Enzyme Inhibition

DOI: 10.1002/ange.201407807

A Reversible and Selective Inhibitor of Monoacylglycerol Lipase Ameliorates Multiple Sclerosis**

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Dedicated to the memory of Carlos F. Barbas III

Abstract: *Monoacylglycerol lipase (MAGL) is the enzyme responsible for the inactivation of the endocannabinoid 2-arachidonoylglycerol (2-AG). MAGL inhibitors show analgesic and tissue-protecting effects in several disease models. However, the few efficient and selective MAGL inhibitors described to date block the enzyme irreversibly, and this can lead to pharmacological tolerance. Hence, additional classes of MAGL inhibitors are needed to validate this enzyme as a therapeutic target. Here we report a potent, selective, and reversible MAGL inhibitor ($IC_{50} = 0.18\ \mu M$) which is active in vivo and ameliorates the clinical progression of a multiple sclerosis (MS) mouse model without inducing undesirable CB_1-mediated side effects. These results support the interest in MAGL as a target for the treatment of MS.*

The activation of the cannabinoid receptors CB_1 and CB_2 regulates many physiological processes. Therefore, modulation of the endogenous cannabinoid system (ECS) holds great therapeutic potential for the treatment of many disorders such as pain, (neuro)inflammation-associated pathologies, obesity, and cancer.[1] However, the use of direct-acting CB_1 receptor agonists is associated with psychotropic effects, a fact that has boosted the search for alternative strategies to activate the ECS without impairing motor and cognitive functions. To this end, one possibility is to increase the levels of the endogenous cannabinoids (eCBs) anandamide (AEA) and 2-arachidonoylglycerol (2-AG) by pharmacological inhibition of the enzymes involved in their degradation.[2,3] AEA is hydrolyzed by the well-characterized fatty acid amide hydrolase (FAAH)[4,5] and its selective inhibition enhances AEA levels and induces analgesia and anxiolytic effects without concomitant psychoactivity.[6–8] However, the analgesic effects of an irreversible inhibitor of FAAH have not been replicated in a phase II clinical trial.[9] Considering that 2-AG is the major brain eCB, and acts as a full agonist for CB_1 and CB_2 receptors, it has become a major focus of attention.[10] The main enzyme involved in its inactivation is monoacylglycerol lipase (MAGL), which accounts for 85% of the 2-AG degradation in mouse brain.[11] It has been suggested that MAGL inhibitors could be useful compounds for the treatment of pain,[12,13] neuropsychiatric disorders,[14] cancer,[15] and neurodegenerative diseases,[16–18] among others.[19] However, potent and selective inhibitors of this enzyme are currently scarce, a fact that has precluded its validation as a therapeutic target.

Although some MAGL inhibitors have been recently disclosed[10,20–23] (Figure 1), they act in an irreversible manner. This permanent inactivation of MAGL can lead to functional

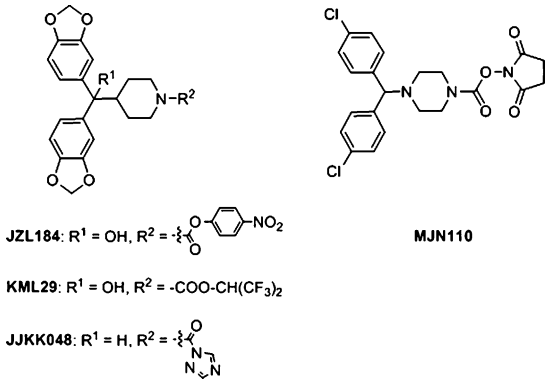

Figure 1. Representative irreversible MAGL inhibitors.

