BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00974.x
www.brjpharmacol.org

RESEARCH PAPER

Inhibition of monoacylglycerol lipase by troglitazone, *N*-arachidonoyl dopamine and the irreversible inhibitor JZL184: comparison of two different assays

E Björklund, E Norén, J Nilsson and CJ Fowler

Department of Pharmacology and Clinical Neuroscience, Umeå University, Umeå, Sweden

Correspondence
CJ Fowler, Department of Pharmacology and Clinical Neuroscience, Umeå University, SE-901 87 Umeå, Sweden. E-mail: cf@pharm.umu.se

Keywords
monoacylglycerol lipase; cannabinoid; troglitazone; rosiglitazone; peroxisome proliferator-activated receptor γ

Received
12 April 2010
Revised
22 June 2010
Accepted
12 July 2010

BACKGROUND AND PURPOSE
Drugs used clinically usually have a primary mechanism of action, but additional effects on other biological targets can contribute to their effects. A potentially useful additional target is the endocannabinoid metabolizing enzyme monoacylglycerol lipase (MGL). We have screened a range of drugs for inhibition of MGL and compared the observed potencies using different MGL enzyme assays.

EXPERIMENTAL APPROACH
MGL activity was screened using recombinant human MGL (cell lysates and purified enzyme) with 4-nitrophenyl acetate (NPA) as substrate. 2-Oleolyglycerol metabolism by rat cerebellar cytosolic MGL and by recombinant MGL was also investigated.

KEY RESULTS
Among the 96 compounds screened in the NPA assay, troglitazone, CP55,940, *N*-arachidonoyl dopamine and AM404 inhibited NPA hydrolysis by the lysates with IC_{50} values of 1.1, 4.9, 0.78 and 3.1 μM, respectively. The potency for troglitazone is in the same range as its primary pharmacological activity, activation of peroxisome proliferator-activated receptor (PPAR) γ. Among PPARγ ligands, the potency order towards human MGL was troglitazone > ciglitazone > rosiglitazone > 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ≈ CAY 10415 > CAY 10514. In contrast to the time-dependent inhibitor JZL184, the potency of troglitazone was dependent upon the enzyme assay system used. Thus, troglitazone inhibited rat cytosolic 2-oleoylglycerol hydrolysis less potently (IC_{50} 41 μM) than hydrolysis of NPA by the human MGL lysates.

CONCLUSIONS AND IMPLICATIONS
'Hits' in screening programmes for MGL inhibitors should be assessed in different MGL assays. Troglitazone may be a useful lead for the design of novel, dual action MGL inhibitors/PPARγ activators.

Abbreviations
NPA, 4-nitrophenyl acetate; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; AM404, *N*-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; BSA, bovine serum albumin; CAY10415 (5-[[4-[2-(5-ethyl-2-pyridinyl)-2-oxoethoxy]phenyl]methyl]-2,4-thiazolidinedione; CAY10514, 8-hydroxy-8-[2-(pentyloxy)phenyl]-5-octynoic acid, methyl ester; CB, cannabinoid; CP55 940 (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; FAAH, fatty acid amide hydrolase; JWH015 (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone; JZL184, 4-nitrophenyl-4-(dibenzo[d][1,3] dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; MGL, monoacylglycerol lipase; 2-OG, 2-oleoylglycerol; PPAR, peroxisome proliferator-activated receptor; URB597, 3′-(aminocarbonyl)[1,1′-biphenyl]-3-3-yl)-cyclohexylcarbamate; WIN 55,212-2 (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

© 2010 The Authors
British Journal of Pharmacology © 2010 The British Pharmacological Society

Introduction

The endocannabinoid system, comprising the endogenous cannabinoids (CB) anandamide (AEA, arachidonoyl ethanolamide) and 2-arachidonoylglycerol (2-AG), their target CB receptors, and their synthetic and degradative enzymes, are involved in a wide number of physiological processes in the body, ranging from cognition to bone metabolism (review, see Pacher *et al.*, 2006). AEA is primarily metabolized by the enzyme fatty acid amide hydrolase (FAAH, also known as FAAH1, Alexander *et al.*, 2009), and selective inhibitors of this enzyme such as URB597 (3′-(aminocarbonyl)[1,1′-biphenyl]-3-3-yl)-cyclohexylcarbamate, Kathuria *et al.* 2003) have shown potentially useful activity in models of pain, inflammation, anxiety and depression (see, e.g. Kathuria *et al.*, 2003; Gobbi *et al.*, 2005; Holt *et al.*, 2005; Jayamanne *et al.*, 2006). 2-AG is also a substrate for FAAH, cyclooxygenase-2, and the hydrolytic enzymes α/β-hydrolase domain (ABHD)12 and ABHD6, but in the mouse brain, the most important hydrolytic enzyme is monoacylglycerol lipase (MGL), which accounts for ~85% of the hydrolysis of this substrate (Goparaju *et al.*, 1998; Dinh *et al.*, 2002; Blankman *et al.*, 2007). JZL184 (4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate, Long *et al.*, 2009a), a selective irreversible inhibitor of MGL, has been shown to produce anti-nociceptive effects in the chronic constriction injury model of neuropathic pain (Kinsey *et al.*, 2009).

The wide range of effects produced by the endocannabinoid system in the body raises the possibility that many drugs that are, or have been, used clinically could interact with this system and that this interaction may contribute to their biological effects in humans over and above their primary pharmacological effect. Compounds directly activating CB_1 receptors would presumably have been discovered by recreational drug abusers, but compounds with inhibitory effects upon FAAH and MGL are not likely to be of interest in this respect since they do not produce cannabis-like behaviours, unless both enzymes are inhibited by the compound in question (Kathuria *et al.*, 2003; Long *et al.*, 2009a,b). There is evidence that non-steroidal anti-inflammatory drugs inhibit FAAH *in vitro* and that these drugs can influence endocannabinoid-mediated responses *in vivo* (Fowler *et al.*, 1997; Gühring *et al.*, 2002; Guindon *et al.*, 2006; Naidu *et al.*, 2009; Bishay *et al.*, 2010). The intravenous anaesthetic propofol is also a FAAH inhibitor, a property that may contribute to its pharmacological effects *in vivo* (Patel *et al.*, 2003).

It is not known, however, whether MGL is also inhibited by drugs that are either currently or previously in our therapeutic arsenal or used experimentally, for pharmacological properties other than MGL inhibition. One way of identifying such compounds is the use of an assay permitting the characterization of a large number of compounds. One such assay, whereby the hydrolysis of 4-nitrophenyl acetate (NPA) by recombinant MGL can be followed spectrophotometrically, has recently been reported (Muccioli *et al.*, 2008). In the present study, we investigated a series of drugs with primary effects in therapeutic areas known to be sensitive to CB actions, such as non-steroidal anti-inflammatory drugs, antidepressants and drugs for the treatment of type 2 diabetes. Active compounds have been characterized further both in the NPA assay and in a standard radiochemical assay using 2-oleoylglycerol (2-OG) as substrate. We report that the peroxisome proliferator-activated receptor (PPAR) γ ligand troglitazone and the endogenous vanilloid receptor agonist, *N*-arachidonoyl dopamine, are relatively potent MGL inhibitors using the NPA assay, but that the potency of troglitazone is dependent upon the MGL assay used.

Methods

Spectrophotometric assay for MGL activity using recombinant human MGL

Assays were carried out in a 96-well microtiter plate (100 μL total volume) using a method based on the assay of Muccioli *et al.* (2008). Human recombinant MGL (either clear lysates or purified enzyme, as indicated) in 10 mM Tris–HCl, 1 mM EDTA, pH 7.4 (added volume 70 μL) was added to each well which also contained test compounds [3 μL in vehicle, DMSO except for *N*-arachidonoyl dopamine (ethanol), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and CAY 10541 (methyl acetate)] or vehicle alone. Buffer (7 μL) was added to each well. To start the hydrolysis, NPA (20 μL, final concentration of 0.25 mM, unless otherwise shown) was added rapidly. Blanks contained buffer alone. The concentration of MGL was chosen to ensure that initial velocities were measured. The samples were incubated at room temperature. The absorbance was measured at 405 nm after 0 min (to rule out effects of the compounds *per se* on the absorbance) and at least two 20 min intervals, thereafter using a Thermomax Microplate Reader (ThermoMax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). For the experiments reported here, the 20 min time point was used to ensure that initial activities were measured but at the same time allow for sufficient

product formation. In a typical experiment, the OD values for the blanks were ~0.045 (with very little variation between assays), while controls were two- to threefold higher than blanks (with a small intra-assay variation). Measurements at 40 min were also made to confirm the inhibition at a higher control : blank ratio.

