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MOLECULAR TOXICOLOGY

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AM404 and VDM 11 non-specifically inhibit C6 glioma cell proliferation at concentrations used to block the cellular accumulation of the endocannabinoid anandamide

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Abstract AM404 [*N*-(4-hydroxyphenyl)arachidonylamide] and VDM 11 [(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide] are commonly used to prevent the cellular accumulation of the endocannabinoid anandamide, and thereby to potentiate its actions. However, it has been reported that AM404 can produce an influx of calcium into cells, which might be expected to have deleterious effects on cell proliferation. In the present study, AM404 and VDM 11 were found to reduce C6 glioma cell proliferation with IC_{50} values of 4.9 and 2.7 μM, respectively. The inhibition of cell proliferation following a 96-h exposure was not accompanied by dramatic caspase activation, and was not prevented by either a combination of cannabinoid and vanilloid receptor antagonists, or by the antioxidant α-tocopherol, suggestive of a non-specific mode of action. Similar results were seen with palmitoylisopropylamide, although this compound only produced significant inhibition of cell proliferation at 30 μM concentrations. AM404 (1 μM), VDM 11 (1 μM) and palmitoylisopropylamide (3–30 μM), i.e. concentrations producing relatively modest effects on cell proliferation per se, reduced the vanilloid receptor-mediated antiproliferative effects of anandamide, as would be expected for compounds preventing the cellular accumulation of anandamide (and thereby access to its binding site on the vanilloid receptor). It is concluded that concentrations of AM404 and VDM 11 that are generally used to reduce the cellular accumulation of anandamide have deleterious effects upon cell proliferation, and that lower concentrations of these compounds may be more appropriate to use in vitro.

Keywords Anandamide · Uptake inhibitors · Cannabinoid receptors · Vanilloid receptors · Cell proliferation · Glioma cells

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Introduction

Anandamide (arachidonoylethanolamide, AEA) is an endogenous compound that exerts a wide variety of actions in the body as a result of activation of cannabinoid (CB) and vanilloid (VR) receptors (Mechoulam et al. 1998; Högestätt and Zygmunt 2002). As with all endogenous signalling molecules, pathways for AEA removal are also present in the body. Thus, extracellular AEA is removed by cellular accumulation followed by metabolism to arachidonic acid (Deutsch and Chin 1993). Whilst the enzyme responsible for AEA metabolism, fatty acid amide hydrolase (FAAH), has been fully characterised (see Fowler et al. 2001; Patricelli and Cravatt 2001 for reviews), there is some controversy as to whether the cellular accumulation of AEA is a facilitated or a passive diffusion process (Patricelli and Cravatt 2001; Glaser et al. 2002). Nevertheless, it is clear that the arachidonic acid analogues AM404 [*N*-(4-hydroxy-phenyl)arachidonylamide] and VDM 11 [(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide], as well as the palmitoylethanolamide analogues PIA (palmitoylisopropylamide) and PCA (palmitoylcyclohexylamide), are able to reduce the rate of cellular accumulation of AEA (Beltramo et al. 1997; De Petrocellis et al. 2000; Jonsson et al. 2001) and that this effect, be it due to a primary effect on a putative uptake site or secondary, for example, to inhibition of FAAH (Deutsch et al. 2001), will potentiate the effect of extracellular

anandamide at CB receptors. In addition, the reduction of intracellular AEA by these compounds would be expected to reduce the effectiveness of exogenous AEA at VR, given that the binding site for AEA on VR is intracellular (Jordt and Julius 2002), and such a result has been seen in transfected cells (De Petrocellis et al. 2001).