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[**] This work was supported by grants from the Spanish Ministerio de Economía y Competitividad (MINECO, SAF2010-22198, SAF2013-48271, and SAF2010-17501), Comunidad de Madrid (S2010/BMD-2353 and S2010/BMD-2308), the Spanish Multiple Sclerosis Network (RD201/0032/0008), the Swedish Research Council (grant no. 12158, medicine), and the Research Funds of the Medical Faculty, Umea University. G.H.-T. and A.C. are Juan de la Cierva and Ramon y Cajal Scholars, respectively, funded by MINECO and the European Social Fund. M.C. is a recipient of a Laboratories For Chemical Biology Umeå postdoctoral fellowship. D.Z. is a predoctoral fellow funded by MINECO (FPU program). We also thank Linda Gabrielsson and Mona Svensson for their expertise in conducting the COX and mouse brain MAGL/FAAH experiments.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201407807.

antagonism of the ECS, pharmacological tolerance, and receptor desensitization that eventually impairs the antinociceptive properties of the inhibitor.[24] Such problems with tolerance are less likely to occur with reversible MAGL inhibitors. To our knowledge, the only compounds described as potent reversible MAGL inhibitors are the naturally occurring terpenoids pristimerin and euphol.[25] The related compounds α- and β-amyrin also inhibit MAGL,[26] but to our knowledge the effects of these compounds upon the ECS in vivo have not been investigated. Accordingly, there is an

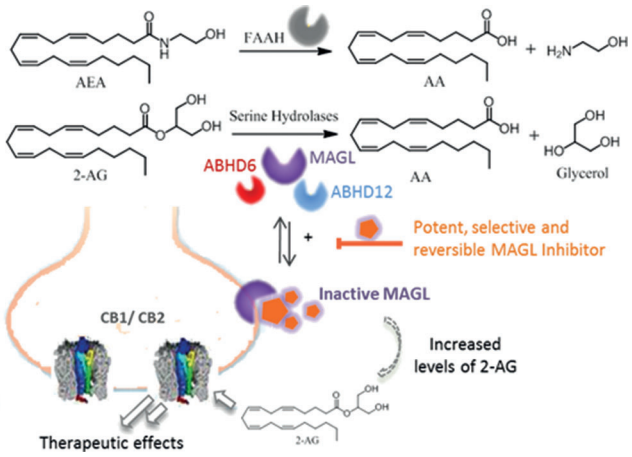


Figure 2. A potent, selective, and reversible MAGL inhibitor would increase the local 2-AG levels inducing therapeutic effects without CB_1 side effects. AA = arachidonic acid.

important need for the development of potent, selective, and reversible MAGL inhibitors that can be used to establish whether the beneficial effects of MAGL inhibition can be produced without concomitant undesirable side effects mediated by central CB_1 activation (Figure 2). Towards this aim, we identified compounds **1** and **2** as reversible dual MAGL/FAAH inhibitors (Figure 3).[27] Using them as a starting point, here we describe the structural exploration that led to the identification of compound **21**, which was characterized as a potent, selective, and reversible MAGL inhibitor. Moreover, derivative **21** is active in vivo in the experimental autoimmune encephalitis (EAE) mouse model of multiple sclerosis (MS), in which it clearly ameliorates the course of the disease without inducing undesirable CB_1-agonist-like activity.

Our previous studies[27] indicated that it was possible to replace

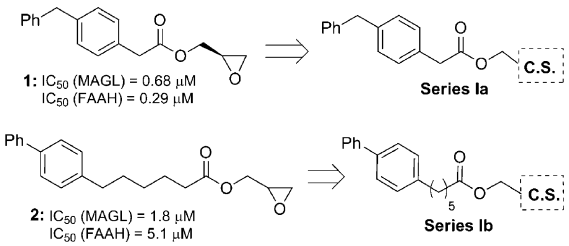
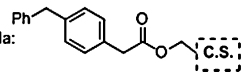


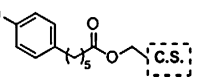
Figure 3. Design of new MAGL inhibitors.

