



Dual inhibition of MAGL and type II topoisomerase by *N*-phenylmaleimides as a potential strategy to reduce neuroblastoma cell growth

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ABSTRACT

The endocannabinoid system is implicated in numerous physiopathological processes while more and more pieces of evidence wave the link between this complex machinery and cancer related phenomenon. In these lines, we confirmed the effects of 2-arachidonoylglycerol (2-AG), the main endocannabinoid, on neuroblastoma cells proliferation *in vitro*, and proved that some *N*-phenylmaleimide compounds that were previously shown as MAGL inhibitors can also inhibit type 2 topoisomerase. We also shed light on their antiproliferative effects on a neuroblastoma cell line. In order to establish a link between MAGL inhibition, topoisomerase inhibition and the effects on N1E-115 cells, we tested combinations of maleimides or known endocannabinoid metabolism inhibitors and 2-AG, the major MAGL substrate, on N1E-115 cells. However, none of the inhibitors tested, except the carbamate CAY10499, managed to increase 2-AG's effects. Even the MAGL reference inhibitor JZL184 failed to induce a stronger inhibition of proliferation.

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1. Introduction

Numerous strategies are being explored in order to reduce or block the development of cancer. For instance, one can act on the tumor environment (e.g. acting on the blood flow (Rowell et al., 1990)) or more directly on cancer cells by reducing their growth (Sheng et al., 1997) or inducing their death (Alexandre et al., 2006). The later strategies resulted in the development of cytostatic (Tsoukala et al., 2010) and cytotoxic (Liu et al., 2011) compounds.

Topoisomerases are one of the classes of enzymes that have been targeted in order to reduce cancer cell growth (Holden, 2001). Because they unwind and wind DNA in order to allow protein synthesis, inhibiting their action affects cell growth and ultimately induces cell death. Among topoisomerases, topoisomerase II cuts both strands of the DNA simultaneously and is a major target for antineoplastic agents, which can interfere with one or many steps of the topoisomerase catalytic cycle (Holden, 2001; Hande, 2003). Topoisomerase II poisons, such as etoposide, stabilize the covalent DNA-topoisomerase complex formed during the enzyme action (Hande, 1998), whereas catalytic inhibitors, such as bisdioxopiperazines

(ICRF-187, ICRF-193) (Tanabe et al., 1991), aclarubicin (Sehested and Jensen, 1996), or merbarone (Jensen et al., 2002), act on one of the steps of topoisomerase catalytic cycle. Among topoisomerase II inhibitors, small molecules such as *N*-methylmaleimide and *N*-ethylmaleimide have been identified as potent catalytic inhibitors of purified human topoisomerase II alpha (Jensen et al., 2002). On the other hand, *N*-ethylmaleimide and more lipophilic analogs have been reported as monoacylglycerol lipase (MAGL) inhibitors (Matuszak et al., 2009; Saario et al., 2005). MAGL is a key enzyme, responsible for the degradation of esters endocannabinoids such as 2-arachidonoylglycerol (2-AG), and its activity tightly modulates this endocannabinoid levels (Chanda et al., 2010).

Interestingly, the endocannabinoid system has been suggested to interfere with cell growth and proliferation (Saghafi et al., 2011; Guindon and Hohmann, 2011; Park et al., 2011; Torres et al., 2011). The endocannabinoid system consists of cannabinoid receptors, their endogenous ligands (i.e. endocannabinoids such as anandamide and 2-AG) and proteins for their synthesis and inactivation (Muccioli, 2010). The activation of cannabinoid receptors by exogenous cannabinoids is known to control cancer cell viability (Jacobsson et al., 2001; Santoro et al., 2009). Similar effects were shown for the endocannabinoids. For instance, anandamide, exerts anti-proliferative and pro-apoptotic effects, mostly, but not only, by activation of CB₁ and CB₂ cannabinoid receptors (Jacobsson

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et al., 2001; Mimeault et al., 2003). However, the effects of 2-AG are perhaps less well defined. Numerous studies report an inhibition of tumor cell growth on a variety of cell lines like for instance colorectal carcinomas, C6 glioma cells, breast or prostate cancer cells (Jacobsson et al., 2001; De Petrocellis et al., 2000; Ligresti et al., 2003). Still, other studies have demonstrated that exogenously added 2-AG could exert proliferative effects (Endsley et al., 2007; Nithipatikom et al., 2011). Indeed, it was suggested that the hydrolysis of this endocannabinoid by MAGL produces arachidonic acid and further downstream eicosanoids that in turn promote cell growth (Endsley et al., 2007). Overall these elements point to the inhibition of MAGL, the main enzyme involved in 2-AG metabolism, as a potential target to reduce cancer cell growth. Supporting this view, MAGL was recently described as the regulator of a fatty acid network regulating, among others, cancer cell growth (Nomura et al., 2010). Thus overexpression of the enzyme resulted in increased pathogenicity of cancer cells, whereas its inhibition reduced cancer growth. Recent data obtained on colorectal cancer cells where MAGL was knocked down through siRNA, clearly indicated that under these conditions the cell proliferation was diminished and the apoptosis enhanced (Ye et al., 2011).

Based on these elements, and in particular the potential dual inhibition of topoisomerase and MAGL that could be obtained using *N*-substituted maleimides, we decided to investigate if interacting with both topoisomerase II and the endocannabinoid system is beneficial to reduce cell growth of N1E115 cells, a neuroblastoma cell line. To achieve this goal, we tested different maleimide derivatives against topoisomerase II and MAGL activities, and then assayed them on N1E115 neuroblastoma cells in culture. These cells seemed an appropriate model since (i) they have been shown to possess a functional endocannabinoid system (Bosier et al., 2007; Hamtiaux et al., 2011) that has been extensively characterized and (ii) as they are commonly used in *in vitro* tests for the cytotoxicity of chemicals on cancer cells (Hainsworth et al., 2008; Mundy et al., 2010; Park et al., 2009).