As a result of their ability to reduce the accumulation of AEA, AM404 and VDM 11 have been widely used to investigate the properties of exogenously administered AEA and to uncover the presence of endocannabinoid tone in vivo (for review see Fowler and Jacobsson 2002). However, the specificity of AM404 has been questioned. Zygmunt et al. (2000) reported that AM404 is able to relax rat isolated hepatic arteries contracted with phenylephrine, with a pEC_{50} value of 7.4 ± 0.1, corresponding to an EC_{50} value of 0.04 μM. This effect could be antagonised by the VR antagonist capsazepine, but not by the CB_1 receptor antagonist SR141716A. These authors also found that 3 μM AM404 produced a large ion current, blockable with capsazepine, in inside-out membrane patches of *Xenopus* oocytes injected with VR1 cDNA (Zygmunt et al. 2000). More recently, Chen et al. (2001) reported that 5 μM AM404 produced an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a variety of different cell types. In Madin-Darby canine kidney (MDCK) cells (which, to our knowledge, have not been reported to express VR), the increased $[Ca^{2+}]_i$ was prevented by removal of extracellular Ca^{2+} and by the Ca^{2+} entry blocker La^{3+}, but not by Ni^{2+} or a variety of L-type Ca^{2+}-channel blockers (Chen et al. 2001). Since AM404 inhibits the accumulation of AEA with an IC_{50} value ranging from 1 to 14 μM, depending upon the cell type and experimental design used (Beltramo et al. 1997; Piomelli et al. 1999; Jarrahian et al. 2000; De Petrocellis et al. 2000; Rakhshan et al. 2000; Jacobsson and Fowler 2001), these data would suggest that the specificity of AM404 is poor.

AM404-induced influx of Ca^{2+} (Chen et al. 2001) may result in deleterious effects upon cell survival, given that an uncontrolled Ca^{2+} influx is associated with apoptotic cell death (see for example Kim et al. 2002 for a recent mechanistic study). It is thus important to determine whether this compound, at concentrations that are generally used to inhibit AEA accumulation in vitro, affects cell proliferation, and whether such effects are also seen with other compounds affecting AEA accumulation. Consequently, in the present study, the effects of AM404, VDM 11, PIA and PCA upon the proliferation of C6 glioma cells have been determined. C6 glioma cells are useful in this respect, since uncontrolled Ca^{2+} influx produced by the Ca^{2+} ionophore A23187 is known to produce apoptotic cell death in these cells (Wernyj et al. 1999; Ray et al. 1999; Morita and Wong 2000). A poster summarising this paper was presented at the recent Society for Neuroscience meeting (Fowler et al. 2002).

Materials and methods

Compounds

PIA (palmitoylisopropylamide, C16:0) and PCA (palmitoylcyclohexylamide, C16:0) were synthesized as described earlier by Lambert et al., (1999). AEA (arachidonoylethanolamide, anandamide) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). AM404 [*N*-(4-hydroxyphenyl)arachidonylamide], VDM 11 [(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide], AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-*1H*-indol-3-yl](4-methoxyphenyl) methanone), AM251 [*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*1H*-pyrazole-3-carboxamide] and capsazepine were purchased from Tocris Cookson (Bristol, UK). CyQUANT cell proliferation assay kits were bought from Molecular Probes (Eugene, OR, USA). All cell culture media, sera and supplements were from Gibco/Life Technologies (Täby, Sweden).

Cell cultures

Rat C6 glioma cells (passage range 41–68) were obtained from the American Type Culture Collection (Rockville, MD, USA) and were grown in 75-cm^2 culturing flasks at 37°C, and under 5% CO_2 in air at normal atmospheric pressure. The cells were cultured in Ham's F10 medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin plus 100 μg/ml streptomycin (1% PEST). The culture media were changed three times a week.

C6 cell proliferation assay

The method described by Jacobsson et al. (2001) was used. C6 glioma cells were plated on flat-bottomed 96-well plates at a initial density of 2500 cells/well in cell culture media supplemented with 1% FBS and 1% PEST and incubated at 37°C for 6 h under an atmosphere of 5% CO_2 in air. After 6 h the cells were incubated with the compounds at different concentrations. AM630, AM251 and calpeptin were dissolved in dimethylsulfoxide (DMSO) and all other substances were dissolved in ethanol. Half of the media was replaced daily for 4 days with fresh substances dissolved in media (see Jacobsson et al. 2001). In all but one experiment, solvent concentrations were kept constant between different wells of the same experiment. In the final experiment (investigating the effects of VR plus CB antagonists upon the antiproliferative effects of the compounds), either ethanol/DMSO or ethanol alone was used as the solvent controls, since the addition of DMSO at the final concentration used did not affect the observed cell proliferation.