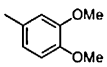
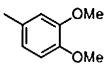
the arachidonic acid chain of 2-AG with 2-(4-benzylphenyl)-acetate and 6-(biphenyl-4-yl)hexanoate subunits (compounds **1** and **2**, respectively, Figure 3), whereas the glycerol moiety could be mimicked with a methyloxirane group. These modifications led to dual FAAH/MAGL inhibitors with IC_{50} values in the low micromolar range (Figure 3). In an attempt to improve not only MAGL inhibition but also selectivity against FAAH, we decided to explore whether the small oxirane heterocycle of compounds **1** and **2** could be replaced by different oxygenated cyclic subunits (C.S.), keeping as optimized lipophilic moieties either the 4-benzylphenyl core (series Ia) or the 1,1′-biphenyl scaffold (series Ib) (Figure 3).

Compounds **3**–**16** were prepared as described in the Supporting Information (SI) and were evaluated for their capacity to inhibit the 2-AG and the AEA hydrolytic activities in brain homogenates. In the case of 2-AG, and as previously described,[27] the most stable analogue, 2-oleoylglycerol (2-OG), was used as a surrogate (see SI for experimental details). First, we explored the influence of

Table 1: Influence of the cyclic subunit (C.S.) on the capacity of the compounds to inhibit 2-AG and AEA hydrolysis.

Series Ia: 

Series Ib: 

Cmpd.	C.S.	Hydrolysis inhibition IC_{50} [μM][a,b,c] 2-AG[d]	AEA	Cmpd.	C.S.	Hydrolysis inhibition IC_{50} [μM][a,b,c] 2-AG[d]	AEA
3		41	1.8	**10**		11	3.3
4		48	6.4	**11**		2.5 [93±2%]	5.8 [91±2%]
5		20	3.5	**12**		10 [87±5%]	12
6		59	12	**13**		5.7 [68±2%]	7.1 [82±5%]
7		>100 [25±5%]	6.8 [96±2%]	**14**		>100 [27±2%]	12 [60±4%]
8		18	0.63 [97±1%]	**15**		5.6	0.49
9		9.3 [79±5%]	2.1	**16**		3.6 [60±3%]	0.62 [91±2%]

[a] The IC_{50} values were derived from the mean pI_{50} values. [b] When the data was better fitted to an inhibition curve with a residual activity, the percentage of inhibitable component is given in the table as [percentage of maximum inhibition ± standard error of the mean (s.e.m.)]. [c] When compounds did not produce ≥50% inhibition at the highest concentration tested (100 μM), the percentage of inhibition seen at that concentration is indicated. [d] In these experiments, the most stable analogue 2-OG was used as a surrogate of 2-AG (see SI for further details).

the cyclic subunit (C.S.) in the capacity of the compounds to inhibit the hydrolysis of both eCBs (Table 1).

The introduction of different oxygenated rings in the previously optimized lipophilic moieties revealed that, in general, compounds of series I b (**10**–**16**) were more potent at inhibiting 2-AG hydrolytic activity than their corresponding analogues of series I a (**3**–**9**). This effect is especially notable in compounds **11** versus **4**, and **13** versus **6** (Table 1). However, all compounds of series I b are still dual inhibitors, as they display comparable IC_{50} values in both assays (see, for example, derivatives **11**–**13**) or even better inhibition of AEA than of 2-AG hydrolysis (as is the case for derivatives **14**–**16**).

Hence, we focused our efforts on series I b by broadening the exploration of the polar C.S. to increase selectivity. Considering that FAAH has a relatively narrow binding pocket compared to that of MAGL,[28] we envisioned that compounds with bulkier C.S. groups could fit into the MAGL but not into the FAAH active site. This idea led us to explore benzofuran and benzodioxole scaffolds in compounds **18**–**22** (Table 2). Also, the requirement of the oxygen was assessed by the small cyclopropane derivative **17** which shows around a 10-fold decrease in its ability to inhibit 2-AG hydrolysis when compared to its corresponding parent compound **2**.

