21 h under nitrogen atmosphere; HCl (37%) was added and the mixture refluxed for 10 min. Neutralization by sodium bicarbonate solution, solvent evaporation and flash chromatography (SiO2; dicrotomethane : methanol 98:2) afforded a yellow oil, which was further purified by semi-preparative HPLC (Supelco Discover Bio-Wide Pore, 25 cm × 10 mm, 10 microm, 3 mL·min⁻¹ flow rate, acetonitrile : water 85:15, 1 = 254 nm), giving 2-octadec-9-enyl-isothiazol-3-one (yield 7%) as a colourless oil. 1H NMR (300 Mz, CDC13): δ;
0.88 (t, 3H, J = 6.36 Hz), 1.28 (m, 22H), 1.72 (m, 2H), 2.01 (m, 4H), 3.78 (t, J = 7.26, 2H), 5.34 (m, 2H), 6.25 (d, 1H, J = 6.24), 8.03 (d, 1H, J = 6.21). APCI-MS m/z 352.4 [MH]+. Anal. (C_{21}H_{37}NOS) C, H, N.

2-octyl-benzo[d]isothiazol-3-one (4) was prepared by adding benzo[d]isothiazol-3-one (Sigma-Aldrich, 10 mmol) and anhydrous potassium carbonate (16 mmol) to a mixture of 1-chloroorcane (Sigma-Aldrich, 10 mmol) and sodium iodide (16 mmol), which had been refluxed for 30 min in anhydrous DMF, and the reaction vessel was heated by microwaves in two steps of 10 min, setting the temperature to 100°C and 120°C respectively (CEM Discover apparatus). After cooling and filtration the mixture was diluted with water and ice and extracted with dichloromethane. Solvent evaporation afforded a crude oil, which was eluted by flash chromatography (SiO_2, dichloromethane : methanol 99.5:0.5) and distilled under reduced pressure (b.p. 240°C at 1 mmHg). \(^{1}\)H NMR (CDCl_3, 300 MHz): \(\delta\) 0.82–0.87 (m, 3H), 1.24–1.40 (m, 10H), 1.74 (q, 2H, J = 7.4 Hz), 3.87 (t, 2H, J = 7.3 Hz), 7.38 (ddd, 1H, J = 7.9, 6.9, 1.4 Hz), 7.53 (ddd, 1H, J = 8.1, 1.3, 0.8 Hz), 7.58 (ddd, 1H, J = 8.1, 6.8, 1.2 Hz), 8.03 (d, 1H, J = 7.9 Hz). Anal. (C_{21}H_{37}NOS) C, H, N.

**Results**

**MGL inhibition by cysteine trapping compounds**

Previous studies have shown that rat cerebellar membranes contain an MGL-like activity that is weakly inhibited by the cysteine-reactive agent N-ethylmaleimide, but is highly sensitive to inhibition by the lipophilic maleimide NAM (Saario et al., 2005). We replicated these results using recombinant purified rat MGL overexpressed in *E. coli* (NEM, IC\(_{50}\) = 2.7 ± 0.5 μM; NAM, IC\(_{50}\) = 46 ± 7 nM; n = 3) (Figure 1A). Based on computational (Saario et al., 2005) and structural data (Zvonok et al., 2008), the inhibitory effect of NAM has been attributed to the ability of this compound to react with C242 and form a Michael addition product. To further examine the functional role of cysteine residues, we screened a series of structurally different cysteine traps for their ability to inhibit MGL activity (Table 1). All compounds contained an electrophilic centre that could covalently bind sulphydryl groups. Ony only, however, 2-octyl-4-isothiazolin-3-one (octhilinone, 1) was potent in inhibiting MGL, that is, IC\(_{50}\) in nM range (n = 5; Figure 1B). By contrast, compounds 5 (an epoxysuccinate) and 6 (an alkyldene-thioxothiazolidinone) displayed weak inhibitory activities (IC\(_{50}\) in μM range; n = 3), while compounds 7 (a thiadiazole) and 8 (a propoxyphthalide) were inactive at all concentrations tested (up to 1 mM, Table 1).

Figure 1B shows the results of a preliminary SAR study, which investigated the effects of select chemical modifications of octhilinone on MGL inhibition. Substitution of the n-octyl group with a methyl group (2) resulted in a threefold decrease in potency compared with the parent compound (Table 1; P < 0.05, n = 4). By contrast, introduction of a larger and more lipophilic oleoyl group at the same site (3) resulted in a twofold increase in potency (Table 1; P < 0.05, n = 4). Replacement of the isothiazolinone moiety with a benzisothiazolinone group (4) also yielded a potent inhibitor (Table 1; n = 3).