On the fifth day, unless otherwise stated (Fig. 1), the plates were frozen at −80°C after the media had been removed by gently turning the microplates on paper towels. On the day of analysis, the cell density was measured using the CyQuant cell proliferation assay kit, which quantifies the total nucleic acid content of the samples. The plates were placed at room temperature for 30 min before addition of reagents and incubation for further 5 min. Fluorescence was measured (excitation/emission 485/520 nm) in a FLUOstar Galaxy microplate reader (BMG Labtechnologies GmbH, Offenberg, Germany). Background fluorescence data were subtracted, and the data were and presented as percentages of control, unless otherwise stated. A series of calibration curves indicated that the relationship between cell density and fluorescence deviated very slightly from linear at high cell densities. This was taken into account for the data shown in Fig. 1. However, for the remaining data, this small deviation is not taken into account when the data are simply expressed as percentages of control. This means that the degree of inhibition of cell proliferation is slightly underestimated, i.e. the effects of the compounds upon cell proliferation are, if anything, slightly greater than those shown in the figures.

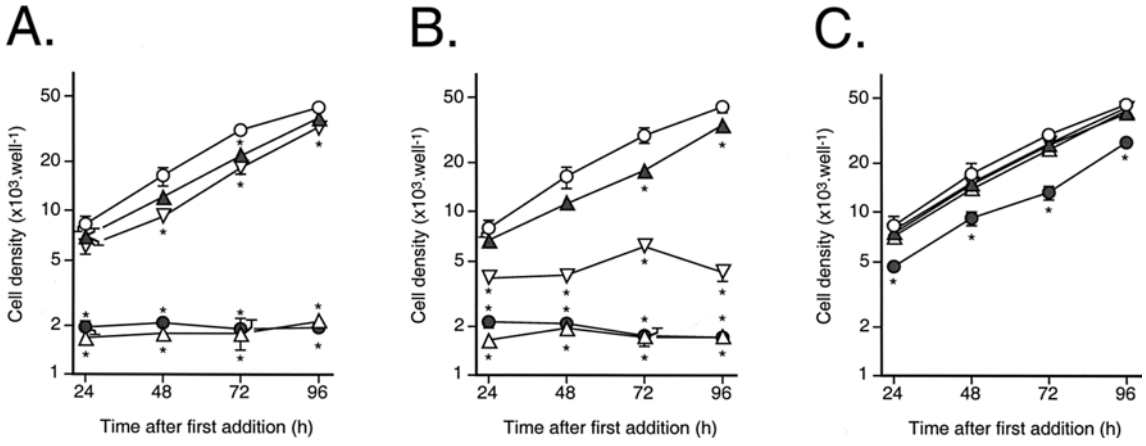

Fig. 1A–C Effects of AM404 (**A**), VDM 11 (**B**) and PIA (**C**) upon the proliferation of C6 glioma cells. Concentrations of the compounds used were 0 (i.e. ethanol carrier, *open circles*), 1 (*solid triangles*), 3 (*open inverted triangles*), 10 (*open triangles*) and 30 μM (*solid circles*). Data are means ± SEM (when not enclosed by the symbols), $n = 3$. $^*P < 0.05$, Bonnferroni-Dunn post hoc test following significant one-way factorial ANOVA versus the corresponding controls at the same time point

Measurement of caspases activity

Activity of caspases was determined using a commercial assay kit that can detect the activities of caspases 2, 3, 6, 7, 8, 9 and 10 (Homogenous caspases assay, fluorimetric; Roche Molecular Biochemicals, Mannheim, Germany). Briefly cells were cultured and treated with test compounds as described above, albeit using Costar 96-well black clear-bottom plates. After 48 or 96 h of incubation, the medium was removed and the plates were frozen. On the day of assay, the plates were thawed (1 h at room temperature) and aliquots (100 μl) of medium were added. Substrate (Asp-Glu-Val-Asp-Rhodamine 110) in lysate buffer (100 μl) was added and the samples were then incubated for 2 h at 37°C. Free rhodamine 110 produced by the action of caspases upon this substrate was detected fluorimetrically using excitation and emission wavelengths of 485 and 520 nm, respectively. Activities in the samples are expressed as percentages of the corresponding activity for a positive control (lysate from apoptotic U937 cells treated with camptothecin) provided with the kit.