In general, all these new derivatives, with the exception of **22**, were able to inhibit 2-AG hydrolysis (Table 2). Furthermore, compound **21** exhibited the best profile, with a submicromolar IC_{50} value for MAGL inhibition and more than 50-fold selectivity versus FAAH [IC_{50} (MAGL) = 0.24 μM versus IC_{50} (FAAH) = 18 μM; see Figure 4 A for representative plots]. Finally, and in an attempt to obtain further insights on the structural requirements needed for potent and selective MAGL inhibition, we varied the distance between the biphenyl group and the heterocycle (analogues **23** and **24**), the ester was replaced by an amide (**25**), and the polarity and bulkiness of the lipophilic moiety was modified (compounds **26** and **27**) (Table 3). Together, these results suggest that the heterocyclic moiety of the inhibitor can modulate the selectivity between MAGL and FAAH. Then, we performed docking calculations to propose a likely binding mode for **21**

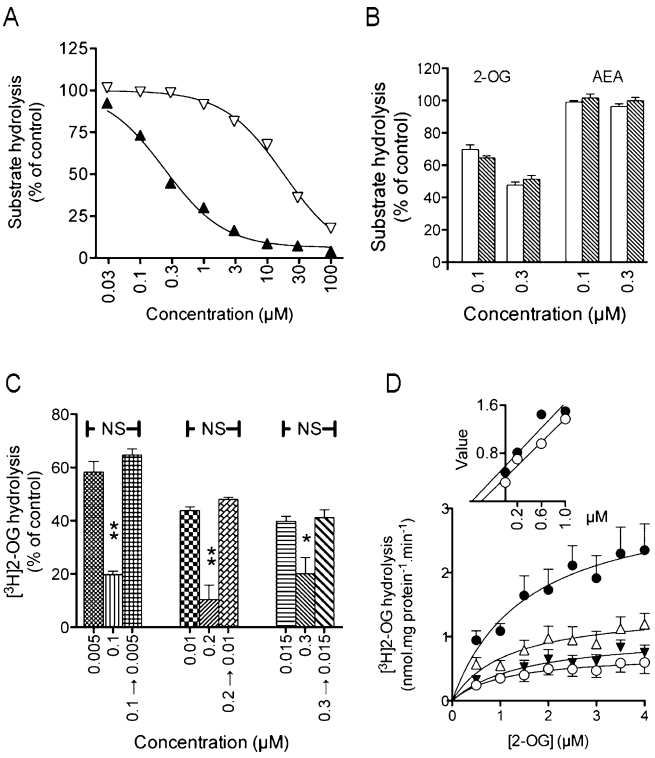
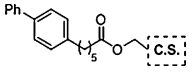
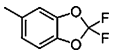


Figure 4. A) Inhibition of the hydrolysis of AEA (▽) and 2-AG (▲) by compound **21**. The figure shows combined data for different independent dose–response experiments, shown are means (n = 4–10). The s.e.m. values are all enclosed within the symbols. B) The data show no preincubation (white bars) and 60 min preincubation (gray bars) expressed as means and s.e.m. (n = 4). C) Bars show the hydrolysis for preincubation followed by dilution (means and s.e.m., n = 3), and indicates the inhibition to be reversible. D) Hydrolysis kinetics (mean ± s.e.m., n = 3), indicative of noncompetitive inhibition with a K_i value of 0.40 μM.

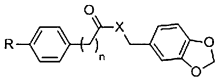
Table 2: Influence of the cyclic subunit (C.S.) on the capacity of the compounds of series I b to inhibit 2-AG and AEA hydrolysis.

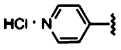
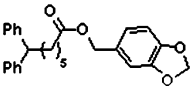


Cmpd.	C.S.	Hydrolysis inhibition IC_{50} [μM][a,b,c] 2-AG[d]	AEA
17		13	27
18		11 [70±6%]	4.6
19		1.7 [70±3%]	2.8 [52±2%]
20		11 [60±4%]	8.7
21		0.24 [94±1%]	18
22		>100 [1±9%]	1.5 [88±4%]

[a–d] See footnotes for Table 1.