Thus we looked for a potential synergistic effect on inhibition of cell growth of the inhibition of both MAGL and topoisomerase II by comparing

- (a) Substituted maleimides with dual inhibitory profile.
- (b) Substituted maleimides with MAGL inhibition and no or low topoisomerase II inhibition.
- (c) Inactive related compounds.

2. Results

2.1. Effects of etoposide and 2-AG, alone or together, on N1E115 neuroblastoma cell viability

First, we sought to confirm in our model, i.e. the N1E115 neuroblastoma cell line, that both the reference topoisomerase II inhibitor, etoposide, and the endogenous cannabinoid receptors ligand, 2-AG, were able to decrease the cellular viability. Thus we used the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into a formazan dye by the mitochondrial reductases of metabolically active cells (MTT assay) as a measure of the cell viability. Increasing concentrations of etoposide (Fig. 1A) and 2-AG (Fig. 1B) were incubated with the cells for 24, 48 or 72 h before the MTT assay was performed. After 72 h of incubation both drugs have a significant dose-dependent effect on N1E115 cell viability thus confirming previous observations for etoposide (Hadjidaniel and Reynolds, 2010; Lange et al., 2003), and for 2-AG (Muccioli et al., 2008), respectively. Although etoposide was more active in reducing cell growth compared to 2-AG, it seems that topoisomerase II and the endocannabinoid system are two potential targets to reduce the cell viability of this neuroblastoma cell line.

Along this line, we incubated etoposide, at 0.1 μ M, a concentration at which it exerts roughly 50% of its activity, with 2-AG. The co-incubation resulted in an additive effect compared to the effect of each drug alone (Fig. 1C).

2.2. Presence and inhibition of monoacylglycerol lipase in N1E115 and screening of *N*-phenylmaleimides and derivatives on a purified recombinant hMAGL

Because 2-AG effects on cell viability observed here could have been reduced by its potential catabolism by lipases we looked for the expression by N1E115 cells of MAGL, the enzyme mainly involved in 2-AG hydrolysis and thus inactivation. Using RT-PCR we found that this neuroblastoma cell line indeed expresses MAGL (Fig. 2A). In line with the mRNA expression results, when using a radiolabeled 2-AG analog, [3 H]-2-oleoylglycerol ([3 H]-2-OG), as substrate we found a significant 2-OG hydrolytic activity in those cells (Fig. 2B), attributed to MAGL activity. The well-known MAGL inhibitors CAY10499 (Long et al., 2009) and JZL-184 (Morris and Lindsley, 1999) were assayed against [3 H]-2-OG hydrolysis in N1E115 neuroblastoma homogenates and intact cells (Fig. 2C and D), used at 1 and 10 μ M, both compounds decrease 2-OG hydrolysis, CAY10499 being more potent in intact cells.

A limited set of *N*-phenylmaleimides **1–6** and *N*-phenylsuccinimide **10** were selected on the basis of their previously reported dose-dependent inhibition of MAGL activity. Three additional compounds (**7–9**) have been synthesized within this family of compounds and have been characterized regarding MAGL inhibition using a recombinant hMAGL, as previously reported (Matuszak et al., 2009). Here, all 10 compounds have been tested using the same batch of protein preparation. The IC₅₀ values are in the micromolar to submicromolar range (Fig. 3, Table 1). The succinimide derivative (**10**) had no effect on the enzymatic activity.

2.3. Screening of *N*-phenylmaleimides and derivatives on human topoisomerase II activity

We assayed the nine maleimide derivatives **1–9** and the succinimide **10** for their inhibitory properties against human topoisomerase II activity. Topoisomerases are enzymes that cut and then ligate back the DNA, thus when using a plasmid as substrate in an *in vitro* assay one can use the apparition of linear DNA as a marker of topoisomerase inhibition. Indeed, in the presence of an inhibitor ligation is blocked and the DNA accumulates in a linear form that can then be quantified following electrophoretic migration. As reported in the literature we found that etoposide at 20 and 50 μ M extensively blocked topoisomerase II activity (Terada et al., 1993; Zheng et al., 2010; Wickstrom et al., 2007). Representative blots for the effects of maleimides **1–9** are presented in Fig. 4A and B. The quantitative data, compared to the effect of etoposide (50 μ M), are reported in Fig. 4C. Interestingly, some derivatives were as potent (**8 and 9**) or even more potent (**3**) than etoposide in inhibiting topoisomerase II activity. Also, as expected *N*-phenylsuccinimide (**10**) did not alter topoisomerase II activity since it lacks the double bond that allows the addition of *N*-phenylmaleimides to the enzymes and thus their inhibition (Matuszak et al., 2009; Saario et al., 2005).

2.4. Effect of *N*-phenylmaleimides and derivatives on N1E115 cell proliferation

Looking at the inhibitory values obtained on hMAGL and htopoisomerase II activities, we delineated three series of compounds to be assayed on cell proliferation. The first series is made of substituted maleimides with dual inhibitory profile, i.e. compounds **3** and **6**. The second one consisted in maleimides with MAGL