Radiochemical assay for 2-OG hydrolysis by rat brain extracts

All animal care and experimental procedures complied with national guidelines and laws and were approved by the local animal ethical committee. Assays were carried out essentially as described in Ghafouri *et al*. (2004), but with a charcoal separation (Boldrup *et al*., 2004) in place of a chloroform : methanol extraction. Briefly, cerebella from adult Wistar or Sprague-Dawley rats that had been obtained previously and stored frozen at –80°C were thawed and homogenized in 0.32 M sucrose containing 50 mM sodium phosphate, pH 8. Homogenates were centrifuged at 100 000 *g* for 60 min at 4°C and the supernatants ('cytosolic fractions') were collected and stored at –80°C in aliquots until used for assay. Protein concentrations for the assays were 3 μg/assay, the fractions being diluted with 10 mM Tris–HCl, 1 mM EDTA, pH 7.4.

The radiochemical assays contained enzyme source (cytosol or recombinant MGL), 10 mM Tris–HCl, 1 mM EDTA and test compound (10 μL, in vehicle [DMSO for troglitazone and JZL184; ethanol for *N*-arachidonoyl dopamine) in a volume of 175 μL. After pre-incubation, when appropriate, 25 μL of a solution of [^{3}H]2-OG (4 μM, to give an assay concentration of 0.5 μM) in 10 mM Tris buffer, pH 7.4 containing 1% (w v^{-1}) fatty acid-free bovine serum albumin (BSA, assay concentration 0.125% w v^{-1}) was added and the samples were incubated for 10 min at 37°C. Thereafter, reactions were stopped by the addition of 400 μL of active charcoal mixture (80 μL charcoal + 320 μL 0.5 M HCl). After vortex mixing and phase separation, aliquots of the aqueous phase were counted for tritium content by liquid scintillation spectroscopy with quench correction. Blanks were prepared in the same manner but without enzyme source. FAAH assays with JZL184 were carried out using homogenates of whole brain (minus cerebella, 0.6 μg protein per assay) from adult Wistar or Sprague-Dawley rats using the method of Boldrup *et al*. (2004) and [^{3}H]AEA (labelled in the ethanolamine part of the molecule, final concentration 0.5 μM) as substrate.

Analysis of data

K_m^{app}, V_{max}^{app}, pI_{50} and IC_{50}, values, linear regressions and confidence limits were determined using the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA). The pI_{50}, and thereby IC_{50} values, were calculated using the built-in programme 'sigmoidal dose–response (variable slope)' from the data expressed as % of vehicle controls using top (i.e. uninhibited) values of 100% and bottom (residual activity) values that were either set to zero or allowed to float. The two curves were compared using Akaike's informative criteria and the preferred model thereafter used.

Compounds

2-Oleoylglyceerol [glycerol – 1,2,3-^{3}H] (1.48 TBq.mmol^{-1}) and AEA [ethanolamine-1-^{3}H] (specific activity 2.22 TBq.mmol^{-1}) were purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO, USA). Human recombinant His-tagged MGL expressed in *Escherichia coli* (hMGL), either as clear lysates, catalogue nos. #10008354 and #705194; (according to the manufacturers, the two lysate preparations are the same enzyme preparation, but are used as part of different enzyme assay kits. The #10008354 is now discontinued. We have indicated the catalogue number of the lysate used in the Figure legends) or as the enzyme that had been further purified using a nickel column, catalogue no. #10007812; non-radiolabelled AEA, troglitazone, rosiglitazone, ciglitazone, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, CAY10415 (5-[[4-[2-(5-ethyl-2-pyridinyl)-2-oxoethoxy]phenyl]methyl]-2,4-thiazolidinedione, compound 10 of Tanis *et al*. 1996), CAY10514 (8-hydroxy-8-[2-(pentyloxy)phenyl]-5-octynoic acid, methyl ester, Compound 14a of Caijo *et al*. 2005), JZL184 (4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl (hydroxy)methyl)piperidine-1-carboxylate) and *N*-arachidonoyl dopamine were obtained from the (Cayman Chemical Co., Ann Arbor, MI, USA). AM404 (*N*-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), CP 55 940 ((-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol), JWH015 ((2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone) and WIN55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) were obtained from Tocris Bioscience (Ellisville, MO, USA). NPA, fatty acid-free BSA, 4-nitrophenol and non-radioactive 2-OG were obtained from the Sigma Chemical Co., St. Louis, MO, USA.

Results

Preliminary screen of selected compounds for inhibition of MGL using the NPA assay

The NPA assay was used with the clear lysates of recombinant human His-tagged MGL expressed in

E. coli ('hMGL lysate') as enzyme source. Initial experiments indicated that the OD_{405} for the NPA hydrolysis product, 4-nitrophenol, was linear over the range 0.01–0.15 mM (r^2 = 0.998, seven concentrations tested) and that the OD_{405} measured when the hMGL lysates were incubated with NPA showed the appropriate dependency upon the incubation time and enzyme concentration (data not shown). Over a range of NPA concentrations (0.08–0.64 mM), the hydrolysis produced by the hMGL lysates was saturable, with a K_m value of 0.23 mM (data not shown), consistent with the study of Muccioli *et al.* (2008) who used a different hMGL preparation. Finally, the rate of NPA hydrolysis showed the expected pH optimum (~7), consistent with the literature (Di Marzo *et al.*, 1999; Goparaju *et al.*, 1999), and was reduced by the alternative substrates, 2-AG and 2-OG, but not by AEA, which is not a substrate for this enzyme (Dinh *et al.*, 2002) (data not shown).

A series of 96 compounds available at the department were tested at two concentrations, 3 and 10 μM. The compounds tested included a number of agents known to interact with the CB system, non-steroidal anti-inflammatory agents, compounds with different actions upon targets in the brain (such as, for example, antipsychotic and antidepressant drugs) and some naturally occurring compounds. The initial screen was carried out with single assays, albeit with separate controls for the 3 μM and 10 μM concentrations, to allow the identification of 'hits', and the values should be considered in this light. Four compounds were found to produce more than 60% inhibition at concentrations of both 3 and 10 μM: the CB receptor agonist CP55,940, the endogenous transient receptor potential vanilloid 1 (TRPV1) agonist *N*-arachidonoyl dopamine, the FAAH inhibitor and TRPV1 antagonist, *N*-arachidonoyl serotonin, and the PPARγ ligand, troglitazone (see Table 1 for a selection of the compounds). Three compounds produced >60% inhibition at 10 but not 3 μM concentrations: the AEA uptake inhibitor AM404, the lipase (including diacylglycerol lipase) inhibitor tetrahydrolipstatin, and the CB_2 receptor-selective agonist JWH015. Among other compounds tested, it was noted that in some cases, the compounds increased the observed rate of NPA hydrolysis, a case in point being JWH133 (see Table 1). The high value for JWH133 was not due to assay interference, as this compound, at the concentrations tested, did not affect the OD at the 0 min time point. In a repeat experiment, activation was also seen when the compound was pre-incubated with the enzyme for 30 or 60 min prior to addition of substrate, but the degree of activation was very variable, and it was not seen in the absence of a pre-incubation phase (see legend to Table 1). This property of the compound was not studied further, given its limited robustness and given that the concentrations used are much higher than needed to interact with CB_2 receptors (Huffman *et al.*, 1999), but it may reflect some form of allosteric action on the enzyme. The raw data for the compounds corresponding to the major groups described above are given for reference purposes in Table 1.

Inhibition of MGL by troglitazone and the other 'hits' in the primary screen

Six of the 'hits' described above were characterized further using hMGL lysates and NPA as substrate. Concentration–response curves for troglitazone, CP55,940, *N*-arachidonoyl dopamine and AM404, over the range 0.1–10 μM were undertaken and pI_{50} values (IC_{50} values in brackets) of 5.95 ± 0.04 (1.1 μM), 5.31 ± 0.07 (4.9 μM), 6.11 ± 0.08 (0.78 μM) and 5.51 ± 0.09 (3.1 μM), respectively, were found (Figure 1A). For WIN55,212-2 and JWH015, there was a large variability at the highest concentration tested, presumably related to the solubility of the compounds, precluding accurate determinations of their IC_{50} values. The inhibition of MGL by troglitazone was compared with that for other PPARγ ligands (Figure 1B; note that the datasets for troglitazone in the two panels of Figure 1 are different, but show very similar potencies), and the potency order was found to be troglitazone > ciglitazone > rosiglitazone > 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ≈ CAY 10415 > CAY 10514 (Table 2). This differs considerably from their potency order as inhibitors of FAAH or as activators of PPARγ (see Table 2).