Statistics

The pI_{50} values [$-\log_{10}(IC_{50}$ value)] of the data expressed as percentage of control were analysed using the built-in equation "sigmoid dose-response (variable slope)" of the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA). "Top" and "bottom" values were set to 100% and 0%, respectively. One-way factorial analyses of variance (ANOVAs), with Bonferroni-Dunn post hoc tests, were conducted using the Statview computer programme (SAS institute Inc., Cary, NC, USA). Two-tailed unpaired *t*-tests were conducted using the GraphPad Prism programme (San Diego, CA, USA).

Results

Time-courses of the antiproliferative effects of AM404, VDM 11 and PIA

The effects of AM404, VDM 11 and PIA upon cell proliferation were measured 24, 48, 72 and 96 h after administration (Fig. 1). AM404 produced a concentration-dependent reduction in cell proliferation that was seen with 24 h of exposure to 10 and 30 μM concentrations and after 48 h at 3 μM (Fig. 1A). The lowest concentration of AM404 tested, 1 μM, produced a significant, albeit small, reduction in cell proliferation at 72 h, but not at the other time points. When all experiments in the entire study using a 96-h incubation time were summarised, 1, 3, 10 and 30 μM AM404 gave cell densities of 94 ± 3% ($n = 12$), 80 ± 3% ($n = 18$), 10 ± 1% ($n = 12$) and 10 ± 1% ($n = 6$) of control, respectively (means ± SEM; pI_{50} 5.31 ± 0.045, corresponding to a mean IC_{50} value of 4.9 μM). The time-course of the effect of AM404 upon cell proliferation contrasts with that seen for AEA, which, under the same assay conditions, does not produce any reduction of cell proliferation at 24 h, but does reduce it after 72 and 120 h (Jacobsson et al. 2001).

VDM 11 was more potent than AM404 at reducing C6 glioma cell proliferation, with significant reductions being found after 24 h for 3, 10 and 30 μM and after 72 h for 1 μM (Fig. 1B). When all experiments in the entire study using a 96-h incubation time were summarised, 1, 3, 10 and 30 μM VDM 11 gave cell densities of 88 ± 3% ($n = 18$), 45 ± 8% ($n = 12$), 9 ± 1% ($n = 9$) and 10 ± 1% ($n = 6$) of control, respectively (pI_{50} 5.56 ± 0.036, corresponding to a mean IC_{50} value of 2.7 μM).

In contrast to the two arachidonoyl-based compounds, PIA did not affect cell proliferation at any of the time-points measured at concentrations of 1, 3 and 10 μM, whereas 30 μM produced a reduction by 24 h (Fig. 1C). When all experiments in the entire study using a 96-h incubation time were summarised, 0.3, 1, 3, 10 and 30 μM PIA gave cell densities of 99 ± 5% ($n = 5$), 96 ± 4% ($n = 8$), 100 ± 3% ($n = 14$), 98 ± 2% ($n = 23$) and 74 ± 4% ($n = 23$) of control, i.e. IC_{50} value > 30 μM. PCA was more efficacious in this respect, and gave cell densities of 95 ± 5% ($n = 6$), 92 ± 4% ($n = 6$), 80 ± 5% ($n = 6$), 62 ± 4% ($n = 12$) and 38 ± 4% ($n = 12$) of control following a 96-h incubation at concentrations of 0.3, 1, 3, 10 and 30 μM, respectively (pI_{50} 4.76 ± 0.015, corresponding to a mean IC_{50} value of 17 μM).

Effects of AM404, VDM 11 and PIA upon induction of caspases

Parallel with the time-course experiments shown in Fig. 1, the effect of the treatments upon the induction of caspases was investigated. Very low levels of caspase activities (relative to the positive control lysate of apoptotic U937 cells treated with camptothecin provided with the kit) were seen after either 48 h or 96 h of treatment with AM404, VDM 11 or PIA. In Fig. 2, the caspase activities are shown following 96 h of treatment. Reduced activities were seen following treatment with either AM404 or VDM 11 or with 30 μM PIA, but these effects essentially mirrored those of the respective compounds upon cell proliferation (shown as *thick bars* in Fig. 2). The apparent difference at high concentrations of AM404 and VDM 11 presumably mirrors the fact that the activities of caspases in these samples are near the detection limit of the assay. Similarly, the very low activities of caspases following 48 h of treatment preclude interpretation of the data, which are therefore not shown here. Thus, the most conservative interpretation of the data is that antiproliferative effects of 1 and 3 μM AM404, 1 μM VDM 11 and 30 μM PIA are not accompanied by any dramatic induction of caspases.