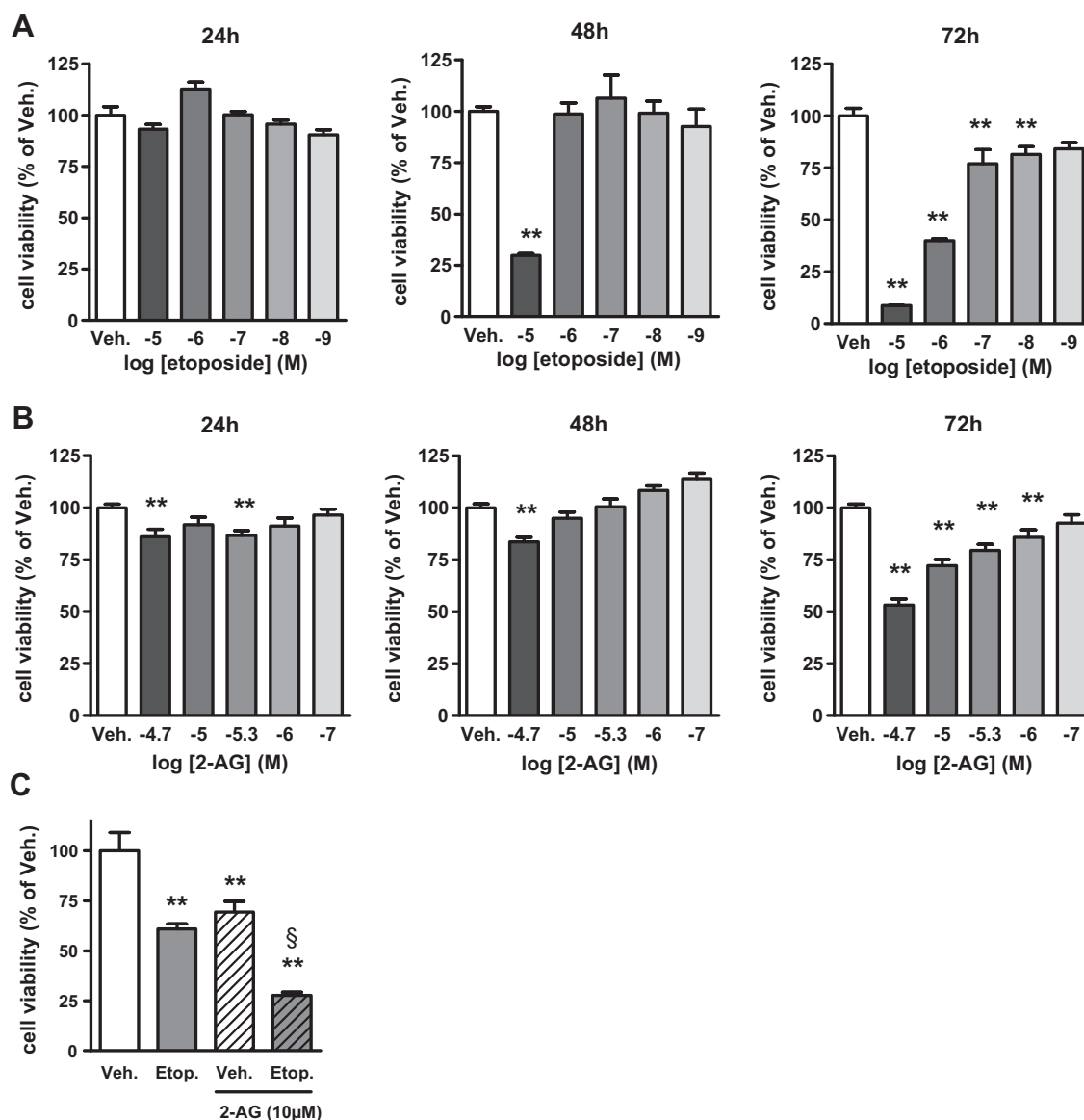


Fig. 1. Effects of etoposide and 2-arachidonoylglycerol on N1E115 cells growth. Etoposide (A) and 2-arachidonoylglycerol (B) effects on cell (2000 cells/well) growth were assayed at increasing concentrations (1 nM to 10 μ M) following 24, 48 and 72 h of incubation. Co-incubation of etoposide (0.1 μ M) and 2-arachidonoylglycerol (10 μ M), (C) reveals an additive effect on cell viability. The results of the MTT assay are expressed as % of viable cells compared the vehicle-treated cells. Data are the mean $\pm$ SEM of three experiments performed in triplicate. (One-way ANOVA, Dunnett post-test * p < 0.05, ** p < 0.01, *** p < 0.001.)

inhibition and no or low topoisomerase II inhibition such as compound **5**. The third one consists on inactive compounds on both enzymes (**10**).

First, we incubated N1E115 cells with increasing concentrations of **1** to confirm that also for these compounds 72 h of incubation were necessary in order to detect an effect on cell proliferation (Supplemental Fig. 1). Then, we assayed all the selected compounds, following 72 h of incubation, using an MTT assay (Fig. 5). Beside compounds **1**, **3** and **6** none of the compounds had a significant effect on cell viability when assayed above 100 μ M.

Based on the effect of 2-AG and etoposide (Fig. 1), a stronger effect was expected for the maleimides that combine MAGL and topoisomerase inhibition. Thus we asked whether a potentially more extensive MAGL inhibition would actually decrease cell viability and therefore assayed three known inhibitors of MAGL including the potent and selective JZL184. We found that when tested at 10 μ M only MAFP and CAY10499 had a small effect on N1E115 cells viability, whereas JZL184 had no effect (Fig. 6A). Note

that the co-incubation of these MAGL inhibitors with 2-AG did not result in a synergistic effect (Fig. 6B).

3. Discussion

Controlling cancer cells growth is one of the main goals of a successful cancer therapy. Because those cells possess mechanisms allowing them to grow faster and to escape drug chemotherapy, it is a common practice to combine several drugs having different modes of action in the course of the treatment. Here, we sought to explore the feasibility of simultaneously acting on two enzymes controlling cell growth, namely topoisomerase II and MAGL, using a single drug designed to target both enzymes which would be based on the maleimide scaffold. This is because among the compounds that have been described to inhibit topoisomerase II, one series of molecules was proved to have also an impact on MAGL activity. Indeed, Jensen et al. (2002) demonstrated that *N*-methylmaleimide