The mode of inhibition by troglitazone of NPA hydrolysis by hMGL lysates was investigated. The inhibition showed no time dependency (Figure 2A), but dilution experiments suggested that it was not rapidly reversible. In the latter experiments, troglitazone was incubated with the human MGL lysates (catalogue no. #705194) for 60 min at room temperature prior to addition of substrate and either a fivefold dilution or no dilution prior assay for activity. For a fully reversible compound, the inhibitory potency at, say, 10 μM prior to dilution should be the same as that seen for an undiluted sample using 2 μM inhibitor, whereas for an irreversible or tight-binding compound, the inhibitory potency should resemble that seen for an undiluted sample using 10 μM inhibitor. For the irreversible compound JZL184, used as a positive control, the % of vehicle control activity (means ± SEM, n = 3) seen after pre-incubation with 50 nM compound followed by fivefold dilution was 7 ± 0.8, which was similar to that seen with the undiluted samples incubated with 50 nM compound (5 ± 1) and much lower than seen with the undiluted samples incubated

Table 1

Effects of 76 compounds with different biological actions upon the activity of human recombinant MGL (lysate assay, 0.25 mM NPA as substrate)

Compound	Primary pharmacological action	MGL activity (% of control) 3 μM	10 μM
CB/TRPV1 receptor ligands and related compounds			
ACEA	CB_1 receptor agonist	126	81
AM251	CB_1 receptor inverse agonist	69	62
AM404**	Blocks endocannabinoid reuptake	63	**18**
AM630	CB_2 receptor inverse agonist	90	55
N-arachidonoyl serotonin	FAAH and TRPV1 inhibitor	**37**	**32**
N-arachidonoyl dopamine**	Endogenous TRPV1 ligand	**15**	**–6**
Capsazepine	TRPV1 antagonist	90	63
CP 55,940**	Non-selective CB receptor agonist	**28**	**26**
JWH015**	CB_2 receptor agonist	53	**37**
JWH133[a]	CB_2 receptor agonist	172	170
(±)-2-Methylarachidonyl-2′-fluoroethylamide	CB_1 receptor agonist	89	99
Resveratrol	Antioxidant, CB_1 receptor ligand	101	86
RHC-80267	DAG lipase inhibitor	102	71
(±)SLV 319	CB_1 receptor inverse agonist	99	79
Tetrahydrolipstatin	Lipase inhibitor (incl. DAG lipase)	95	**37**
Win 55,212-2 mesylate**	Non-selective CB receptor agonist	67	**25**
Non-steroidal anti-inflammatory drugs			
Acetylsalicylic acid*, diclofenac, dipyrone, fenbufen*, fenoprofen, flufenamic acid, ketoprofen*, meloxicam*, nabumetone, niflumic acid, nimesulide, sulindac, suprofen	Inhibition of cyclooxygenase	range 76–147	range 95–161
Antidepressants			
Amitriptyline, clomipramine, desipramine, imipramine, nortriptyline, maprotiline	Monoamine reuptake inhibitors	range 82–120	range 78–122
Phenelzine	Monoamine oxidase inhibitor		
Other CNS-active drugs and related compounds			
Haloperidol, cis-(Z)-flupenthixol	Dopamine receptor antagonists	range 86–116	range 72–116
Spiperone*	DA/5-HT_{1A} receptor antagonists		
Ketanserin, pirenperone*	5-HT_2 receptor antagonists		
Promethazine	Histamine H_1 receptor antagonist		
Diazepam, flurazepam, temazepam	Benzodiazepines		
Sodium barbital	Barbiturate		
Nipecotic acid	GABA reuptake inhibitor		
(+)MK801	NMDA receptor antagonist		
Naloxone	Opioid receptor antagonist		
Physostigmine sulphate	Acetylcholinesterase inhibitor		
Other drugs and related compounds			
Lidocaine, tetracaine	Na^+-channel blockers	range 87–170	range 90–114
Verapamil	L-type Ca^{2+} channel blocker		
Lovastatin	HMG-CoA reductase inhibitor		
S(–)Atenolol, R(+)atenolol	ß-Adrenoceptor antagonists (S>R)		
Captopril	ACE inhibitor		
Theophylline, 8-phenyltheophylline	Adenosine receptor antagonists		
Cromolyn sodium	'Mast cell stabiliser'		
Progesterone	Sex steroid hormone		
Troglitazone**	PPARγ ligand	**21**	**2**

Table 1

Continued.

Compound	Primary pharmacological action	MGL activity (% of control) 3 μM	10 μM
Naturally occurring, biologically active compounds			
Coumestrol	Non-steroidal phytoestrogen	range 74–99	range 45–143
Baicalein, fisetin, luteolin, morin, phloretin, quercetin	Flavonoids; antioxidant and other properties		
Other compounds (a selection)			
3-Hydroxytyramine		Range 92–126	range 76–103
Benzamide			
Hydrocinnamic acid			
N-Acetyl-D-sphingosine			
8-Methyl-*N*-vanillyl-6-noneamide			
Thimerosal		61	45

Data are for single experiments, albeit run on different plates for the 3 μM and the 10 μM test concentrations, with 'hits' (>60% inhibition at the test concentration shown) given in bold text. Selected compounds (marked with asterisks) were retested at a later date at a concentration of either 10 μM (inactive compounds, *) or over a concentration range (**, compounds shown in Figure 1A) to confirm the reliability, but the values shown here are from the initial experiments.

[a]JWH133 was also retested at a later stage, and the % of control activities seen after 0, 30 and 60 min pre-incubation, respectively, were: 3 μM, 100 ± 17, 129 ± 17 and 133 ± 25; 10 μM, 98 ± 20, 146 ± 8 and 159 ± 21 (means ± SEM, $n = 3$).

with 10 nM JZL184 (43 ± 3). A similar pattern was seen with troglitazone: the % of control activity (means ± SEM, $n = 3$) seen after pre-incubation with 10 μM compound followed by fivefold dilution was 34 ± 3, which was similar to that seen with the undiluted samples incubated with 10 μM compound (33 ± 2) and much lower than seen with the undiluted samples incubated with 2 μM troglitazone (77 ± 4).

The saturation curves for NPA incubated with troglitazone (no pre-incubation phase) are shown in Figure 2B. The K_m^{app} values calculated from the mean data with 0 and 0.75 μM troglitazone were 0.17 and 0.70 mM, respectively. The corresponding V_{max}^{app} values (calculated using a *post hoc* calibration curve for 4-nitrophenol) were 0.44 and 0.57 nmol substrate hydrolysed per minute and assay tube, respectively, each assay tube containing 0.01 μL of the lysate preparation. At higher troglitazone concentrations, the K_m^{app} values were greater than the highest substrate concentration tested, and so could not be determined with accuracy. A slope re-plot with 0 and 0.75 μM troglitazone gave an approximate K_i value of 0.34 μM. This is in good agreement with the value of K_i 0.45 calculated from the IC_{50} value of 1.1 μM (Table 2) assuming a competitive interaction and a K_m value of 0.17 mM.

Assay dependency of the inhibition of MGL by troglitazone, N-arachidonoyl dopamine and JZL184

In order to investigate whether the inhibition of MGL by the compounds was assay, substrate and/or species dependent, two assay systems were compared: (i) NPA as substrate, hMGL lysates or purified hMGL as enzyme sources, assays run at room temperature; (ii) 2-OG as substrate, rat cytosol, hMGL lysates or purified hMGL as enzyme sources; 0.125% $w \cdot v^{-1}$ fatty acid-free BSA present, assays run at 37°C.

