

Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity

Luciano De Petrocellis^{a,1}, Tiziana Bisogno^{b,1}, John B. Davis^c, Roger G. Pertwee^d,
Vincenzo Di Marzo^{b,*}

^aEndocannabinoid Research Group, Istituto di Cibernetica, C.N.R., Via Toiano 6, 80072, Arco Felice, Napoli, Italy

^bEndocannabinoid Research Group, Istituto per la Chimica di Molecole di Interesse Biologico, C.N.R., Via Toiano 6, 80072, Arco Felice, Napoli, Italy

^cNeuroscience Research, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW, UK

^dDepartment of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK

Received 2 August 2000; revised 12 September 2000; accepted 12 September 2000

Edited by Shozo Yamamoto

Abstract Some synthetic agonists of the VR1 vanilloid (capsaicin) receptor also inhibit the facilitated transport into cells of the endogenous cannabinoid anandamide (arachidonylethanolamide, AEA). Here we tested several AEA derivatives containing various derivatized phenyl groups or different alkyl chains as either inhibitors of the AEA membrane transporter (AMT) in intact cells or functional agonists of the VR1 vanilloid receptor in HEK cells transfected with the human VR1. We found that four known AMT inhibitors, AM404, arvanil, olvanil and linvanil, activate VR1 receptors at concentrations 400–1000-fold lower than those necessary to inhibit the AMT. However, we also found three novel AEA derivatives, named VDM11, VDM12 and VDM13, which inhibit the AMT as potently as AM404 but exhibit little or no agonist activity at hVR1. These compounds are weak inhibitors of AEA enzymatic hydrolysis and poor CB₁/CB₂ receptor ligands. We show for the first time that, despite the overlap between the chemical moieties of AMT inhibitors and VR1 agonists, selective inhibitors of AEA uptake that do not activate VR1 (e.g. VDM11) can be developed. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Capsaicin; Vanilloid; Anandamide; Cannabinoid; Calcium; Transporter

1. Introduction

Two receptors for the psychoactive ingredient of marijuana, Δ^9 -tetrahydrocannabinol (THC), the CB₁ and CB₂ cannabinoid receptors, have been found (see [1] for review). Arachidonylethanolamide (AEA), better known as anandamide,

acts as an endogenous CB₁ receptor agonist [2]. Termination of the activity of AEA by cells is achieved through a two-step process including: (i) re-uptake by intact cells via the ‘anandamide membrane transporter’ (AMT) and (ii) hydrolysis of the amide bond, catalyzed by the enzyme ‘fatty acid amide hydrolase’ (FAAH) ([3–6], and [7] for a recent review). Another receptor for a bioactive plant lipid component, capsaicin (Table 1), which is responsible for the pungent taste of ‘hot’ chili peppers [8], was recently characterized. This receptor, also known as the ‘vanilloid’ receptor, is a ligand-gated unselective cation channel, and was named VR1 [9].

The chemical similarity between AEA and the potent ligand of vanilloid receptors, olvanil [10] (Table 1), suggested that certain vanilloids might interact with cannabinoid receptors, the AMT, or FAAH, and that, conversely, AEA might activate VR1 receptors. Both these hypotheses found strong support in recent experimental reports. It was found that olvanil has an affinity for CB₁ ($K_i = 1.6 \mu\text{M}$) only slightly lower than that observed for VR1 receptors ($K_i = 0.4 \mu\text{M}$) [11,12]. Olvanil also inhibits the AMT in rat basophilic leukemia (RBL-2H3) cells ($\text{IC}_{50} = 9 \mu\text{M}$) without influencing FAAH activity [12]. Capsaicin was inactive or very weakly active in these assays. A chemical ‘hybrid’ between AEA and capsaicin, named arvanil (Table 1), was found to be a more potent ligand of CB₁ receptors ($K_i = 0.25\text{--}2.6 \mu\text{M}$) and inhibitor of the AMT ($\text{IC}_{50} = 3.6 \mu\text{M}$) than olvanil [13]. This compound and olvanil are partial agonists at CB₁ receptors [12,14]. Another capsaicin homologue and AMT inhibitor ($\text{IC}_{50} = 8 \mu\text{M}$), linvanil (Table 1), enhances AEA actions in vitro [15] and exhibits low affinity for CB₁ receptors ($K_i = 3.4 \mu\text{M}$) [13]. Arvanil and linvanil appear to be agonists at VR1 receptors, but no full assessment of their functional activity has been performed to date. Interestingly, not only do some VR1 ligands inhibit the AMT but also the AMT inhibitor *N*-(4-hydroxyphenyl)-arachidonylethanolamide (better known as AM404 [16], (Table 1)) is a full agonist at VR1 with affinity and efficacy at least 10-fold higher than those for the AMT [17–19]. Parallel to these findings, AEA was shown to act as a full agonist at both rat and human VR1 receptors, either native, in perivascular and dorsal root sensory neurons, or over-expressed in human embryonic kidney (HEK) cells and *Xenopus* oocytes [20,21]. The possibility that AEA represents the physiological endogenous ligand of VR1 receptors is being currently debated [19,23–25]. These observations (a) impose that great caution be taken in the interpretation of data obtained by using AEA or inhib-