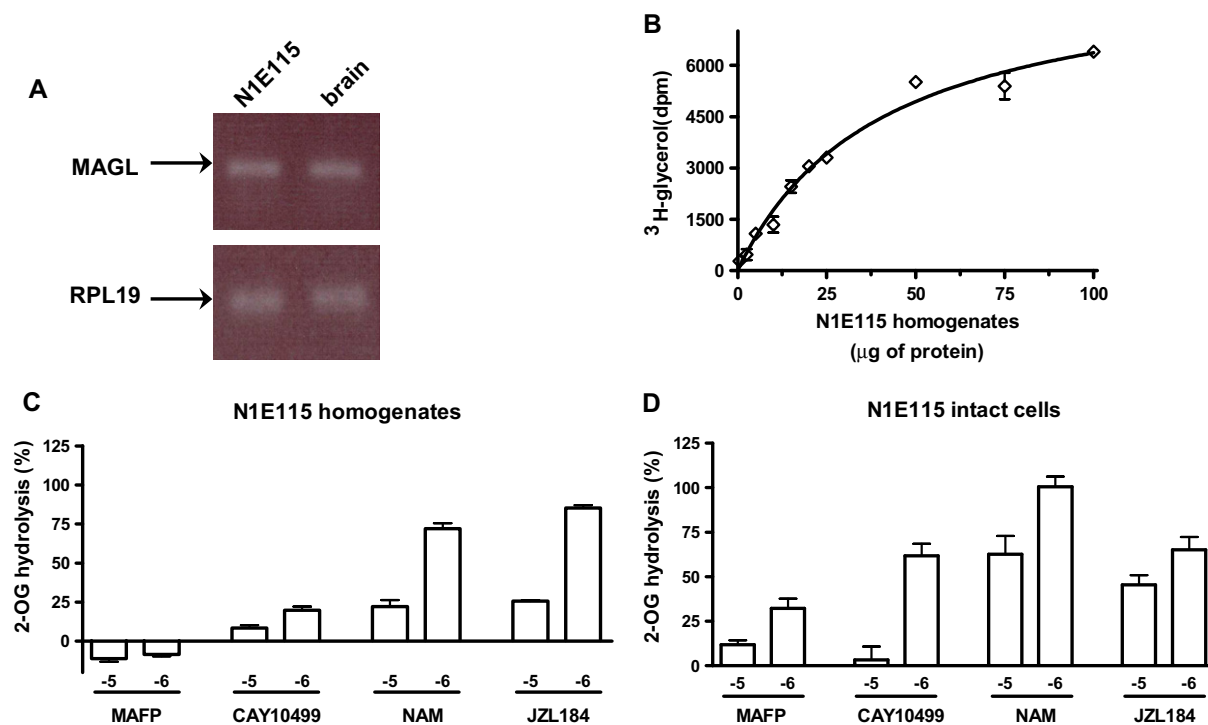


Fig. 2. Evidence for the presence and activity of MAGL in N1E115 cells. Detection of MAGL gene by conventional RT-PCR in either N1E-115 or in mouse brain (RPL19 gene used as control gene) (A). Hydrolysis of [^3H]-2-oleoylglycerol by increasing amounts of N1E-115 cells homogenate (B). Inhibition of [^3H]-2-oleoylglycerol hydrolysis by N1E-115 cells homogenate (C) or by intact cells (D) coincubated with different endocannabinoid metabolism inhibitors (10^{-5} and 10^{-6} M).

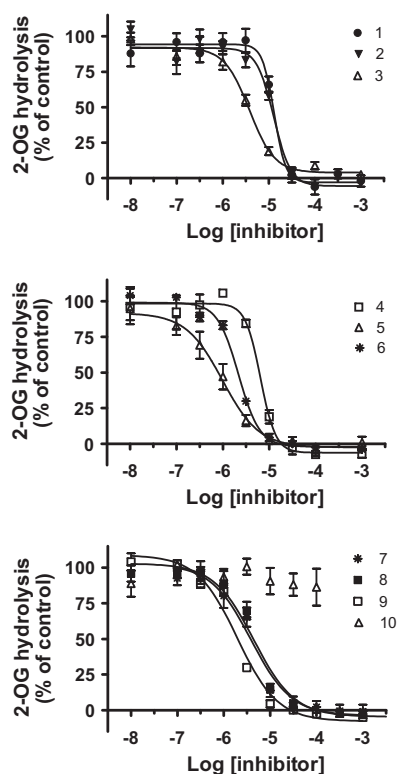


Fig. 3. MAGL inhibition by the maleimide derivatives used in this study. Increasing concentrations of maleimides 1–9 and succinimide 10 were assayed against hMAGL activity using [^3H]-2-oleoylglycerol as substrate. Data are the mean of at least four experiments performed in duplicate.

and *N*-ethylmaleimide were able to inhibit topoisomerase II activity, while we showed that *N*-phenylmaleimides were MAGL

inhibitors (Matuszak et al., 2009). Therefore we decided to look for maleimide derivatives that would combine inhibitory activities at both topoisomerase II and MAGL. This dual approach was strengthened by the observation that addition of 2-AG to N1E115 neuroblastoma cells reduces cell viability and that co-incubation with etoposide has additive effects. Increasing 2-AG local levels by inhibiting its MAGL-mediated degradation was the rational to test the inhibitory potential of several *N*-phenylmaleimides. Maleimides compounds inhibit MAGL by covalently binding cysteine residues close to the active site and thus decrease the amount of substrate hydrolyzed by the enzyme (Matuszak et al., 2009; Saario et al., 2005).

Although the effect of etoposide on N1E115 cells growth was no surprise, since IC_{50} values ranging between 0.5 and 100 μM have already been obtained using neuroblastoma cell lines (Demorow et al., 2007), the data obtained using exogenous 2-AG are more interesting since quite disparate data can be found in the literature. Indeed, depending on the cell line, 2-AG either promotes (Nithipatikorn et al., 2011; Fowler et al., 2003) or inhibits (Jacobsson et al., 2001; Bifulco et al., 2004) cell growth. Here, 2-AG dose-dependently reduced N1E115 neuroblastoma cells growth. Note that the relatively high concentrations needed to obtain an effect are similar to those reported by others (Ligresti et al., 2003; Di Marzo et al., 2000; Fonseca et al., 2010) and might be due to the degradation of 2-AG and to its binding to the culture wells.