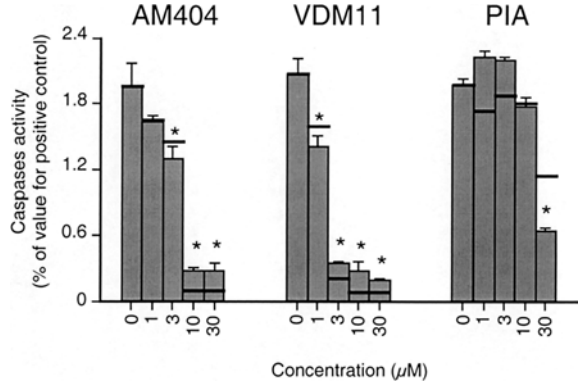

Fig. 2 Effects of AM404, VDM 11 and PIA upon the caspases activity of C6 cell lysates following incubation for 96 h. Data are means ± SEM, $n = 3$, and are expressed as percentages of the activity found for a positive control lysate (apoptotic U937 cells treated with camptothecin provided with the assay kit). The *thick bars* indicate the corresponding mean nucleic acid contents of parallel samples (taken from Fig. 1) normalised to the caspases activity for the controls for each treatment $^*P < 0.05$, following significant one-way factorial ANOVA and Bonnferroni-Dun post hoc test versus the corresponding control

Effects of VR plus CB receptor blockade, α-tocopherol and calpeptin upon the antiproliferative effects of AM404, VDM 11, PIA and PCA

In the experiments described in this section, the test compounds (e.g. AM404 etc.) and putative blockers (e.g. α-tocopherol) were co-incubated with the cells for the 96-h period, with the appropriate changes of medium as described in the Materials and methods Section. The effects of a combination of the CB_1 receptor antagonist AM251, the CB_2 receptor antagonist AM630 and the VR antagonist capsazepine upon the antiproliferative effects of AM404, VDM 11, PIA and PCA are shown in Fig. 3A. The combination, at the concentrations used here, completely block the antiproliferative effects produced by AEA (Jacobsson et al. 2001). In contrast, they were without significant effect upon the antiproliferative effects of AM404, VDM 11, PIA and PCA. A similar result, with the exception of that at 30 μM PCA, was seen when the cells were co-incubated with 10 μM α-tocopherol (Fig. 3B), a concentration that completely prevents the antiproliferative effects of 3 μM AEA (Jacobsson et al. 2001).

In a single experiment, the effects of 1 μM calpeptin upon the antiproliferative effects of AM404 and VDM