*Corresponding author. Fax: (39)-81-8041770.
E-mail: vdimarzo@icmib.na.cnr.it

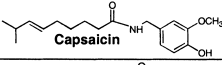
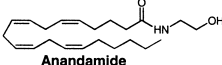
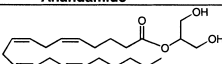
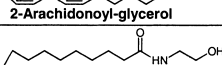
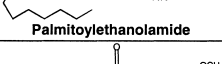
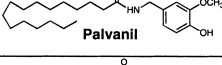
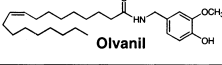
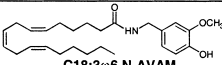
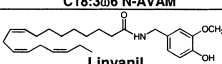
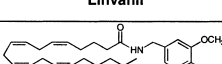
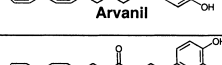
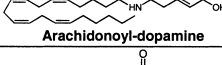
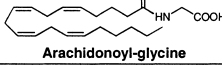
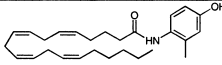
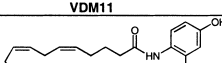
¹ These authors contributed equally to this work.

Abbreviations: AEA, arachidonylethanolamide; AMT, anandamide membrane transporter; FAAH, fatty acid amide hydrolase; HEK, human embryonic kidney; N-AVAM, *N*-acyl-vanillyl-amine; hVR1, human vanilloid receptor type 1

itors of the AMT in pharmacological studies in vivo, particularly as VR1 receptors have been recently found in brain regions, such as the basal ganglia, hippocampus and hypothalamus, that also contain CB₁ receptors and AEA [25–28]; and (b) prompt the development of more selective AMT inhibitors, a task that we have undertaken in this study. Furthermore, these data indicate a partial overlap between the structural requirements of VR1 and AMT binding sites [25], which here we have investigated further.

Table 1

Chemical structures of the fatty acid derivatives tested in this study and their effect on hVR1-mediated Ca²⁺ influx into hVR1-HEK293 cells and on the anandamide membrane transporter (AMT) in RBL-2H3 and C6 glioma cells

Substance	VR1, max. stimulation (% ionomycin)	VR1, potency, EC ₅₀ , μ M	AMT, C6 glioma IC ₅₀ , μ M	AMT, RBL-2H3 IC ₅₀ , μ M
 Capsaicin	66.8 \pm 5.4	0.026 \pm 0.011	N.D.	46.9 \pm 2.1 ^a
 Anandamide	69.0 \pm 4.3	0.63 \pm 0.25	18.2 \pm 2.4	14.5 \pm 1.3
 2-Arachidonoyl-glycerol	23.6 \pm 4.6	NM	50.1 \pm 3.3	39.8 \pm 4.3
 Palmitoylethanolamide	8.6 \pm 3.1	NM	ND	>50
 Palvanil	69.5 \pm 5.2	0.005 \pm 0.003	ND	>50 ^a
 Olvanil	67.2 \pm 9.1	0.0005 \pm 0.0003	19.9 \pm 3.7	9.0 \pm 2.0 ^a
 C18:3 ω 6 N-AVAM	72.8 \pm 4.9	0.0007 \pm 0.0004	ND	5.0 \pm 1.0 ^a
 Linvanil	60.7 \pm 12.8	0.003 \pm 0.002	12.9 \pm 1.4	8.0 \pm 2.0 ^a
 Arvanil	75.4 \pm 4.7	0.0005 \pm 0.0002	11.2 \pm 2.1	3.6 \pm 0.7 ^a
 Arachidonoyl-dopamine	74.8 \pm 4.6	0.026 \pm 0.012	17.5 \pm 1.7 ^b	21.5 \pm 9.1 ^b
 Arachidonoyl-glycine	<5.0	NM	>50	>50
 VDM11	17.0 \pm 7.0	NM	10.2 \pm 1.3	11.2 \pm 1.0
 VDM12	68.4 \pm 0.6	39.8 \pm 14.7	25.1 \pm 3.7	13.2 \pm 2.0
 VDM13	<5.0	NM	12.0 \pm 2.0	12.0 \pm 1.0
 AM404	64.3 \pm 8.0	0.026 \pm 0.013	10.2 \pm 0.7	8.1 \pm 2.6
HU-210	30.1 \pm 7.7	1.17 \pm 1.08	ND	ND