Here, we synthesized *N*-substituted maleimides, and modified the *N*-substituent of their reactive moiety in order to detect potential dual inhibitors of topoisomerase II and MAGL (Table 1). We found that derivatives 3, 7–8 are as potent inhibitors of topoisomerase II activity as the reference inhibitor etoposide. We also confirmed that the maleimide double-bond is essential for their inhibiting activity as *N*-phenylsuccinimide (10) had almost no activity. This suggests the involvement of a cysteine residue, or alternatively an active serine residue, as target for maleimide derivatives. We previously postulated a similar mode of action

Table 1
Summary of the maleimide derivatives properties on topoisomerase II and MAGL activities and on N1E115 cells growth.

cpd	1	2	3	4	5	6	7	8	9	10
TOPO II (% of inhibition @ 20 μ M)	64.8%	74.4%	100%	27.0%	26.1%	71.2%	63.1%	30.6%	37.1%	18.6%
MAGL (IC ₅₀ in μ M)	15.9 $\pm$ 2.9 ^a	14.1 $\pm$ 4.1	3.80 $\pm$ 0.56	5.37 $\pm$ 1.24	0.73 $\pm$ 0.25	1.90 $\pm$ 0.21	4.71 $\pm$ 0.76	2.83 $\pm$ 0.53	5.00 $\pm$ 0.42	No effect

for the inhibition of MAGL by these compounds (Matuszak et al., 2009). Here, again, the absence of the maleimides double-bond completely abrogated MAGL inhibition observed with **1**. The *N*-substituent of **5** mimics the arachidonic chain of 2-arachidonoyl-glycerol which could explain its better activity against MAGL compared to the other maleimides derivatives. Thus **5** can be seen as a rather good MAGL inhibitor with poor activity against topoisomerase II. Conversely, **2** is a relatively poor MAGL inhibitor but shows interesting potential against topoisomerase II. Finally, maleimide **6** shows interesting activities against both targets.

When looking at the effect of these compounds on N1E115 neuroblastoma cell growth it appears that there is no direct correlation between the inhibition of topoisomerase II by our maleimides and the inhibition of cell growth. Thus for instance **9** completely blocked topoisomerase II activity when tested at 20 μ M and exerted a potent cytotoxic effect at 10 μ M. Conversely, **3** while completely blocking topoisomerase II activity (at 20 μ M), only decreased by 20% the cell growth when assayed at 10 μ M. Also, etoposide only inhibited by 30% topoisomerase II activity (when tested at 20 μ M) but was quite potent in inducing cell toxicity.

On the other hand, maleimides **1**, **5** and **6** are more effective than 2-AG in affecting neuroblastoma cell growth (compare Fig. 1B with Fig. 5). Here, again however there is no direct correlation between MAGL inhibition and cellular toxicity. Indeed, there is one order of magnitude between the MAGL inhibitions induced by **1** (IC₅₀ = 13 μ M) and **5** (IC₅₀ = 0.7 μ M) but both compounds have a similar effect on cell growth. This is further confirmed by the absence of cytotoxic effect induced by the potent MAGL selective inhibitor JZL184 on N1E115 cells (IC₅₀ of 6 nM in the same conditions (Matuszak et al., 2009). Two other reference MAGL inhibitors – MAFP and CAY10499 – had a limited effect on N1E115 cell growth. The discrepancy between the effects of those three inhibitors could be due to a difference in cell penetration or by other enzymes targeted by MAFP and CAY10499. Since all three inhibitors have been shown to inhibit MAGL or to increase endocannabinoid levels in intact cells the later hypothesis seems more likely. We noticed that 2-AG, a compound for which controversial effects on cell death are found in the literature (Maccarrone et al., 2000; Pasquariello et al., 2009; Siegmund et al., 2007; Deutsch et al., 1997) had a significant influence on the cell growth, but none of the tested inhibitor, even JZL184, managed to enhance its *in vitro* effects. In this context, Fatty Acid Amide Hydrolase (FAAH) could be seen as a prime candidate since we showed that the FAAH inhibitor URB597 reduces N1E115 cell viability (Hamtiaux et al., 2011) and it is known that MAFP and CAY10499 are also FAAH inhibitors (Long et al., 2009; Christian, 2001). Another element against the involvement of MAGL in the effect of maleimides on cell growth is the fact that compounds **1**, **5** and **6** are less potent MAGL inhibitors and yet are more effective in reducing cell growth than the above mentioned reference inhibitors. Since these maleimides derivatives are ineffective as FAAH inhibitors it is yet unclear by which mechanism(s) they reduce N1E115 cells growth.

Nevertheless, the results of a recent study on MAGL inhibition in human cancer cells add further support to MAGL as a potential therapeutic target in cancer (Nomura et al., 2010). Indeed, using both genetic (shRNA) and pharmacological (JZL184) blockade of MAGL Cravatt's group demonstrated that its inhibition could reduce cancer cell growth *in vitro* and *in vivo*. Thus, although MAGL inhibition does not seem to allow a reduction in N1E115 cell growth, it remains an attractive target. The pharmacological tools developed here allowing targeting both MAGL and topoisomerase II (e.g. derivative **6**) should therefore represent an interesting starting point in developing more potent drugs to reduce cancer cell growth.