**Mechanism of MGL inhibition by octhilinone**

We utilized a rapid dilution assay (Copeland, 2005) to assess whether octhilinone inhibits MGL through a reversible or irreversible mechanism. Purified MGL was first preincubated with a concentration of octhilinone that was 10-fold higher than its IC\(_{50}\) value, and then diluted 100-fold (to 10% of its IC\(_{50}\) value). As shown in Figure 2A, the dilution produced a partial recovery of enzyme activity. By contrast, when MGL was incubated with the irreversible serine-reacting probe MAFP, MGL activity did not recover after dilution (Figure 2A). The partial reversibility of the effect of octhilinone is not consistent with the chemical reactivity of a Michael addition product, suggesting that octhilinone may form instead a reducible disulphide bond with a cysteine residue in MGL.

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**Table 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC(_{50}) (nM)</th>
<th>Percentage of activity (nM)</th>
<th>SEM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (octhilinone)</td>
<td>2.7 ± 0.5</td>
<td>97.4 ± 0.7</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>2 (methyl-octhilinone)</td>
<td>2.7 ± 0.5</td>
<td>97.4 ± 0.7</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>3 (oleoyl-octhilinone)</td>
<td>2.7 ± 0.5</td>
<td>97.4 ± 0.7</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>4 (benzisothiazolinone)</td>
<td>2.7 ± 0.5</td>
<td>97.4 ± 0.7</td>
<td>0.3</td>
<td>3</td>
</tr>
</tbody>
</table>

**Figure 1** Inhibition of monoacylglycerol lipase (MGL) activity by cysteine-reactive agents. Concentration–response curves for the inhibition of purified MGL overexpressed in *Escherichia coli* by (A) the maleimides N-arachidonylemaleimide (NAM) and NEM and by (B) the isothiazolinones octhilinone (1), 2-methyl-4-isothiazolin-3-one (2) and 2-oleoyl-4-isothiazolin-3-one (3). Results are expressed in percentage of activity in the presence of vehicle (dimethylsulphoxide, final concentration 1%) (mean ± SEM, n = 3–5).

**Figure 2**

(A) Inhibition of purified MGL overexpressed in *E. coli* by (A) the maleimides N-arachidonylemaleimide (NAM) and NEM and by (B) the isothiazolinones octhilinone (1), 2-methyl-4-isothiazolin-3-one (2) and 2-oleoyl-4-isothiazolin-3-one (3). Results are expressed in percentage of activity in the presence of vehicle (dimethylsulphoxide, final concentration 1%) (mean ± SEM, n = 3–5).
### Table 1  Inhibition of purified recombinant MGL by cysteine trap compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>$IC_{50}$</th>
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</thead>
<tbody>
<tr>
<td>N-arachidonylmaleimide</td>
<td><img src="image" alt="Structure" /></td>
<td>$46 \pm 7 \text{nM}$</td>
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<tr>
<td>NEM</td>
<td><img src="image" alt="Structure" /></td>
<td>$3 \pm 0.3 \mu M$</td>
</tr>
<tr>
<td>1(Octhinone)</td>
<td><img src="image" alt="Structure" /></td>
<td>$88 \pm 12 \text{nM}$</td>
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<td><img src="image" alt="Structure" /></td>
<td>$239 \pm 68 \text{nM}$</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="Structure" /></td>
<td>$43 \pm 8 \text{nM}$</td>
</tr>
<tr>
<td>4</td>
<td><img src="image" alt="Structure" /></td>
<td>$59 \pm 7 \text{nM}$</td>
</tr>
<tr>
<td>5</td>
<td><img src="image" alt="Structure" /></td>
<td>$20 \pm 7 \mu M$</td>
</tr>
<tr>
<td>6</td>
<td><img src="image" alt="Structure" /></td>
<td>$28 \pm 0.5 \mu M$</td>
</tr>
<tr>
<td>7</td>
<td><img src="image" alt="Structure" /></td>
<td>$&gt;100 \mu M$</td>
</tr>
<tr>
<td>8</td>
<td><img src="image" alt="Structure" /></td>
<td>$&gt;1 \text{mM}$</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM ($n = 3–4$). 
[Correction added after online publication 30 June 2009: in the chemical structure of N-arachidonylmaleimide a missing oxygen was added]
shown). As expected, incubation with DTT produced no change in the potency of MAFP (Figure 2C). We interpret these results to indicate that octhilinone inhibits MGL activity by interacting with a cysteine residue in this enzyme.