In the former case, both potency (EC₅₀) and efficacy (as % of the response obtained with 4 μ M ionomycin) are reported. For the AMT, data for potency of inhibition (IC₅₀) of [¹⁴C]anandamide uptake are shown. Data are means \pm S.D. of *n* = 3 separate experiments. NM, efficacy was too little to measure EC₅₀; ND, not determined. N-AVAM, *N*-acyl-vanillyl-amine.

^aData are from [13].

^bData are from [33].

2. Materials and methods

2.1. Transfected cells

Over-expression of hVR1 cDNA into human embryonic kidney (HEK) 293 cells was carried out as described previously [22]. Cells were grown as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% fetal calf serum and 0.2 mM glutamine, and maintained under 95%/5% O₂/CO₂ at 37°C.

2.2. Compounds

Capsaicin, AM404, AEA, palmitoylethanolamide and 2-arachidonoyl-glycerol were purchased from Cayman Chemicals (Ann Arbor,

MI, USA). HU-210 was a gift from Dr. R. Mechoulam, Hebrew University of Jerusalem, Israel. *N*-acyl-vanillyl-amines (N-AVAMs) were synthesized as described in [12,13]. Arachidonoyl-glycine was a gift from Dr. S. Burnstein, University of Massachusetts, USA. Arachidonoyl-dopamine, VDM11, VDM12 and VDM13 (Table 1) were synthesized from the corresponding amines and arachidonoyl-chloride (all from Sigma) in di-methyl-formamide, in the presence of 1.1 equivalents of triethylamine for 18 h at 4°C. The reaction was stopped by adding water and by extracting the products with diethyl ether. The compounds were purified by direct phase high pressure liquid chromatography, and chemical structures confirmed by means of proton nuclear magnetic resonance and infrared spectroscopy.

2.3. Ca^{2+} influx assays

The effect of the substances on the influx of Ca^{2+} into cells was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+} . Four days prior to experiments cells were transferred into six-well dishes coated with poly-L-lysine (Sigma) and grown in the culture medium mentioned above. The day of the experiment the cells (50–60 000 per well) were loaded for 2 h at 25°C with 4 μ M Fluo-3 methylester (Molecular Probes) in DMSO containing 0.04% Pluronic. After the loading, cells were washed with Tyrode pH = 7.4, and trypsinized to be suspended in the cuvette of the fluorescence detector (Perkin-Elmer LS50B) under continuous mixing. Experiments were carried out by measuring cell fluorescence at 25°C (λ_{EX} = 488 nm, λ_{EM} = 540 nm) before and after the addition of the test compounds at various concentrations. Capsazepine (1–5 μ M) or EGTA (4 mM) were added 30 or 10 min, respectively, before the test compounds. Data are expressed as the concentration exerting a half-maximal effect (EC_{50}) calculated by using GraphPad software. The efficacy of the effect was determined by comparing it to the analogous effect observed with 4 μ M ionomycin.

2.4. FAAH activity assays

N18TG2 cells were cultured as described previously ([12,13] and references cited therein). The effect of compounds on the enzymatic hydrolysis of [14 C]AEA (6 μ M) was studied by using membranes prepared from N18TG2 cells incubated with increasing concentrations of compounds in 50 mM Tris-HCl, pH 9, for 30 min at 37°C [12,13]. [14 C]Ethanolamine produced from [14 C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ 2:1 (by vol).