In conclusion, we confirmed the effects of some maleimides on type II topoisomerase that had been previously described with

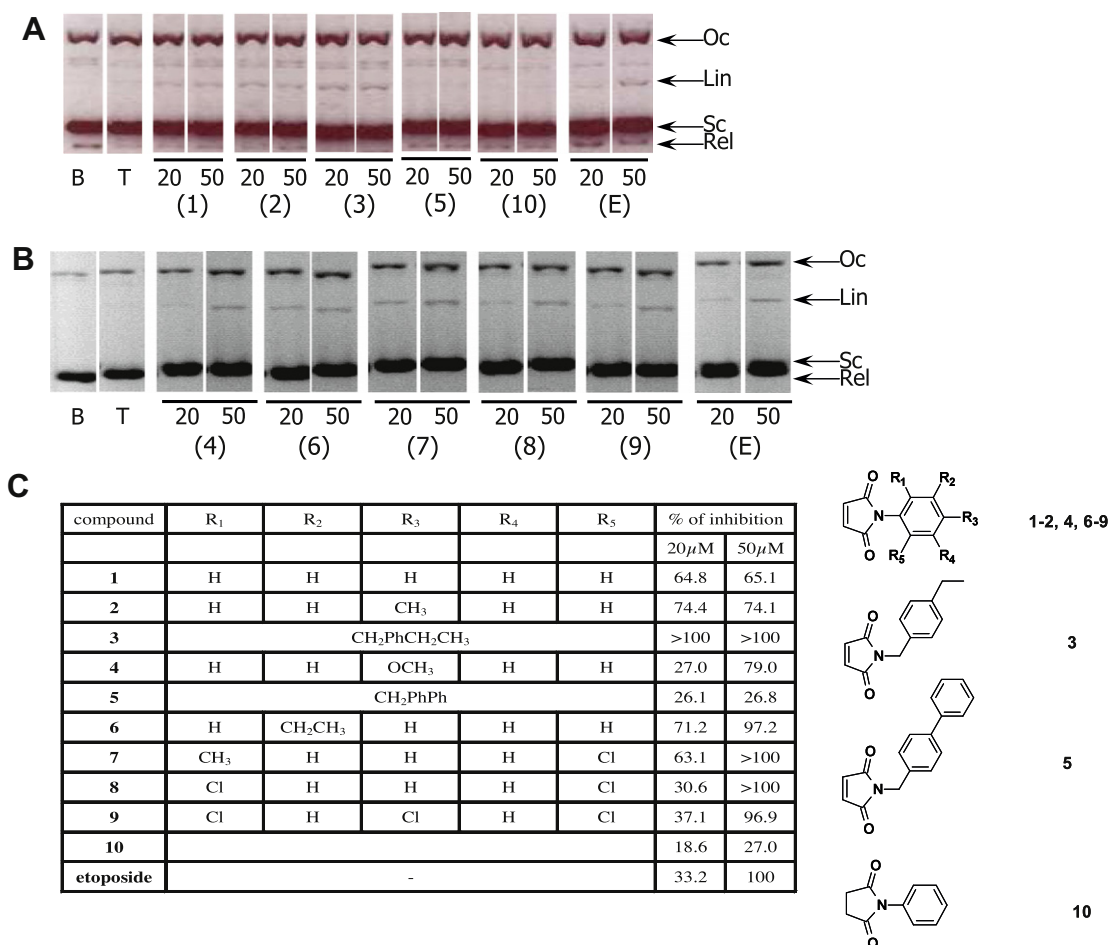


Fig. 4. Topoisomerase II inhibition by maleimide derivatives compared to inhibition by etoposide. Topoisomerase II activity was assayed using supercoiled DNA as substrate in the presence of test compounds (20 and 50 μM) (A and B). Etoposide (E) was used as reference inhibitor and tested at 20 and 50 μM. Lanes B and T are loaded respectively with supercoiled DNA alone or supercoiled DNA and topo II. For each gel, the band corresponding to the apparition of the linear form of DNA (Lin) was quantified, and the data expressed as percentage of etoposide's effect (when used at 50 μM) (C). Oc: open circular form of DNA; Lin: linear form of DNA; Sc: supercoiled form of DNA; Rel: relaxed form of DNA.

N-phenylmaleimide by Jensen et al. and proved a significant cytotoxic effect on cancer cells, but no synergy between topoisomerase II inhibition and MAGL inhibition could be clearly demonstrated. The implication of 2-arachidonoylglycerol in cancer phenomena is still controversial, and depending on its localization, depending on the cells that are the main actors in a biological process, this endogenous fatty acid ester could act in favor or against the cancer cell growth. However, it remains a target of interest and it has been recently proved to have antiproliferative effects (Nithipatikom et al., 2011).

4. Experimental section

4.1. Chemistry

All reagents were purchased from commercial sources (Sigma–Aldrich or Acros) and were used without further purification. Solvents were of analytical grade. Nuclear magnetic resonance (¹H NMR, ¹³C NMR) spectra were recorded at room temperature on a Bruker Avance 400 operating at 400 MHz for ¹H and 100 MHz for ¹³C. Chemical shifts (δ) are reported relative to the tetramethylsilane peak set at 0.00 ppm. Signals were abbreviated as s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet. In the case of multiplets, the signals are reported as intervals. Mass spectra were recorded on a Finnigan MAT 44S, with an ionization voltage of 70 eV.

4.1.1. Compounds 1–7

The synthesis and characterization of compounds 1–6 have been previously reported (Matuszak et al., 2009). Compound 10, 1-phenyl-2,5-pyrroledione, was purchased from Sigma (Sigma–Aldrich, Bornem, Belgium).

4.1.2. 1-(2-Chloro-6-methylphenyl)-1H-pyrrole-2,5-dione (7)

Compound 7 was similarly synthesized starting from 2-chloro-6-methylaniline and maleic anhydride, and was obtained with a yield of 76%. GC–MS *m/z* 222 (MH⁺). ¹H NMR (CDCl₃) δ 6.90 (s, 2H), 7.26 (m, 3H), 7.35 (d, 1H), 7.45 (d, 2H). ¹³C NMR (CDCl₃) δ 18.43, 127.82, 128.18, 129.40, 130.61, 133.67, 134.67, 139.90, 168.92.

4.1.3. 1-(2,6-Dichlorophenyl)-1H-pyrrole-2,5-dione (8)

Compound 8 was synthesized as 7, starting from 2,2-dichloroaniline and maleic anhydride, and was obtained with a yield of 73%.