**Localization of cysteine residues in MGL**

There are six cysteine residues in rat and mouse MGL, four of which are conserved in their human orthologue (Figure 3A). A homology model of MGL constructed using as a template the crystal structure of chloroperoxidase L from *S. lividans* (Figure 3B) (Saario et al., 2005) positions C242 on a conserved loop of the enzyme, in close proximity of a histidine residue (H269) that is part of the catalytic triad (S122, D239, H269) (Karlsson et al., 1997). Residues C201 and C208 are located within the lid domain, wherefrom they might be able to extend their sulphydryl groups towards the substrate-binding site, while residues C32 and C55 are positioned in two structurally conserved regions: C32 in the second β-strand and C55 in a loop before the beginning of the first α-helix. C301 is not included in the model because it occupies a region of MGL that is not present in the chloroperoxidase template. Consistent with previous studies (Saario et al., 2005), our computational analyses identify three cysteines (C201, C208 and C242) which, based on their location on the tertiary structure of the enzyme, may exert regulatory roles in MGL function and might, therefore, be targeted by isothiazolinone-based compounds.

**Site-directed mutagenesis**

To test these possibilities, we generated a series of MGL mutants in which individual cysteines were replaced with glycines. We expressed the mutants in HeLa cells and evaluated the impact of each mutation on MGL activity. Wild-type (WT) and mutant proteins displayed comparable levels of heterologous expression (Figure 4A). Nevertheless, the C242G mutation produced a striking reduction in MGL activity (Figure 4B), which was due to a decrease in maximal reaction velocity (V\text{MAX} in pmol·min⁻¹·µg⁻¹); WT, 58 ± 7; C242G, 4 ± 1; n = 3–4) rather than a change in Michaelis constant (K\text{M} in µM; WT, 115 ± 32; C242G, 98 ± 28; n = 3–4). Mutations affecting C201 and C208 also caused significant decreases in enzyme activity, albeit smaller than that produced by C242G (Figure 4C). By contrast, mutations to cysteines located distal to the active site of MGL (C32G, C55G and C301G) had no effect on MGL activity (Figure 4B). The results highlight the important functional role of cysteine residues proximal to the MGL active site, as previously suggested by computational (Saario et al., 2005) and structural studies (Zvonok et al., 2008).

**Interaction of octhilinone with cysteine 208**

We next examined whether cysteine mutations influence the ability of octhilinone to inhibit MGL. As illustrated in Figure 5, mutating C208 to glycine reduced the inhibitory potency of octhilinone, compared with WT MGL (WT, IC_{50} = 151 ± 17 nM; C208G, IC_{50} = 722 ± 74 nM, P < 0.0001; n = 4) (Figure 5A), whereas mutation of all other cysteines produced no significant effect (Figure 5B). Interestingly, the C208G mutation had no effect on the inhibitory potency of NAM, which was selectively reduced instead by mutation of C242 or C201 (WT, IC_{50} = 30 ± 3 nM; C242G, IC_{50} = 107 ± 17 nM, P < 0.05; C201G, IC_{50} = 442 ± 79 nM, P < 0.01; n = 3) (Figure 5C).

**Discussion**

In the present study, we have further characterized the role of cysteine residues in MGL function and identified a novel family of highly potent isothiazolinone-based inhibitors of MGL activity. Preliminary SAR studies on one of these compounds, octhilinone, revealed that increasing lipophilicity of

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Figure 2  Reversibility of monoacylglycerol lipase (MGL) inhibition by octhilinone. (A) Rapid dilution assays of purified MGL in the presence of vehicle (dimethylsulphoxide, final concentration 2%), octhilinone or methyl arachidonylfluorophosphonate (MAFP). See text for details. (B) Scheme illustrating the formation of a disulphide adduct between octhilinone and cysteine. (C) Effects of dithiothreitol (DTT, 10 µM) of octhilinone and MAFP towards purified MGL. Results are from individual experiments, each performed in duplicate.
the carbon side chain enhanced MGL inhibitory potency. Indeed, substitution of the $n$-octyl group with a more lipophilic oleoyl chain increased potency, while substitution with a methyl group decreased it. A role for lipophilicity in modulating the inhibitory potency of cysteine traps towards MGL has been previously noted by Saario et al. (2005), who explored the SAR of maleimide-based compounds such as NAM.