In order to establish the degree of assay variation for a well-characterized compound, we used the selective irreversible MGL inhibitor JZL184 (Figure 3). In all assays, the compound inhibited the substrate hydrolysis in a time-dependent manner, consistent with its irreversible mode of action (Long *et al.*, 2009a). JZL184 inhibited NPA hydrolysis by the hMGL lysates with pI_{50} values of 7.17 ± 0.04 (IC_{50} value 68 nM) and 8.33 ± 0.04 (IC_{50} value 4.7 nM) following 0 and 60 min of pre-incubation, respectively. When 2-OG was used as substrate, the potency was about fourfold lower (Figure 3A). In their original study, Long *et al.* (2009a) used recombinant mouse MGL with 100 μM 2-AG as substrate and reported IC_{50} values of 6 nM, following a 30 min pre-incubation period. For the 2-OG assays, the control activity was affected by the

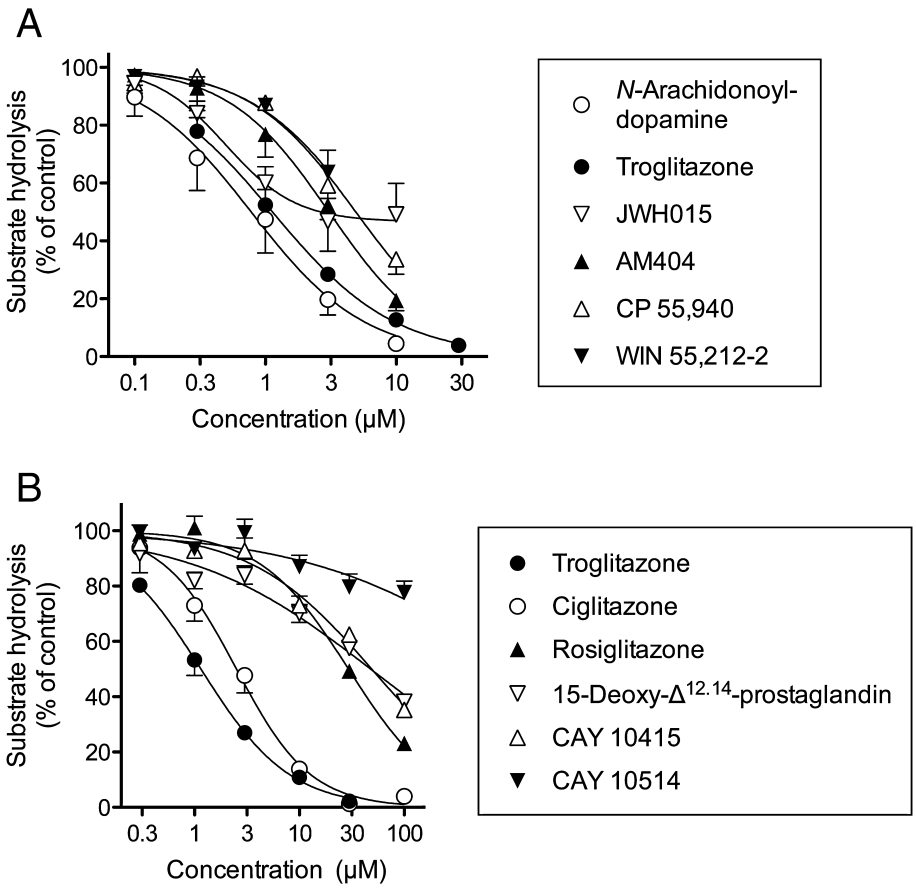


Figure 1

Inhibition of the hydrolysis of 4-nitrophenyl acetate by human recombinant monoacylglycerol lipase lysates by (A) 'hits' from the initial screen of a small compound library and (B) peroxisome proliferator-activated receptor (PPAR) γ agonists. Shown are means and SEM (when not enclosed by the symbols), n = 3–4. The catalogue numbers of the lysates were for Panel A, #705194 and for Panel B 10008354.

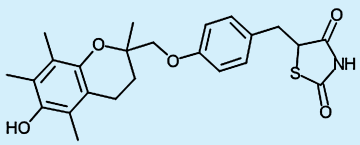
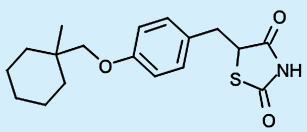
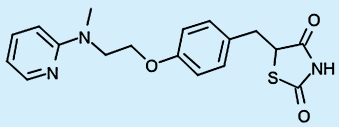
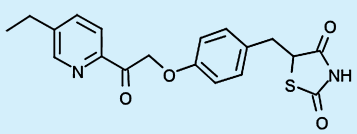
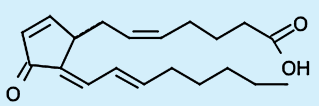
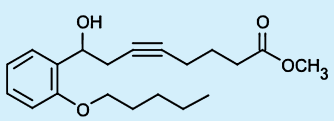
pre-incubation phase; thus, the rate of hydrolysis of 2-OG by the pre-incubated lysates was 36 ± 0.8% of the corresponding activity seen for the samples that were not pre-incubated. This was even more apparent when the purified hMGL was used: the rate of hydrolysis of the pre-incubated control lysates was only 14 ± 3% of that seen in the absence of pre-incubation. A reduction in activity was also seen with pre-incubation in the NPA assays, and the reduction was more variable. However, the remaining activity after the pre-incubation phase was sensitive to inhibition by JZL184, with pI_{50} values of 7.78 ± 0.12 (IC_{50} value 17 nM) and 8.02 ± 0.08 (IC_{50} value 9.6 nM) being found for NPA and 2-OG as substrates, respectively (Figure 3B). Finally, JZL184 inhibited rat cytosol 2-OG hydrolysis with pI_{50} values of 6.46 ± 0.04 (IC_{50} value 350 nM), 7.91 ± 0.10 (IC_{50} value 12 nM, residual activity 16 ± 6%) and 8.24 ± 0.02 (IC_{50} value 5.8 nM, residual activity 9 ± 1%) following 0, 30 and 60 min pre-incubation times, respectively (Figure 3C). In a single experiment, the effect of JZL184 upon 2-OG hydrolysis by the hMGL lysates was investigated at room temperature, rather than at 37°C. Approximate IC_{50} values of 850 and 14 nM were found following pre-incubation times of 0 and 60 min, respectively, in line with the data at 37°C (data not shown). Taken together, the results show there is a time dependency in the sensitivity of MGL to JZL184, but the potencies for a given pre-incubation time are reasonably consistent in the different assays.

In the absence of a pre-incubation phase, *N*-arachidonoyl dopamine inhibited the hydrolysis by the hMGL lysates of NPA and 2-OG with pI_{50} values of 6.11 ± 0.08 (IC_{50} value 0.78) and 5.66 ± 0.03 (IC_{50} value 2.2 μM), respectively), while the compound was less potent as an inhibitor of the hydrolysis of 2-OG by rat cytosol (pI_{50} value 4.70 ± 0.04, IC_{50} value 20 μM) (Figure 4A).

For troglitazone (no pre-incubation), there was a large variation in the inter-assay potencies. With NPA as substrate, the compound inhibited the

Table 2

Inhibition of NPA hydrolysis in hMGL lysates by PPARγ ligands

Compound	Structure	hMGL pI_{50} [IC_{50} (μM)]	rat brain FAAH IC_{50} (μM)	PPARγ EC_{50} (μM) (h/m)
Troglitazone		5.94 ± 0.03 (1.2)	~200 μM[a]	0.55/0.78[b]
Ciglitazone		5.60 ± 0.05 (2.5)	84 μM[a]	–/3[c]
Rosiglitazone		4.53 ± 0.04 (29)	~200 μM[a]	0.043/0.076[b]
CAY 10415		4.31 ± 0.04 (49)		
15-deoxy-$\Delta^{12,14}$-prostaglandin J_2		4.32 ± 0.06 (48)	87 μM[a]	–/2[d]
CAY 10514		<4 (>100)		0.64/–[e]

The pI_{50} values and hence IC_{50} values were calculated from the raw data shown in Figure 1B, using hMGL lysates (catalogue no. #10008354) and 0.25 mM NPA. [a]The FAAH data are for rat brain homogenates, 0.5 μM [^{3}H]AEA as substrate, and an assay pH of 7.4, and are taken from Lenman *et al.* (2007). The PPARγ values for troglitazone, ciglitazone, rosiglitazone, 5-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and CAY10514 are for transactivation assays using human (h) or mouse (m) chimeric receptors and are from [b]Willson *et al.* (2000); [c]Willson *et al.* (1996); [d]Kliewer *et al.* (1995) and [e]Caijo *et al.* (2005). Note that CAY10514 showed a low efficacy compared with rosiglitazone, which in the same study reported to have an EC_{50} value of 0.004 μM. Comparable data are not (to our knowledge) reported for CAY10415. However, this compound increases insulin-stimulated inccorporation of [^{14}C]acetate into lipid in differentiated mouse 3T3-L1 cells at 3–10 μM concentrations (Tanis *et al.*, 1996).

purified hMGL with a threefold lower potency than seen for the lysate, and a pI_{50} value of 4.61 ± 0.07 (IC_{50} value 25 μM, residual enzyme activity 21 ± 7%) was seen for rat cytosols with 2-OG as substrate (Figure 4B). Rosiglitazone was also less potent as an inhibitor of 2-OG hydrolysis by the cytosols than of NPA hydrolysis by hMGL lysates, producing 31 ± 1% inhibition at the highest concentration tested (100 μM) for the cytosols (data not shown) as compared to an IC_{50} value of 29 μM for the hMGL lysates (Table 2). Most surprisingly, troglitazone was a very poor inhibitor of the hMGL lysate when assayed radiochemically with 2-OG as substrate (Figure 4B). This was not due to the assay temperature used, as a single experiment with 2-OG carried out at room temperature gave an IC_{50} value of approximately 62 μM for the inhibition by troglitazone of the hMGL lysate-catalysed hydrolysis of 2-OG (data not shown).