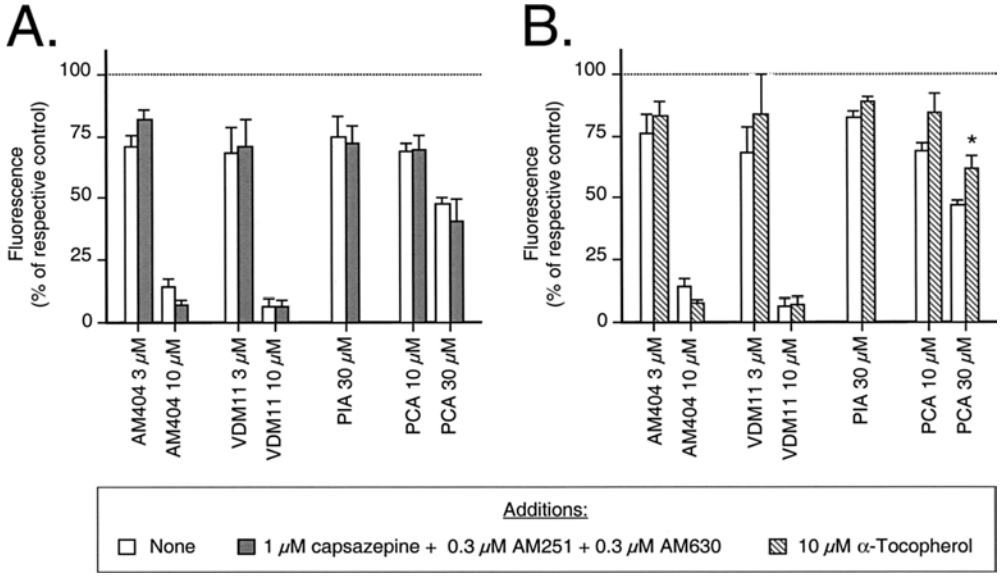

Fig. 3A,B Effects of a combination of CB_1 receptor antagonist AM251, CB_2 receptor antagonist AM630 and VR antagonist capsazepine (**A**), or of the antioxidant α-tocopherol (**B**) upon the antiproliferative effects of AM404, VDM 11, PCA and PIA. The incubation time was 96 h. Data are expressed as percentages of control cells not treated with anandamide but treated with the same concentration of antagonist or α-tocopherol as appropriate, and are means ± SEM, $n = 3$–7. $^*P < 0.05$, two-tailed unpaired *t*-test versus the corresponding control (otherwise $P > 0.1$)

11 were investigated. The cell density (as percentage of the corresponding control containing the same calpeptin or carrier concentration) for the carriers and calpeptin, respectively, were: 84 and 91% for 3 μM AM404, 7 and 7% for 10 μM AM404, 81 and 76% for 3 μM VDM 11, and 4 and 5% for 10 μM VDM 11 (means of triplicate determinations each for three separate 24 well plates, assayed concomitantly). Thus, no obvious effect of calpeptin was seen.

Effects of AM404, VDM 11 and PIA upon the antiproliferative effects of AEA

In a series of experiments, the abilities of AM404, VDM 11 and PIA to prevent the inhibition of cell proliferation by AEA was investigated. Initial experiments confirmed the concentration-dependent inhibition of cell proliferation by AEA after 96 h of incubation, and demonstrated that the potency was identical for ambient ethanol concentrations of 0.5, 1 and 2% (data not shown). However, in contrast to the robust effects of AM404, VDM 11 and PIA, AEA showed a different potency among different experiments, and in some experiments no antiproliferative effects of AEA could be detected. Nevertheless, when all experiments in a long series ($n=36$) were summarised for 3 μM AEA, the cell proliferation was $58\pm4.9\%$ of control (mean $\pm$SEM, $P<0.001$, one-sample test versus 100%, 95% confidence interval 48–68%). Since the present experiments were undertaken in order to investigate whether AM404, VDM 11 or PIA reduce the antiproliferative effects of AEA, only experiments where AEA per se gave a reduction in cell proliferation were included. The data are expressed as percentages of the corresponding value in the absence of AEA for the same concentration of AM404, VDM 11 or PIA, as appropriate, to negate the effects of these compounds per se on cell proliferation.

The general pattern was the same for all three compounds, namely that they reduced the antiproliferative effect of AEA (Fig. 4). Thus, AM404 at concentrations of 1 and 3 μM significantly reduced the antiproliferative effects of 2 and 3 μM AEA (Fig. 4A), and a similar pattern was seen with VDM 11 (Fig. 4B). In the experiment shown in Fig. 4C, PIA at concentrations of 3, 10 and 30 μM greatly reduced the antiproliferative effects of 1–5 μM AEA. A similar result was seen in a subsequent experiment with 10 μM PIA, although in this case there was a much larger variability among samples, and the effects were not significant (at 3 μM AEA, controls $63\pm10\%$, PIA $107\pm31\%$; at 5 μM AEA, controls $47\pm5\%$, PIA $70\pm11\%$; means $\pm$SEM, $n=3$). However, when the results of these experiments were combined with the corresponding data from Fig. 4C to increase the power of the sample, significant differences were seen (at 3 μM AEA, controls $50\pm8\%$, PIA $95\pm15\%$, $P<0.05$; at 5 μM AEA, controls, $46\pm3\%$, PIA $77\pm6\%$, $P<0.01$; means $\pm$SEM, $n=6$).