2.5. AEA transporter assays

The effect of compounds on the uptake of AEA by RBL-2H3 cells was studied by a modification of the method described previously [6,12,13], and analogous to the protocol described in [5,16] except for the use of a higher concentration (4 μ M) of [14 C]AEA. Cells were incubated with [14 C]AEA for 5 min at 37°C, in the presence or absence of varying concentrations of the inhibitors. Residual [14 C]AEA in the incubation media after extraction with $CHCl_3/CH_3OH$ 2:1 (by vol), determined by scintillation counting, was used as a measure of the AEA that was taken up by cells. We applied the same protocol also to C6 rat glioma cells, which also contain a membrane transporter for AEA [3]. Data are expressed as the concentration exerting 50% inhibition of AEA uptake (IC_{50}) calculated with GraphPad.

2.6. CB_1 and CB_2 receptor binding assays

Displacement assays for CB_1 receptors were carried out by using [3 H]SR141716A (0.4 nM, 55 Ci/mmol, Amersham) as the high affinity ligand, and the filtration technique previously described [12–14], on membrane preparations (0.4 mg/tube) from frozen male CD rat brains (Charles River, Italia), and in the presence of 100 μ M PMSF. Specific binding was calculated with 1 μ M SR141716A (a gift from Sanofi Recherche, France) and was 84.0%. The spleen from CD rats were used to prepare membranes (0.4 mg/tube) to carry out CB_2 binding assays by using [3 H]WIN55,212-2 (0.8 nM, 50.8 Ci/mmol, NEN-DuPont) as described previously [14], and again in the presence of 100 μ M PMSF. Specific binding was calculated with 1 μ M HU-348 (a gift from Prof. R. Mechoulam and Pharmos) and was 75.0%. In all cases, K_i values were calculated by applying the Cheng-Prusoff equation to the IC_{50} values (obtained by GraphPad) for the displacement of the bound radioligand by increasing concentrations of the test compounds.

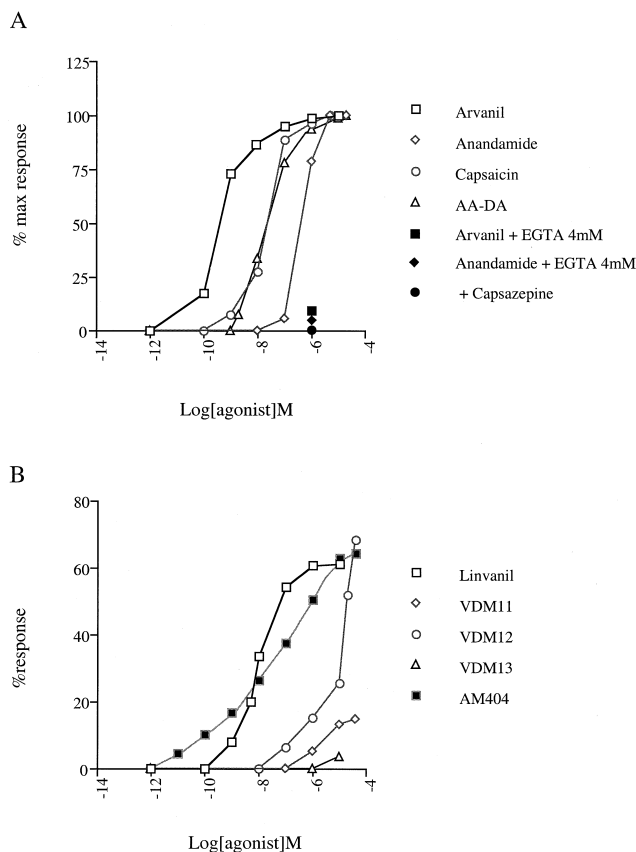


Fig. 1. (A) Dose-response curves for the effects of capsaicin, anandamide, arvanil and arachidonoyl-dopamine (AA-DA) on hVR1-mediated Ca^{2+} influx into hVR1-HEK293 cells. In order to show the difference in potency among various agonists the effect is expressed as % of maximal response obtained for each agonist. The maximal responses for each agonist (as % of the effects observed with 4 μ M ionomycin) are reported in Table 1. Data are means of $n=3$ different experiments. Error bars are not shown for the sake of clarity and were never higher than 10% of the means. The effect of EGTA (4 mM) on the response obtained with anandamide and arvanil is shown. Capsazepine (5 μ M) reduced the effect of all agonists (including those shown in panel B) to 0%. (B) Dose-response curves for the effects of linvanil, AM404, VDM11, VDM12 and VDM13 on hVR1-mediated Ca^{2+} influx into hVR1-HEK293 cells. In order to show the difference in both potency and efficacy among various agonists, the effect is expressed as % of the response obtained with 4 μ M ionomycin. Data are means of $n=3$ different experiments. S.D. bars are not shown for the sake of clarity and were never higher than 10% of the means.