Three possible reasons for the discrepancy in potencies seen for troglitazone in the different assays using the same enzyme source were investigated:

1 The effect of troglitazone upon NPA hydrolysis is an artefact of the assay. For all compounds, the initial reading at t = 0 min was made to ensure that the compounds did not produce a signal at

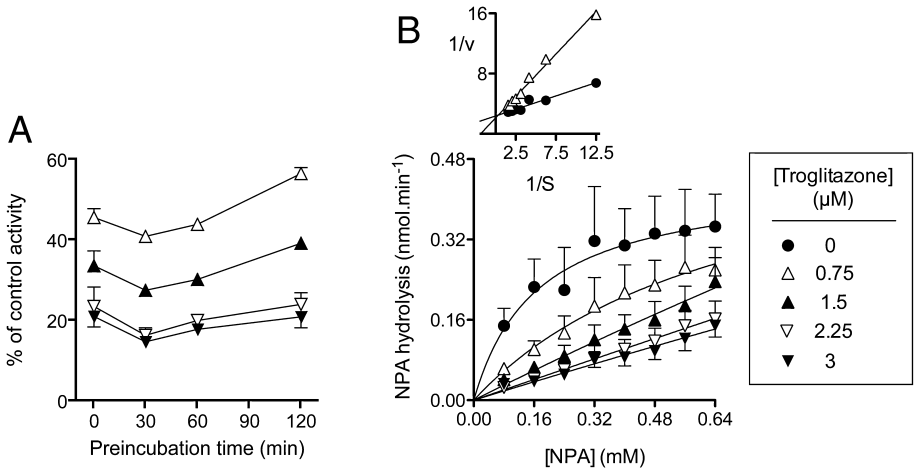


Figure 2

Mode of inhibition by troglitazone of 4-nitrophenyl acetate (NPA) hydrolysis by human monoacylglycerol lipase (MGL) lysates (catalogue number #10008354). In Panel A, the lysates were pre-incubated with troglitazone for the times shown at room temperature prior to addition of NPA (0.25 mM) and assay for activity. In Panel B, no pre-incubation phase was used. Shown are means and SEM (when not enclosed by the symbols), $n = 3$, except for the data in Panel B with 0.32 mM NPA, where $n = 2$. The inset in Panel B shows a double reciprocal plot of the mean data with 0 and 0.75 μM troglitazone, to illustrate the competitive nature of the inhibition.

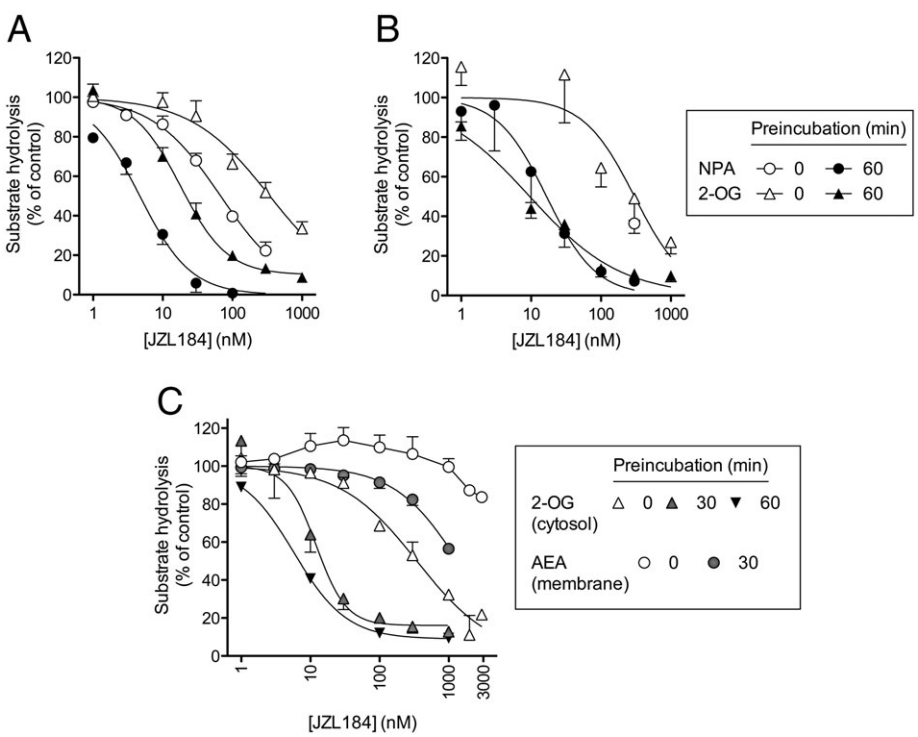


Figure 3

Inhibition by JZL184 of the hydrolysis of 4-nitrophenyl acetate (NPA) or 2-oleoylglycerol (2-OG) by A, human monoacylglycerol lipase (MGL) lysates (catalogue no. #705194); B, purified human MGL; C, rat cerebellar cytosol fractions. Shown are means and SEM (when not enclosed by the symbols), $n = 3$–9. For comparative purposes, the effect of the compound upon the hydrolysis of anandamide (AEA) (0.5 μM) by rat brain membrane preparations is shown to illustrate the MGL-selectivity of the compound.

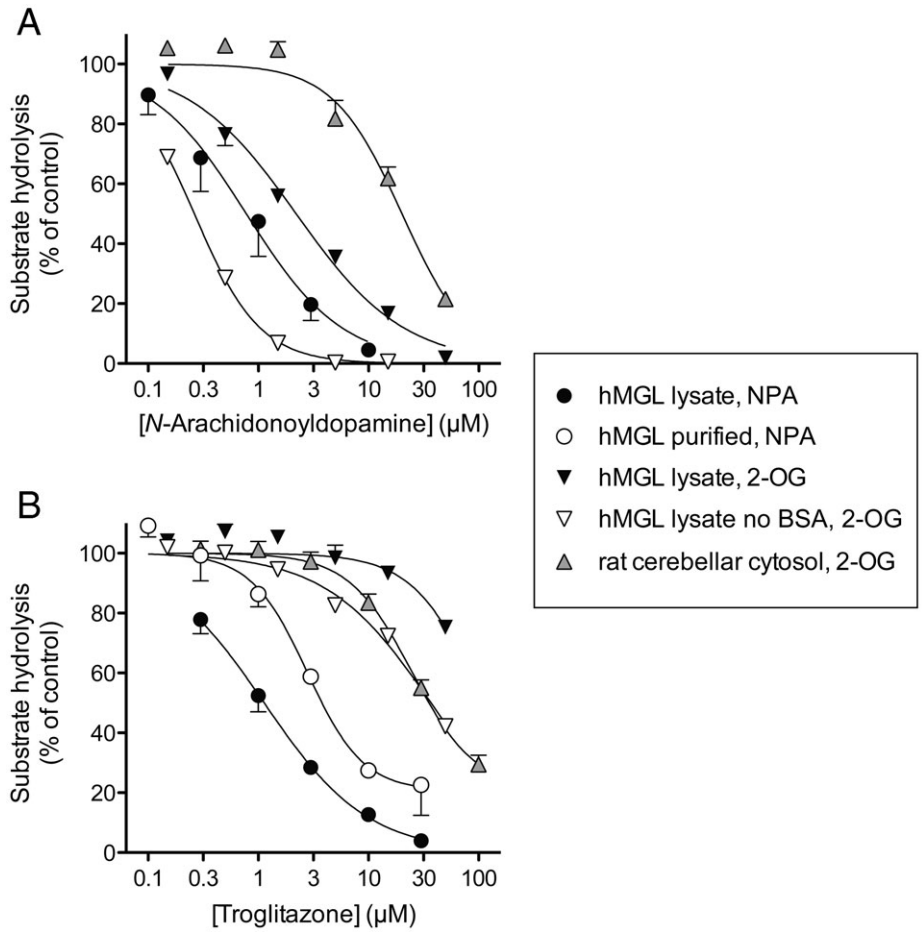


Figure 4

The potencies of *N*-arachidonoyl dopamine (A) and troglitazone (B) towards monoacylglycerol lipase (MGL) in different assays. The compounds were not pre-incubated with the enzyme preparations prior to addition of substrate. Shown are means and SEM (when not enclosed by the symbols), $n = 3–6$. The catalogue number of the lysates was #705194. Note that the curves for the human MGL lysate with NPA as substrate are the same as shown in Figure 1A for the two compounds.

the OD used, but it is possible that troglitazone can quench the signal produced by NPA hydrolysis in the samples. However, this would have been expected to affect equally the signal seen in assays with the lysates and the purified enzyme, and would not have been likely to produce the effects it did in the dilution experiments or to give a competitive type of inhibition. Nonetheless, the possibility was checked by adding troglitazone to assay mixtures containing the lysate and NPA after a 20 min incubation period and reading the plate immediately and 2 min later. Over the concentration range tested (0.1–30 μM), troglitazone was without any effect on the observed absorbance at 405 nM (data not shown), indicating that effect of troglitazone is not due to quenching of the 4-nitrophenol signal.