Table 3: Capacity of analogues of compound **21** to inhibit 2-AG and AEA hydrolysis.



Cmpd.	R	n	X	Hydrolysis inhibition IC_{50} [μM][a,b,c] 2-AG[d]	AEA
21		5	O	0.24 [94±1%]	18
23		3	O	10 [58±8%]	17
24		1	O	24 [61±3%]	9.2
25		5	NH	15 [63±9%]	80
26		5	O	43	15
27				51	4.5

[a–d] See footnotes for Table 1.

and to rationalize why other derivatives, such as **22** and **27** as representative examples, were largely inactive at this enzyme. These models (see SI for details) indicate that inhibitor **21** can establish favorable polar and hydrophobic interactions with MAGL whereas neither compound **22** nor **27** are completely accommodated in the enzyme (Figure S1). In addition, **22** and **27** nicely fit in the FAAH enzyme cavity while derivative **21** shows a different binding mode with less polar interactions and a different orientation of the benzodioxole ring, making the overall interaction less favorable (Figure S2). These models would be in agreement with the observed better inhibition of FAAH of **22** and **27** compared to **21**.

Further exploration of the cyclic subunit, different ester replacements, and modifications on the biphenyl ring, has been included, for the sake of clarity, in Tables S1 and S2 in the Supporting Information. As none of the modifications performed allowed us to improve the profile of derivative **21**, we assessed whether this inhibitor would fulfill the desired features in terms of inhibition mechanism, kinetics, and selectivity. First, we confirmed that **21** inhibited MAGL in a reversible manner, as shown by preincubation and dilution experiments. As expected for a noncovalent inhibitor, the enzyme activity is not affected by preincubation with the compound (Figure 4B). In addition, data from dilution experiments (Figure 4C) are consistent with a reversible character of the inhibitor, since the enzyme activity is fully recovered after dilution of an initially inhibitory concentration of the compound. Kinetic studies indicated that **21** acts as a noncompetitive inhibitor (Figure 4D) with a K_i value of 0.4 μM. In agreement with this result, NMR experiments showed that the human recombinant MAGL (hrMAGL) was not able to hydrolyze compound **21** whereas it did hydrolyze, in the same time interval, competitive inhibitors such as epoxide **2** (Figure S3). In order to estimate the half-life of enzyme inhibition we carried out time course NMR studies. After 13 h the enzyme activity is completely restored as the inhibitor has been hydrolyzed by the enzyme (Figure S4).

Then, we assessed the selectivity of **21** against the CB_1 and CB_2 receptors as well as other enzymes involved in the degradation of 2-AG, mainly ABHD6 and ABHD12.[11] We carried out competitive activity-based protein profiling (ABPP) experiments in COS-7 cells transiently transfected with the human serine hydrolases MAGL, ABHD6, and ABHD12 in the presence of the serine hydrolase directed probe fluorophosphonate-rhodamine[29] (FP-Rh, see SI for details). These experiments confirmed MAGL inhibition after treatment with compound **21** (Figure 5A). Under the same conditions, quantification of the fluorescence intensity did not show significant differences between ABHD12 and ABHD6 enzymes (Figure 5B), a result that highlights the selectivity of **21** over the rest of enzymes responsible for the degradation of 2-AG. In addition, MAGL inhibitor **21** does not bind CB_1 or CB_2 receptors ($K_i > 10$ μM). Furthermore, we studied the selectivity of **21** in a broad panel that includes a variety of receptors and enzymes. Compound **21** did not inhibit significantly any of the analyzed targets (Table S3 and Figure S5). Taken together, all these data show that compound **21** fulfills the sought requirements of potency, reversibility, and selectivity and, hence, it is a potentially useful tool to validate MAGL as a useful target for drug development. However, for that to be the case, the compound needs to show in vivo activity.