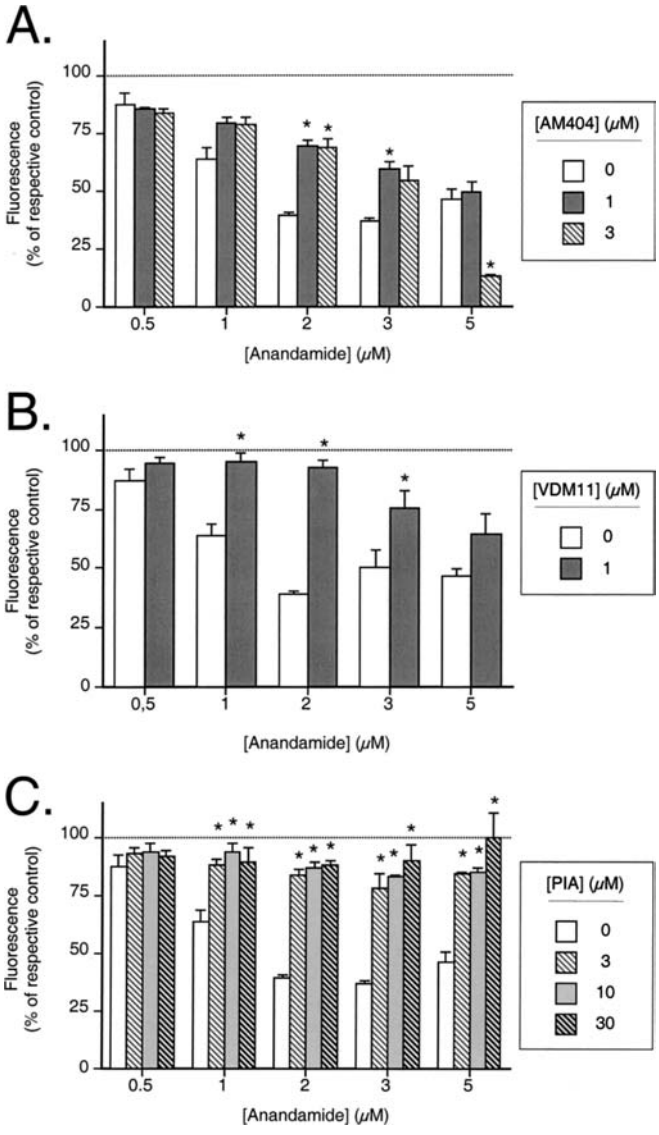

Fig. 4A–C Effects of AM404 (**A**), VDM 11 (**B**) and PIA (**C**) in modulating the antiproliferative activity of anandamide. The incubation time for these experiments was 96 h. Data are expressed as percentages of control cells not treated with anandamide but treated with the same concentration of AM404, VDM 11 or PIA, as appropriate, and are means $\pm$SEM, $n=3$ (**A**, **C**) or $n=3$–9 (**B**). In **A** and **C** (experiments run concomitantly, hence the same bars for the controls at each anandamide concentration), $^*P<0.05$, following significant one way factorial ANOVA and Bonnferroni-Dunn post hoc test versus the corresponding controls at the same time point. In **B**, $^*P<0.05$, two-tailed unpaired *t*-test versus the corresponding control

Discussion

The ability of AM404 and VDM 11 to prevent the cellular accumulation of AEA and thereby prolong the extracellular "life" of this compound has provided an attractive approach to possible endocannabinoid-based therapies. Examples where such an approach may be useful are in the treatment of multiple sclerosis and

Huntington's disease (Baker et al. 2001; Lastes-Becker et al. 2002). Such treatment strategies, however, require that the compounds themselves are not cell-toxic. In view of the finding that AM404 can produce, at concentrations in the range generally used for in vitro experiments, an influx of Ca^{2+} into a variety of cells (Chen et al. 2001), it is important to determine whether these compounds have deleterious effects upon cell proliferation.

The results of the present study indicate that AM404 and VDM11 produce dramatic effects upon cell proliferation. Concentrations of 10 and 30 μM AM404 and VDM 11 completely blocked cell proliferation at all time points measured (24–96 h), whilst concentrations of 1–3 μM AM404 and 1 μM VDM 11 had more modest, albeit significant, effects that are seen first after ≥48 h of exposure. The lack of significant effects of 1 and 3 μM AM404 and 1 μM VDM 11 at the 24-h time-point would suggest that these latter effects are due to a time-dependent slowing of cell proliferation, rather than induction of an initial lag phase.