2 The manner in which the substrate is presented to the enzyme may be of importance, given the difference in lipophilicity between 2-OG and NPA. In order to investigate this possibility, the inhibitory potency of troglitazone was investigated when 2-OG was emulsified using Triton X-100 and compared to that in the standard BSA-containing assay. However, preliminary experiments indicated that the enzyme activity towards 2-OG in the presence of Triton X-100 was very much lower than seen in the standard BSA-containing assay (~12% for both MGL lysates and for rat cytosolic preparations using a Triton X-100 concentration of 5 mM to emulsify the substrate, final assay concentration 0.625 mM, that is, 0.04% w v^{-1}), and in the cytosolic preparations, there was no obvious difference in the sensitivity to troglitazone (data not shown).

3 Differences in fatty acid-free BSA concentration in the two assays affect the observed inhibitory potency of troglitazone. The presence of BSA is

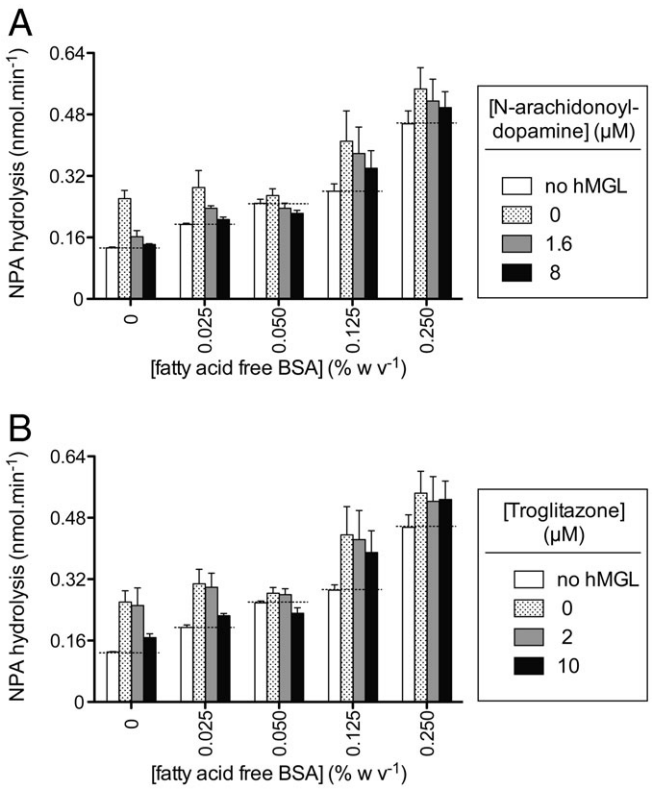


Figure 5

Effect of fatty acid-free bovine serum albumin (BSA) upon the rate of hydrolysis of 4-nitrophenyl acetate (NPA) and its inhibition by (A) *N*-arachidonoyl dopamine, (B) troglitazone. The compounds were not pre-incubated with the enzyme preparations prior to addition of substrate. Note that in the figure, the blank values (containing the same concentration of fatty acid-free bovine serum albumin, but no hMGL lysate) are not subtracted. Shown are means and SEM., $n = 3$. The catalogue number of the lysates was #705194.

likely to reduce the free troglitazone concentration given its propensity to bind to albumin (>99.9% binding to human serum albumin, Shibukawa *et al.*, 1995), and this may result in a reduced observed potency. In order to investigate this possibility, NPA assays were undertaken in the presence of different concentrations of fatty acid-free BSA (0–0.25% w v^{-1}) and with *N*-arachidonoyl dopamine and troglitazone as test compounds. The experiments were complicated by the fact that serum albumins have esterase activity (see Salvi *et al.*, 1997), and this can be clearly seen in the blank values obtained at each fatty acid-free BSA concentration (Figure 5A and B). At the high BSA concentrations, the blank values are so high relative to the MGL activity that accurate determination of inhibitory potencies is not possible. However, the difference between the blank and enzyme-containing controls is approximately the same at 0 and 0.125% w·v^{-1} BSA, and in these cases, the inhibition produced by 8 μM *N*-arachidonoyl dopamine and 10 μM troglitazone, appeared to be greater in the absence than in the presence of 0.125% w·v^{-1} fatty acid-free BSA. Experiments were also undertaken using the hMGL lysates and 2-OG in the radiochemical assay, but lacking fatty acid-free BSA. In these experiments, *N*-arachidonoyl dopamine was a very potent inhibitor of the 2-OG hydrolysis (pI_{50} value 6.58 ± 0.02, IC_{50} 0.26 μM), that is, greater inhibition than seen in the presence of BSA (Figure 4A), although as a caveat it was noted that the vehicle used (ethanol) reduced the enzyme activity, compared to a DMSO vehicle. Troglitazone was also a better inhibitor of 2-OG hydrolysis by the lysates in the absence of BSA (pI_{50} 4.43 ± 0.04, IC_{50} 37 μM), although this value is still lower than seen with the lysates in the NPA assay (Figure 4B).

Discussion

In the present study, we used a high-capacity assay to investigate a series of compounds with respect to their MGL inhibitory properties, and characterized the two most potent in the screen with respect to their inter-assay variability. The screen identified several compounds which inhibited NPA hydrolysis by the hMGL lysates in the low micromolar range. With the exception of troglitazone (see below), the compounds were either arachidonoyl analogues (AM404, *N*-arachidonoyl dopamine) or compounds sharing the ability of 2-AG to interact with CB receptors (CP 55 940; JWH015; WIN 55,212-2). With respect to CP 55,940, JWH015 and WIN 55,212-2, the potencies towards the hMGL in the lysates are lower than the nanomolar concentrations required to interact with CB receptors (see McPartland *et al.*, 2007), and so can best be described as 'off-target' actions of these compounds unlikely to contribute to their pharmacological effects. The same is probably true for *N*-arachidonoyl dopamine, which is a potent TRPV1 receptor agonist, although the IC_{50} value seen here for human MGL is similar to that seen for the interaction of the compound with CB_1 receptors (Bisogno *et al.*, 2000; Chu *et al.*, 2003). The potency of AM404 towards hMGL in the lysates is similar to its potency as an inhibitor of endocannabinoid uptake (Beltramo *et al.*, 1997), and so its effects upon MGL may contribute to the pharmacological actions of this compound *in vivo*. AM404 also inhibits the hydrolysis of 2-OG by rat cerebellar cytosolic fractions (IC_{50} value of ~20 μM, Vandevoorde and Fowler, 2005).

Two other compounds, *N*-arachidonoyl serotonin and tetrahydrolipstatin, were found to be 'hits' in the original screen, but were not investigated further. Tetrahydrolipstatin was also evaluated by Muccioli *et al*. (2008) using the NPA assay and a different hMGL preparation. They found it to be quite a potent inhibitor of hMGL, but not to produce more than ~50% inhibition of the enzyme, even at high (100 μM) concentrations. However, it had an IC_{50} value >10 μM in cytosolic fractions from monkey COS-7 cells when MGL was assayed with 2-AG as substrate (Ortar *et al*., 2008). *N*-arachidonoyl serotonin was reported not to inhibit the hydrolysis of 2-AG by rat cerebellar membranes (Saario *et al*., 2004), so it is possible that the effect seen here may be dependent upon the species and/or assay used. Muccioli *et al*. (2008) also tested a series of non-steroidal anti-inflammatory agents (including ibuprofen, naproxen, flubiprofen and diclofenac sodium), and found <50% (100 μM) and ≤33% (10 μM) inhibition in all cases. This lack of effect of this class of drugs upon hMGL is consistent with our data (see Table 1). Finally, the organic mercurial compound thimerosal produced 55% inhibition at 10 μM in our screen, a finding consistent with the ability of other organic mercurials to inhibit MGL activity (see Tornqvist and Belfrage, 1976; Chau and Tai, 1988; Tarzia *et al*., 2007).