$N$-arachidonylmaleimide reacts with $C_{242}$, which gives a Michael addition to the carbon–carbon double bond (Zvonok et al., 2008). The isothiazolinone group of octhilinone presents a sulphenamide fragment and a conjugated double bond, both potentially able to make an electrophilic attack on a cysteine sulphhydril group. It has been shown that 2-methylisothiazolin-3-one (2) reacts with $N$-acetylcysteine in aqueous solutions, yielding a disulphanyl derivative resulting from attack at the sulphenamide fragment (Alvarez-Sanchez et al., 2002). Yet the close resemblance between the structures of isothiazolinone and maleimide suggests that derivatives of these compounds could inhibit MGL through a Michael addition. To explore the mechanism by which octhilinone inhibits MGL, we first performed rapid dilution assays, which test the reversibility of enzyme–inhibitor interactions (Copeland, 2005). The results showed that octhilinone blocked MGL
activity through a partially reversible mechanism. We hypothesized that this partial reversibility might arise from the formation of a reducible disulphide bond with a cysteine sulphydryl group in MGL, rather than from the generation of a Michael addition product. Accordingly, increasing the reducing potential of the assay buffer by addition of a low concentration of DTT (10 mM) produced a marked decrease in the inhibitory potency of octhilinone. In further support of our hypothesis, the benzisothiazolinone 4, which cannot undergo Michael addition because its conjugated double bond is involved in benzene aromaticity, was as potent as octhilinone at inhibiting MGL activity. Together, the results suggest that the sulphenamide fragment in octhilinone forms a disulphide bond with one or more cysteine residues in MGL.

To identify such residues, we systematically mutated all cysteines in MGL and transiently expressed the mutants in HeLa cells. We found that mutations affecting cysteines that are located in close proximity of the active site caused a loss in basal MGL activity. This result is in apparent contrast with a recent study, which reported that mutating either C208 or C242 does not affect the basal activity of human MGL (Zvonok et al., 2008). The discrepancy might be attributed, however, to the considerable differences in enzyme assay protocols utilized in the two studies. Specifically, we employed an LC/MS-based assay, which quantifies both substrates and products in the MGL reaction, while Zvonok et al. (2008) used a spectrophotometric assay, which measures hydrolysis of the fluorogenic reporter substrate arachidonoyl,7-hydroxy-6-methoxy-4-methylcoumarin ester.

In addition to its effect on baseline MGL activity, mutation of C208 caused a marked decrease in the inhibitory potency of octhilinone. The remaining inhibitory effect of octhilinone on the C208G mutant was likely due to non-selective interactions with other amino acid residues in MGL, because mutations targeting other cysteines did not affect the compound’s inhibitory potency. Thus, the high-potency component of the inhibitory effect of octhilinone on MGL appears to be due to a preferential interaction of this compound with C208. A docking position for a disulphide adduct of octhilinone with C208 is illustrated in Figure 6.

Previous studies have demonstrated that NAM inhibits MGL by interacting with C242 (Saario et al., 2005; Zvonok et al., 2008). Our result with the C242G mutant, which shows a 3.5-fold decrease in NAM potency, supports the conclusion that inhibition of MGL by NAM occurs via C242. However, we also see a significant rightward shift in potency resulting from mutation of C201. This unexpected observation suggests a previously unrecognized interaction between NAM and C201, which deserves further investigation.

In conclusion, the serine lipase MGL may be a crucial component of the presynaptic mechanism by which neurons inactivate the endocannabinoid 2-AG. Thus, targeting this enzyme might have a variety of therapeutic applications, including analgesia, anti-inflammation and neuroprotection (Hohmann et al., 2005; Comelli et al., 2007; Mechoulam and Shohami, 2007; Desroches et al., 2008). The present results underscore the functional importance of select cysteine residues in MGL activity and introduce a novel isothiazolinone-based scaffold, which may be used to design novel MGL inhibitors.

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Conflicts of interest

None.

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Sequence alignments of rat monoacylglycerol lipase (MGL) (P20-W289 segment) and Chloroperoxidase L from *Streptomyces lividans*. rMGL1: first-generation alignment [Saario 2005]: identity score: 17%; similarity score: 32%; gap score: 7% (calculated by GeneDoc: http://www.nrbsc.org/gfx/genedoc). rMGL2: second-generation alignment: identity score: 17%; similarity score: 31%; gap score: 7%. Conserved residues are highlighted in bold, cysteine residues of rMGL are coloured in red. S185-R186 and A203-G204 residues are underlined and coloured in blue. Secondary structure elements of Chloroperoxidase L, derived from the crystallographic coordinates (PDB code 1A88), are denoted by the letters E (b sheet) or H with (a helix). Numbers refers to rMGL1 sequence.

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