Briefly, to a solution of maleic anhydride in diethyl ether, a solution the appropriate aniline (1 eq.) in diethyl ether was added and stirred at room temperature for 1 h. After cooling, the *N*-substituted maleamic acid was recovered by filtration, dried and then added to a solution of anhydrous sodium acetate (0.4 eq.) in acetic anhydride and stirred over a steam bath for 30 min. After pouring the mixture on an ice-water mixture, the precipitated product was recovered by filtration, washed, and dried. The crude *N*-substituted maleimide

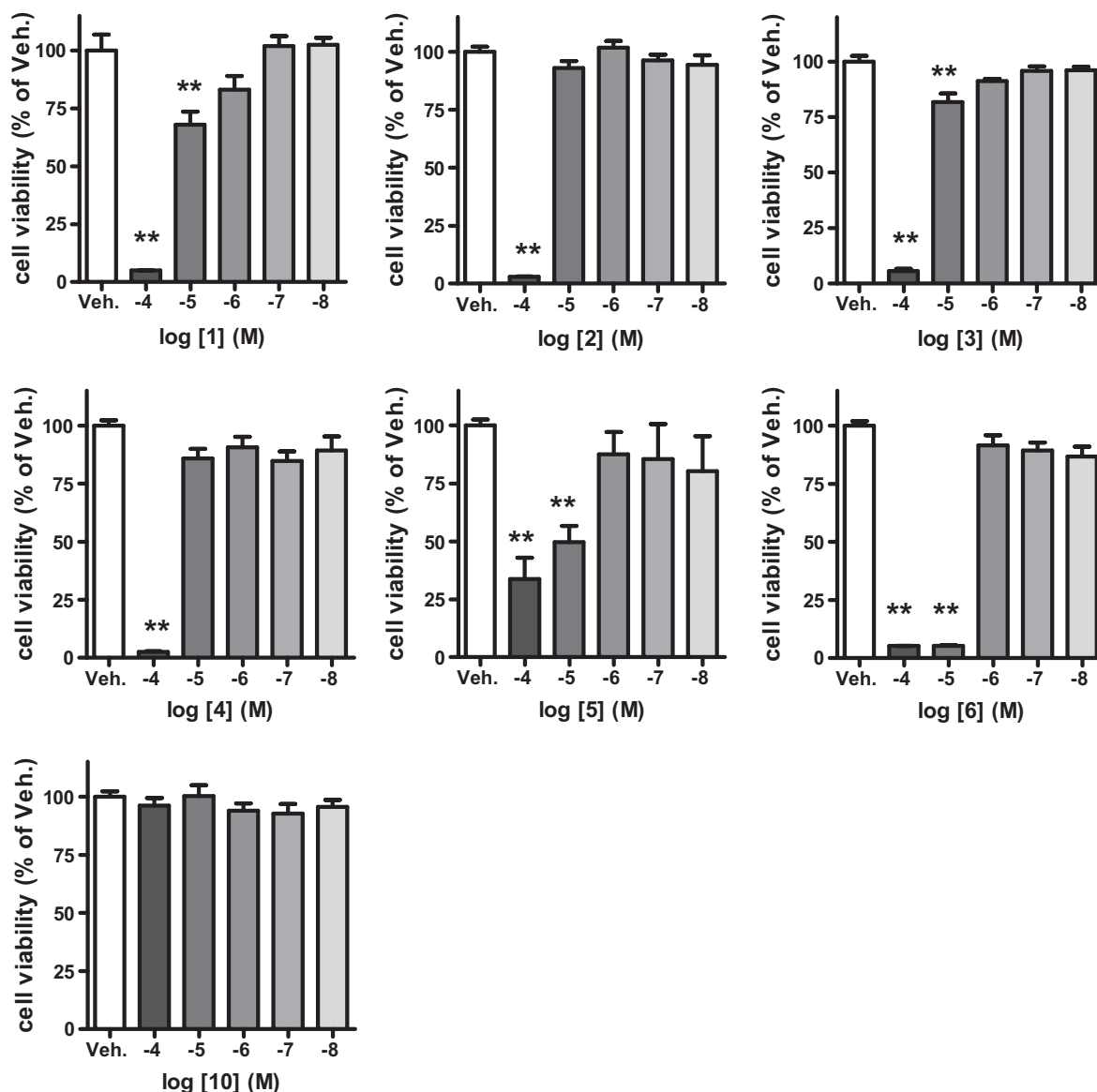


Fig. 5. Effect of maleimide derivatives on N1E115 cells growth. Maleimide derivatives were incubated (72 h) at increasing concentrations with N1E115 cells. Results of the MTT assay are expressed as % of viable cells compared the vehicle-treated cells. Data are the mean $\pm$ SEM of three experiments performed in triplicate. (One-way ANOVA, Dunnett post-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.)

was recrystallized from ethanol to afford the desired product. GC-MS m/z 242 (MH^+). 1H NMR ($CDCl_3$) δ 6.92 (s, 2H), 7.35 (t, 1H), 7.45 (d, 2H). ^{13}C NMR ($CDCl_3$) δ 127.5, 128.6, 131.2, 134.7, 167.8.

4.1.4. 1-(2,4,6-Trichlorophenyl)-1H-pyrrole-2,5-dione (**9**)

Compound **9** was synthesized according to the procedure described previously for **1** (Matuszak et al., 2009) from 2,4,6-trichloroaniline and maleic anhydride as building blocks (yield: 58%). GC-MS m/z 276 (MH^+). 1H NMR ($CDCl_3$) δ 6.92 (s, 2H), 7.48 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 126.33, 128.78, 134.79, 136.20, 136.61, 167.55.

4.2. Pharmacology

Drugs were dissolved at 5 mM in dimethyl sulfoxide and then further diluted in the appropriate assay media. Stock solutions of drugs were kept at $-20^\circ C$ and were freshly diluted to the desired concentration immediately before use.