The finding that the PPARγ agonist troglitazone inhibits NPA hydrolysis by the hMGL lysates extends previous work showing that PPARγ agonists can inhibit FAAH (Lenman *et al*., 2007), and that endocannabinoids and related biological lipids can interact with PPAR subtypes (see O'Sullivan, 2007). PPARγ is a ligand-activated nuclear receptor, and the PPARγ ligands rosiglitazone and pioglitazone are currently used for the treatment of type 2 diabetes. For the thiazolidinedione compounds tested here, the rank order of potencies for inhibition of MGL (troglitazone > ciglitazone > rosiglitazone) was different from that seen for either inhibition of FAAH (ciglitazone > rosiglitazone ≈ troglitazone, Lenman *et al*., 2007) or activation of PPARγ (rosiglitazone >> troglitazone > ciglitazone; Willson *et al*., 1996; 2000). The initial inhibition of NPA hydrolysis in the hMGL lysates by troglitazone was competitive in nature (K_i 0.34 μM). Although the inhibition was not time dependent, dilution experiments indicated that the inhibition was not rapidly reversible.

A degree of variation in potency is expected for a compound when tested in different assay systems, and in the case of JZL184, the primary variable influencing the observed potency is the pre-incubation time, as expected of an irreversible inhibitor. In the case of *N*-arachidonoyl dopamine, the variation in potency seems to be species-related, since the potencies were reasonably similar for the hMGL lysates when assayed with the two different substrates. For troglitazone, however, the pattern was at first sight more surprising, given that the compound inhibited the hydrolysis of NPA much more potently than the hydrolysis of 2-OG when the hMGL lysates were used. However, troglitazone has a very high degree of albumin binding (Shibukawa *et al*., 1995), and the difference in potency appears to be related in part to the concentration of fatty acid-free BSA in the assay medium, since its addition reduced the inhibition by both troglitazone and *N*-arachidonoyl dopamine of NPA hydrolysis by the hMGL lysates. The reverse assay (the use of 2-OG in the absence of fatty acid-free BSA) is more difficult to interpret, since the albumin concentration will affect the level of free substrate, and hence the observed inhibitory potency of a competitive inhibitor like troglitazone. We found that the sensitivity of hMGL to inhibition by *N*-arachidonoyl dopamine was indeed increased in the 2-OG assay when BSA was excluded. We have previously reported that the potency of VDM11 (an arachidonoyl-based uptake inhibitor with a structure close to AM404) and arachidonoyl serinol (serinol is 2-amino-1,3-propanediol) are roughly twice as potent as inhibitors of the hydrolysis of 2-OG by rat cerebellar membranes when assayed in the absence of fatty acid-free BSA compared to when assayed at 0.125% $w{\cdot}v^{-1}$ albumin (Vandevoorde and Fowler, 2005), which is entirely consistent with the present data with *N*-arachidonoyl dopamine and with the known ability of its analogues arachidonate and AEA to bind to albumin (Bojesen and Bojesen, 1994; Bojesen and Hansen, 2003; Oddi *et al*., 2009). Why the sensitivity of the 2-OG hydrolysis to inhibition by troglitazone is not similarly increased in the absence of BSA is unclear, but our current hypothesis is that 2-OG presents itself to the enzyme in a different manner to NPA and by doing so is less amenable to inhibition by troglitazone (but not by *N*-arachidonoyl dopamine or JZL184).

An obvious question raised by the present study is whether the ability of troglitazone to inhibit MGL is seen in man after normal dosing of the drug. Troglitazone was the first thiazolidinedione used clinically for the treatment of diabetes type 2. It was withdrawn from the US market at the request of the FDA in 2000 due to serious hepatotoxicity issues, the mechanism of which is controversial, but may involve the production of reactive metabolites and/or the ability of the compound to impair

mitochondrial function (see Masubuchi, 2006). However, pharmacokinetic data were obtained prior to its withdrawal. In healthy subjects, 7 days of treatment with troglitazone at doses of 200, 400 and 600 mg (doses in the range that was used to reduce plasma glucose in patients with type 2 diabetes, Johnson *et al.*, 1998) produced maximum serum concentrations of 0.90, 1.61 and 2.82 μg/mL, respectively, corresponding to concentrations of 2.0, 3.6 and 6.4 μM, respectively. The corresponding trough plasma concentrations were 0.3, 0.5 and 0.8 μM, respectively (Loi *et al.*, 1999). Although these numbers do not consider the influence of the plasma protein binding of troglitazone (>99.8%, Shibukawa *et al.*, 1995), they are at least in the same range as seen for the inhibition of NPA hydrolysis by the hMGL lysates, which in turn are similar to the potency of the compound for activation of human PPARγ in transactivation assays (EC_{50} value 0.55 μM, Willson *et al.*, 2000). Given the variation in potency of troglitazone for the different MGL assays, a 'best case' speculation is that MGL may have been inhibited upon normal troglitazone dosing. On the other hand, it is highly unlikely that the enzyme is inhibited following normal rosiglitazone dosing to patients with type 2 diabetes, given that the compound is used in lower doses than troglitazone (the maximum plasma concentration obtained after an 8 mg tablet is ~600 ng/mL, corresponding to 1.7 μM; Cox *et al.*, 2000); is an order of magnitude more potent than troglitazone towards PPARγ; and is an order of magnitude less potent than troglitazone as an inhibitor of NPA hydrolysis by the hMGL lysates and of 2-OG hydrolysis by the rat cerebellar cytosol fractions.

In conclusion, the present study has investigated a series of compounds with primary biological actions in areas where the endocannabinoid system is known to operate, and identified the PPARγ ligand troglitazone as a reasonably potent inhibitor of NPA (but not 2-OG) hydrolysis by hMGL lysates. PPARγ ligands may be useful in the treatment of diseases such as arthritis and neuropathic pain (Fehrenbacher *et al.*, 2009; Giaginis *et al.*, 2009), diseases where modulators of the endocannabinoid system are also being explored as treatment strategies (see Yao *et al.*, 2008). It may thus be possible to use troglitazone as a template for the design of novel compounds without its hepatotoxicity but which have potent effects upon both MGL and PPARγ, as potential novel analgesics and anti-arthritis drugs. The study also underlines the importance of investigating MGL inhibitory potency in several different assays, to ensure that the inhibitory properties of the compound are not assay dependent.

Acknowledgements

The authors would like to thank Britt Jacobsson and Eva Hallin for technical assistance with the radiochemical assays. This work was supported by grants from the Swedish Research Council (Grant no. 12158, medicine) and the Research Funds of the Medical Faculty, Umeå University.

Conflicts of interest

None declared with respect to the present work.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edn. Br J Pharmacol 158 (Suppl. 1): S1–S25.

Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, Piomelli D (1997). Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science 277: 1094–1097.

Bishay P, Schmidt H, Marian C, Häussler A, Wijnvoord N, Ziebell S *et al.* (2010). R-flurbiprofen reduces neuropathic pain in rodents by restoring endogenous cannabinoids. PLoS ONE 5: e10628.

Bisogno T, Melck D, Bobrov M, Gretskaya N, Bezuglov V, De Petrocellis L *et al.* (2000). *N*-acyl-dopamines: novel synthetic CB_1 cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity *in vitro* and *in vivo*. Biochem J 351: 817–824.

Blankman JL, Simon GM, Cravatt BF (2007). A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem Biol 14: 1347–1356.

Bojesen I, Bojesen E (1994). Binding of arachidonate and oleate to bovine serum albumin. J Lipid Res 35: 770–778.

Bojesen I, Hansen H (2003). Binding of anandamide to bovine serum albumin. J Lipid Res 44: 1790–1794.

Boldrup L, Wilson SJ, Barbier AJ, Fowler CJ (2004). A simple stopped assay for fatty acid amide hydrolase avoiding the use of a chloroform extraction phase. J Biochem Biophys Methods 60: 171–177.

Caijo F, Mosset P, Grée R, Audinot-Bouchez V, Boutin J, Renard P *et al.* (2005). Synthesis of new carbo- and heterocyclic analogues of 8-HETE and evaluation of their activity towards the PPARs. Bioorg Med Chem Lett 15: 4421–4426.

Chau L-Y, Tai H-H (1988). Monoglyceride and diglyceride lipases from human platelet microsomes. Biochim Biophys Acta 963: 436–444.

Chu CJ, Huang SM, De Petrocellis L, Bisogno T, Ewing SA, Miller JD *et al*. (2003). *N*-oleoyldopamine, a novel endogenous capsaicin-like lipid that produces hyperalgesia. J Biol Chem 278: 13633–13639.

Cox PJ, Ryan DA, Hollis FJ, Harris AM, Miller AK, Vousden M *et al*. (2000). Absorption, disposition, and metabolism of rosiglitazone, a potent thiazolidinedione insulin sensitizer, in humans. Drug Metab Dispos 28: 772–780.

Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner J *et al*. (1999). Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. Eur J Biochem 264: 258–267.

Dinh T, Carpenter D, Leslie F, Freund T, Katona I, Sensi S *et al*. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci USA 99: 10819–10824.

Fehrenbacher J, LoVerme J, Clarke W, Hargreaves K, Piomelli D, Taylor B (2009). Rapid pain modulation with nuclear receptor ligands. Brain Res Rev 60: 114–124.

Fowler C, Tiger G, Stenström A (1997). Ibuprofen inhibits rat brain deamidation of anandamide at pharmacologically relevant concentrations. Mode of inhibition and structure–activity relationship. J Pharmacol Exp Ther 283: 729–734.

Ghafouri N, Tiger G, Razdan RK, Mahadevan A, Pertwee RG, Martin BR *et al*. (2004). Inhibition of monoacylglycerol lipase and fatty acid amide hydrolase by analogues of 2-arachidonoylglycerol. Br J Pharmacol 143: 774–784.

Giaginis C, Giagini A, Theocharis S (2009). Peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands as potential therapeutic agents to treat arthritis. Pharmacol Res 60: 160–169.

Gobbi G, Bambico F, Mangieri R, Bortolato M, Campolongo P, Solinas M *et al*. (2005). Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc Natl Acad Sci USA 102: 18620–18625.

Goparaju S, Ueda N, Taniguchi K, Yamamoto S (1999). Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. Biochem Pharmacol 57: 417–423.

Goparaju S, Ueda N, Yamaguchi H, Yamamoto S (1998). Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. FEBS Lett 422: 69–73.

Gühring H, Hamza M, Sergejeva M, Ates M, Kotalla C, Ledent C *et al*. (2002). A role for endocannabinoids in indomethacin-induced spinal antinociception. Eur J Pharmacol 454: 153–163.

Guindon J, De Léan A, Beaulieu P (2006). Local interactions between anandamide, an endocannabinoid, and ibuprofen, a nonsteroidal anti-inflammatory drug, in acute and inflammatory pain. Pain 121: 85–93.

Holt S, Comelli F, Costa B, Fowler CJ (2005). Inhibitors of fatty acid amide hydrolase reduce carrageenan-induced hind paw inflammation in pentobarbital-treated mice: comparison with indomethacin and possible involvement of cannabinoid receptors. Br J Pharmacol 146: 467–476.

Huffman J, Liddle J, Yu S, Aung M, Abood M, Wiley J *et al*. (1999). 3-(1′,1′.Dimethylbutyl)-1-deoxy-Δ^8-THC and related compounds: synthesis of selective ligands for the CB_2 receptor. Bioorg Med Chem 7: 2905–2914.

Jayamanne A, Greenwood R, Mitchell VA, Aslan S, Piomelli D, Vaughan CW (2006). Actions of the FAAH inhibitor URB597 in neuropathic and inflammatory chronic pain models. Br J Pharmacol 147: 281–288.

Johnson MD, Campbell LK, Campbell RK (1998). Troglitazone: review and assessment of its role in the treatment of patients with impaired glucose tolerance and diabetes mellitus. Ann Pharmacother 32: 337–348.

Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A *et al*. (2003). Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9: 76–78.

Kinsey SG, Long JZ, O'Neal ST, Abdullah RA, Poklis JL, Boger DL *et al*. (2009). Blockade of endocannabinoid-degrading enzymes attenuates neuropathic pain. J Pharmacol Exp Ther 330: 902–910.

Kliewer S, Lenhard J, Willson T, Patel I, Morris D, Lehmann J (1995). A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. Cell 83: 813–819.

Lenman A, Fowler CJ (2007). Interaction of ligands for the peroxisome proliferator-activated receptor γ with the endocannabinoid system. Br J Pharmacol 151: 1343–1351.

Loi CM, Alvey CW, Vassos AB, Randinitis EJ, Sedman AJ, Koup JR (1999). Steady-state pharmacokinetics and dose proportionality of troglitazone and its metabolites. J Clin Pharmacol 39: 920–926.

Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE *et al*. (2009a). Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. Nat Chem Biol 5: 37–44.

Long JZ, Nomura DK, Vann RE, Walentiny DM, Booker L, Jin X *et al*. (2009b). Dual blockade of FAAH and MAGL identifies behavioral processes regulated by endocannabinoid crosstalk *in vivo*. Proc Natl Acad Sci USA 106: 20270–20275.

Masubuchi Y (2006). Metabolic and non-metabolic factors determining troglitazone hepatotoxicity: a review. Drug Metab Pharmacokinet 21: 347–356.

McPartland J, Glass M, Pertwee R (2007). Meta-analysis of cannabinoid ligand binding affinity and receptor distribution: interspecies differences. Br J Pharmacol 152: 583–593.

Muccioli G, Labar G, Lambert D (2008). CAY10499, a novel monoglyceride lipase inhibitor evidenced by an expeditious MGL assay. Chembiochem 9: 2704–2710.

Naidu PS, Booker L, Cravatt BF, Lichtman AH (2009). Synergy between enzyme inhibitors of fatty acid amide hydrolase and cyclooxygenase in visceral nociception. J Pharmacol Exp Ther 329: 48–56.

O'Sullivan SE (2007). Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. Br J Pharmacol 152: 576–582.

Oddi S, Fezza F, Pasquariello N, D'Agostino A, Catanzaro G, De Simone C *et al*. (2009). Molecular identification of albumin and Hsp70 as cytosolic anandamide-binding proteins. Chem Biol 16: 624–632.

Ortar G, Bisogno T, Ligresti A, Morera E, Nalli M, Di Marzo V (2008). Tetrahydrolipstatin analogues as modulators of endocannabinoid 2-arachidonoylglycerol metabolism. J Med Chem 51: 6970–6979.

Pacher P, Bátkai S, Kunos G (2006). The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev 58: 389–462.

Patel S, Wohlfeil E, Rademacher D, Carrier E, Perry L, Kundu A *et al*. (2003). The general anesthetic propofol increases brain *N*-arachidonoylethanolamine (anandamide) content and inhibits fatty acid amide hydrolase. Br J Pharmacol 139: 1005–1013.

Saario S, Savinainen J, Laitinen J, Järvinen T, Niemi R (2004). Monoglyceride lipase-like enzymatic activity is responsible for hydrolysis of 2-arachidonoylglycerol in rat cerebellar membranes. Biochem Pharmacol 67: 1381–1387.

Salvi A, Carrupt PA, Mayer JM, Testa B (1997). Esterase-like activity of human serum albumin toward prodrug esters of nicotinic acid. Drug Metab Dispos 25: 395–398.

Shibukawa A, Sawada T, Nakao C, Izumi T, Nakagawa T (1995). High-performance frontal analysis for the study of protein binding of troglitazone (CS-045) in albumin solution and in human plasma. J Chromatogr A 697: 337–343.

Tanis SP, Parker TT, Colca JR, Fisher RM, Kletzein RF (1996). Synthesis and biological activity of metabolites of the antidiabetic, antihyperglycemic agent pioglitazone. J Med Chem 39: 5053–5063.

Tarzia G, Antonietti F, Duranti A, Tontini A, Rivara S, Traldi P *et al*. (2007). Identification of a bioactive impurity in a commercial sample of 6-methyl-2-p-tolylaminobenzo[d][1,3]oxazin-4-one (URB754). Ann Chim 97: 887–894.

Tornqvist H, Belfrage P (1976). Purification and some properties of a monoacylglycerol-hydrolysing enzyme of rat adipose tissue. J Biol Chem 251: 813–819.

Vandevoorde S, Fowler CJ (2005). Inhibition of fatty acid amide hydrolase and monoacylglycerol lipase by the anandamide uptake inhibitor VDM11: evidence that VDM11 acts as an FAAH substrate. Br J Pharmacol 145: 885–893.

Willson T, Cobb J, Cowan D, Wiethe R, Correa I, Prakash S *et al*. (1996). The structure–activity relationship between peroxisome proliferator-activated receptor γ agonism and the antihyperglycemic activity of thiazolidinediones. J Med Chem 39: 665–668.

Willson T, Brown P, Sternbach D, Henke B (2000). The PPARs: from orphan receptors to drug discovery. J Med Chem 43: 527–550.

Yao B, Hsieh G, Frost J, Fan Y, Garrison T, Daza A *et al*. (2008). *In vitro* and *in vivo* characterization of A-796260: a selective cannabinoid CB_2 receptor agonist exhibiting analgesic activity in rodent pain models. Br J Pharmacol 153: 390–401.