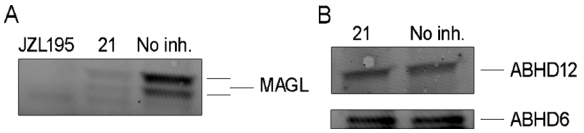


Figure 5. Competitive activity-based protein profiling (ABPP) for compound **21**. Proteomes from COS-7 cells transiently transfected with human cDNAs for A) MAGL, B) ABHD12 (top), and ABHD6 (bottom) were incubated with **21** (10 μM) for 10 min at RT and then with fluorophosphonate-rhodamine (75 nM, 2.5 min). Then, proteins were separated by SDS-PAGE, and analyzed by in-gel fluorescence scanning to reveal enzyme inhibition. Fluorescent gel is shown in grayscale. Note that MAGL migrates as several bands as reported previously.[30]

MS is a chronic, inflammatory autoimmune disease characterized by nerve demyelination. Disease onset usually occurs in young adults and it affects up to 2.5 million people worldwide.[31] Although considerable progress has been made, there is no drug that prevents the progression of the disease in patients with progressive forms of MS, and no means to repair injured axons or protect neurons from further damage. Thus, there is an important unmet need for new therapeutic strategies.[32,33]

It has been proposed that the ECS can improve symptoms commonly associated with progressive MS,[34] and eCBs have been suggested to be neuroprotective in this context.[35] These observations prompted us to examine the capacity of **21** to ameliorate the progression of MS using the EAE mouse model. Before in vivo administration, we determined the inhibition of mouse MAGL and FAAH, and some pharmacokinetic parameters. Compound **21** inhibited mouse brain MAGL and FAAH with IC_{50} values of 0.18 and 59 μM, respectively (Figure S6). The compound bound to serum albumin with a K_d value of 60 μM and after 120 min of incubation in mouse serum, 42% of the compound still could be detected. In addition, the pharmacokinetic profile showed that the compound could be identified in mouse plasma after 4 h. Taken together, these values are sufficient to allow the in vivo use of the compound.

EAE was induced as detailed in the Supporting Information. Treatment started at day 6 post-immunization (p.i.) and consisted of daily injections of compound **21** (5 mg kg^{-1}, intraperitoneal, i.p.) or vehicle for the following 21 days. All mice were examined daily for clinical signs of EAE and were killed at day 27 p.i. Administration of compound **21** clearly ameliorated the progression of the disease, as assessed by the significantly lower clinical score in the MS model (Figure 6A). This improvement correlated with an increase of the 2-AG levels in the spinal cord of treated animals (Figure 6B) and with evident changes at the histological level, as compound **21** significantly decreased leukocyte infiltration and microglial response (Figure 6C,D), prevented axonal damage, and partially restored myelin morphology in EAE mice (Figures S7 and S8).

In order to rule out that the anti-inflammatory activity observed could be due to direct inhibition of the prostaglan-