The concentrations of VDM 11 and AM404 producing dramatic effects on cell proliferation are in the range generally used for uptake inhibition experiments. For example, De Petrocellis et al. (2000) reported that AM404 and VDM 11 inhibited the uptake of 4 μM AEA into C6 glioma cells with IC_{50} values of 10 μM in both cases. AM404, PIA and PCA have also been found to reduce the formation of [^{3}H]-ethanolamine (i.e. a combination of effects on AEA accumulation and FAAH-catalysed hydrolysis) following addition of 3 μM [^{3}H]-AEA to intact C6 glioma cells, with IC_{50} values in the region of 10 μM for AM404 and PIA and 30 μM for PCA (Jonsson et al. 2001). At these concentrations, AM404, VDM 11 and PCA dramatically reduce cell proliferation, whereas PIA is more modest in this respect.

The antiproliferative effects of VDM 11 (1 μM) and AM404 (1–3 μM) were not accompanied by a dramatic activation of caspases after a 96-h exposure, and the effects of 3 and 10 μM of these compounds were not affected by either a combination of VR and CB receptor antagonists, α-tocopherol or, in a preliminary experiment, by calpeptin. A similar VR- and CB receptor-independent mechanism of inhibition of cell proliferation was found for the related compounds arachidonoyl-serotonin and olvanil (*N*-vanillyloleamide) in these cells (Jacobsson et al. 2001). Interestingly, olvanil has also been found to produce a Ca^{2+} influx into MDCK cells (i.e. an effect similar to that seen with AM404; Chen et al. 2001), in a manner not blocked by either capsazepine or AM251 (Jan et al. 2002). In contrast, AEA produces apoptosis (Maccarrone et al. 2000) and a reduction of cell proliferation in C6 cells, which can be blocked by all these treatments (Jacobsson et al. 2001). Apoptosis is also found following treatment of C6 glioma cells with the calcium ionophore A23187 (Ray et al. 1999; Wernyj et al. 1999; Morita and Wong 2000). Taken together, these data would suggest that the antiproliferative effects of AM404, VDM 11 and 30 μM PIA are rather non-specific in nature and not necessarily related to uncontrolled Ca^{2+} influx.

The main aim of the present study has been to determine whether compounds affecting the accumulation of AEA have deleterious effects upon cell proliferation (and hence have potential toxicity problems if such compounds are developed therapeutically) at the concentrations affecting AEA uptake. Our conclusion extends to VDM 11 the conclusion of Chen et al. (2001) who wrote: "Because our data indicate that 2–5 μM AM404 is able to increase $[Ca^{2+}]_i$ in many cell types derived from different important tissues, the concentration of AM404 should be kept below this range when it is used as a selective inhibitor of anandamide transporter". An important question is therefore whether such low concentrations of AM404 and VDM 11 are effective with respect to prevention of AEA accumulation. Compounds like AEA and AM404 are highly lipophilic, and the absolute concentrations of the compounds at the biophase are highly dependent upon the assay conditions used. In particular, arachidonic acid derivatives bind to serum albumin, and thus different concentrations of serum albumin in the different assays may greatly affect the observed potency of the compounds. It is thus important to determine whether the potency of AM404 for affecting cellular AEA accumulation overlaps with unwanted effects when identical assay conditions are used for the two endpoints. The present assay is ideal in this respect since AEA exerts its antiproliferative effects in C6 cells primarily via activation of VR (Jacobsson et al. 2001). Given that AEA exerts its effects upon VR intracellularly (De Petrocellis et al. 2001; Jordt and Julius 2002), the ability of AM404, VDM 11 and PIA to prevent the cellular accumulation of AEA should modulate the observed antiproliferative effects of AEA.

The present data suggest that 1 μM AM404, 1 μM VDM 11 and 3–10 μM PIA, concentrations that have relatively modest effects upon cell proliferation, do indeed block the antiproliferative effect of AEA. In theory, this blockade may be secondary to changes in cellular function produced by the compounds, such as a down-regulation of VR, rather than a blockade of AEA accumulation, given that AM404 and VDM 11 affect C6 functionality, as assessed by cell proliferation at the concentration used. However, this explanation is less likely for PIA since at the concentrations used to block the effects of AEA it has absolutely no effect on cellular proliferation. It is thus reasonable to conclude from these data that it is possible to separate the wanted and unwanted effects of these compounds. Nevertheless, the present study highlights the need not only to determine how these compounds reduce the rate of AEA accumulation (Patricelli and Cravatt 2001; Glaser et al. 2002) but also to develop compounds with a wider therapeutic separation between effects on AEA accumulation and cell proliferation.

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