4.2.1. MAGL activity assay

For the determination of the inhibition potential of tested compounds, the assay was conducted as we previously described (Matuszak et al., 2009). Briefly, 2-OG (10 μM ; [3H]-2-OG 50,000 dpm, American Radiolabeled Chemicals) was incubated at $37^\circ C$ for 10 min in Tris buffer (pH 8.0, 50 mM with 0.15% BSA) in the presence of purified recombinant human MAGL (5 ng) and tested compounds. The incubation was stopped by adding 400 μL of an ice-cold methanol-chloroform (1:1, v/v) mixture and thorough mixing. After centrifugation at 700g for 5 min, radioactivity in the upper aqueous layer was measured by liquid scintillation. Blanks (i.e. tubes containing no enzyme) were made for each experiment (and the values subtracted to all the other values).

4.2.2. Topoisomerase II mediated DNA cleavage assays

The assays were performed as previously described (Bosier et al., 2008). Briefly, supercoiled pKMp27 DNA (0.5 μg) was incubated with four units of human topoisomerase II (TopoGen Inc.) at $37^\circ C$ for 30 min in relaxation buffer (50 mM Tris, pH 7.8,

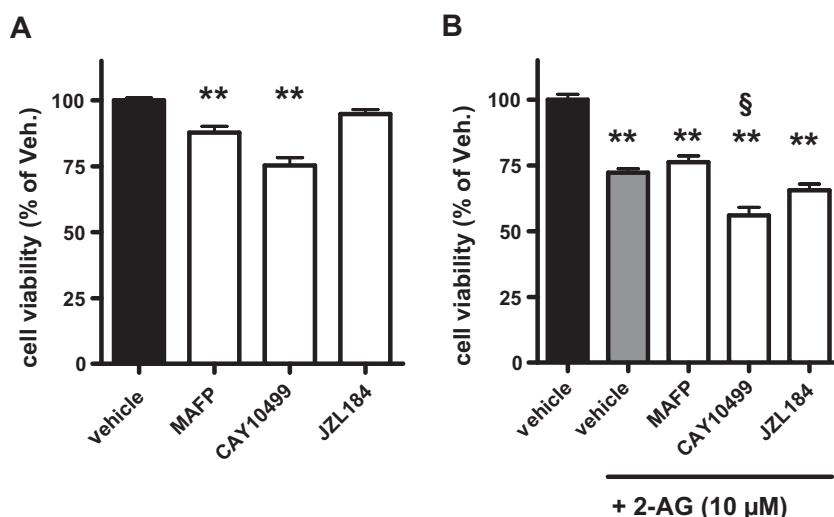


Fig. 6. Effect of reference MAGL inhibitors on N1E115 cells growth. MAGL inhibitors alone (10 μ M) (A) and in combination with 2-arachidonoylglycerol (10 μ M) (B) were incubated 72 h with N1E115 cells (2000 cells/well). The results of the MTT assay are expressed as % of viable cells compared the vehicle-treated cells. Data are the mean $\pm$ SEM of three experiments performed in triplicate. (One-way ANOVA, Dunnett post-test * p < 0.05, ** p < 0.01, *** p < 0.001.)

50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA and ATP) in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μ g/mL. DNA samples were then added to the electrophoresis dye mixture (3 mL) and electrophoresed in a 1% agarose gel containing ethidium bromide (1 mg/mL) at room temperature for 2 h at 120 V. The gel electrophoresis separates the remaining supercoiled plasmid DNA (Sc) from the relaxed DNA (Rel and the linear DNA (Lin). Gels were washed and photographed under UV light. For the quantitative determination of topoisomerase II activity, the band corresponding to the linear DNA on photographic negatives was densitometrically scanned using ImageJ software for integration (Ligresti et al., 2003) (<http://rsb.info.nih.gov/ij/>). The results are expressed based on the activity of the reference drug etoposide assayed at 50 μ M (inhibition value set at 100%).

4.2.3. Viability assayed by MTT conversion

Mouse N1E-115 neuroblastoma cells were expanded at 37 $^{\circ}$ C in a humidified atmosphere containing 5% CO_2 in DMEM/F-12 (1:1) medium supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU/mL), and streptomycin (100 μ g/mL) (Bosier et al., 2008). The inhibition of cell proliferation was determined with the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Hamtiaux et al., 2011). Briefly, 5 h before treatment cells were seeded (2000 cells/well, 96 wells, flat bottom) in DMEM/F-12 (1:1) medium supplemented with 10% serum. After 5 h of incubation at 37 $^{\circ}$ C in a 5% CO_2 humidified atmosphere, test compounds diluted in culture medium were added to each well. The incubation was continued for 24, 48 or 72 h, and then the medium was removed and 100 μ L of MTT was added to each well (0.3 mg/mL, final concentration). Cells were incubated for 2 h at 37 $^{\circ}$ C in a humidified atmosphere before the medium was removed and the formazan dye, produced by metabolically active cells, solubilized in 100 μ L of DMSO. MTT conversion ($A_{650}-A_{570}$) was measured using a microplate spectrophotometer (SpectraMAX Plus, Molecular Devices). Experiments were carried out at least three times and the results are reported as mean $\pm$ SEM. Statistical analyses were performed using GraphPad InStat 3 Version 5.00 (GraphPad Software Inc, USA).

4.2.4. Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from the cultured cell with the TriPure Isolation reagent (Roche). To measure mRNA expression, reverse transcription was performed using the Reverse Transcription System (Promega) and the generated cDNA was amplified by PCR using the following primers sets: MAGL forward: atgtcctgatttcacacctggt; reverse: tcaacctccgactgttccgagaca and for the control gene 60S ribosomal protein L19 (PRL19) forward: gaaggtcaaagggaatgtgttca and reverse: ccttgctgccttcagcttgt. Polymerase chain reactions were performed according to the following parameters: 95 $^{\circ}$ C for 10 min, 95 $^{\circ}$ C for 3 s, 60 $^{\circ}$ C for 26 s, and 72 $^{\circ}$ C for 10 s (45 cycles). After amplification, agarose gel electrophoresis was used to detect the expression of the studied genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ejps.2011.11.011](https://doi.org/10.1016/j.ejps.2011.11.011).

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