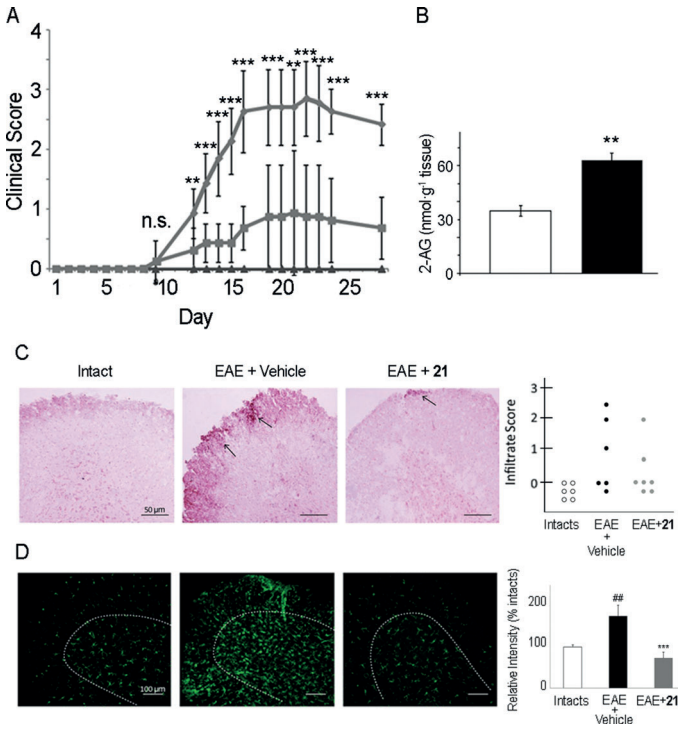


Figure 6. Treatment with MAGL inhibitor **21** significantly ameliorates the clinical signs and disease progression of EAE and increases the 2-AG levels in the spinal cord of treated mice. A) EAE was induced by subcutaneous immunization with MOG_{35-55} peptide. Treatment started at day 6 p.i. and consisted of daily injections of **21** (5 mg kg^{-1}, i.p.) or vehicle (DMSO/saline) for the following 21 days. The mice were examined daily for clinical signs of EAE and disease scores were measured as follows: 0 no disease, 1 limp tail, 2 limp tail and hind limb weakness, 3 hind limb paralysis, 4 hind limb and front limb paralysis, 5 moribund and death. Results are shown as means ± SD for the following groups: control animals (▲, $n=6$), EAE + 21 (■, $n=8$), and EAE + vehicle (◆, $n=8$). B) Levels of 2-AG in the spinal cord of mice treated with vehicle (white bar) and compound **21** (black bar). Tissue was harvested at day 27 p.i. Results are means ± s.e.m. ($n=8$ per group). C) Compound **21** decreases leukocyte infiltration in EAE animals as indicated by the infiltrate score. Arrow points to infiltrates. D) Treatment with **21** attenuates microglial response in EAE animals as quantified by the percentage area occupied by microglia in the spinal cord white matter per field. Representative thoracic spinal cord sections ($n=6$–7 per group) were obtained at day 27 p.i. and stained with hematoxylin-eosin (in C) or with Iba1 (in D). ns, non-significant statistical difference; **, $p<0.01$; ***, $p<0.001$ for EAE + vehicle vs EAE + 21; ##, $p<0.01$ for EAE + vehicle versus intact.

din production, the capacity of both **21** and its metabolite 6-([1,1′-biphenyl]-4-yl)hexanoic acid to inhibit cyclooxygenases (COX) 1 and 2 was determined. At a concentration of 10 μM, the compounds produced very modest effects upon the hydrolysis of arachidonic acid by either enzyme form, and upon the hydrolysis of 2-AG by COX-2 (Figure S9). Furthermore, administration of **21** did not produce catalepsy nor hypokinesia, as assessed by evaluation of spontaneous locomotor activity (Figure S10).

In conclusion, in the present work we describe the design and characterization of a potent reversible MAGL inhibitor with pronounced activity in vivo. Compound **21** is largely selective for MAGL ($IC_{50}=0.18$ and 0.24 μM for mouse and rat MAGL, respectively) against other related targets of the ECS, including FAAH, ABDH6, and ABHD12 enzymes, the CB_1 and CB_2 cannabinoid receptors, and also against a broad panel of receptors and enzymes. The compound is active in vivo, as it enhances the 2-AG levels in spinal cord, and clearly ameliorates the progression of the disease in a mouse model of MS by improving clinical symptoms and decreasing tissue damage in the spinal cords of diseased mice. Importantly, this therapeutic effect is not accompanied by catalepsy or other motor impairments that have been observed after the administration of potent and irrreversible MAGL inhibitors previously described. These results support the interest in MAGL as a target for the treatment of MS and the potential clinical application for MAGL inhibitors in the treatment of this disease.

Received: July 13, 2014
Published online: October 8, 2014

Keywords: endocannabinoids · endogenous cannabinoid system · enzyme inhibitors · monoacylglycerol lipase · multiple sclerosis

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