3. Results and discussion

In the present study we have carried out the full characterization of the functional activity at the human VR1 (hVR1) receptor of several AEA derivatives, including the previously reported N-AVAMs [13] and AM404 [16], as well as the five new synthetic compounds: arachidonoyl-dopamine, arachidonoyl-glycine, VDM11, VDM12 and VDM13 (Table 1). AEA, N-AVAMs, AM404 and arachidonoyl-dopamine behaved as both potent and full agonists at the hVR1, as assessed by measuring their stimulation of Ca^{2+} influx into hVR1-HEK cells (Table 1, Fig. 1). VDM12 was also a full agonist in this assay, although it was almost two orders of magnitude less potent than AEA. Arachidonoyl-glycine, VDM11 and VDM13 were almost totally devoid of VR1 agonist activity.

2-Arachidonoyl-glycerol, palmitoylethanolamide or the synthetic cannabinoid HU-210 only induced a small stimulation of Ca^{2+} influx into hVR1-HEK cells, in agreement with previous studies [20,21]. The effects of all the most potent compounds were abolished by 1–5 μM capsazepine, an antagonist of vanilloid receptors [29], or by 4 mM EGTA (Fig. 1 and data not shown), which chelates extracellular Ca^{2+} thus preventing VR1-mediated Ca^{2+} influx into the cells [30]. Only a small effect on Ca^{2+} influx could be observed with the most potent compounds in non-transfected HEK cells at the highest concentrations used (10^{-4} – 10^{-5} M, data not shown). In this case the effect was not affected by EGTA. These data indicate that all the compounds found here to activate Ca^{2+} influx do so by acting through the hVR1 receptor.

Arvanil and its 18:3 ω 6 homologue are among the most potent activators of hVR1 reported so far, with EC_{50} values of 0.5–0.7 nM, comparable to and lower than those of olvanil and capsaicin, respectively (Table 1). In the N-AVAM series, the rank of potency was as follows: arvanil = olvanil \geq 18:3 ω 6 > linvanil \geq palvanil > capsaicin. Therefore, unlike native rodent vanilloid receptors [31,32], the hVR1 seems to prefer as agonists long chain, unsaturated N-AVAMs to capsaicin. Furthermore, olvanil is respectively as potent as or more potent than capsaicin at activating rat or human VR1 over-expressed in HEK cells, respectively [18]. These data, if supported by further experiments, may suggest different substrate specificities for hVR1, rat VR1 and the possibly heterogeneous population of rat native vanilloid receptors [8,10].

The rank of potency observed here for N-AVAMs on hVR1 is similar to that previously reported for the inhibition of the AMT by the same compounds: arvanil \geq 18:3 ω 6 \geq olvanil \geq linvanil \gg palvanil = capsaicin [13]. Although in the case of the AMT palvanil and capsaicin were almost inactive, these data support the concept of a partial overlap between the ligand recognition properties of the AMT and hVR1 [25]. In support of the importance of the arachidonate moiety for recognition by both hVR1 and the AMT, we found that two AEA derivatives (and AMT inhibitors), arachidonoyl-dopamine [33] and AM404 [16], also potently activate hVR1 (Fig. 1, Table 1). Both compounds were at least 10-fold less potent than arvanil, on Ca^{2+} influx, in agreement with previous reports [34] that the addition or deletion of one methylene group between the amide and the phenol groups in capsaicin causes a 10-fold loss of potency at rat native vanilloid receptors [34]. More importantly, these data confirm that AM404 is much more potent as a VR1 agonist [17–19] than as an inhibitor of the AMT, and emphasize the necessity of more selective AMT inhibitors, particularly for in vivo studies on the mechanisms controlling the duration of AEA action. Interestingly, the dose–response curve for AM404, unlike that of capsaicin or AEA, was not a sigmoid (Fig. 1). This may suggest that this compound does not interact with hVR1 in the same way as the other vanilloid agonists.

Although a necessary prerequisite, a polyunsaturated fatty acid chain is not sufficient alone for potent activation of hVR1. In fact, we found that the other endogenous ligand of cannabinoid receptor, 2-arachidonoyl-glycerol, and another AEA analogue, arachidonoyl-glycine, are almost inactive on hVR1 receptors (Table 1). Interestingly, both compounds were also very weak or inactive as inhibitors of [^{14}C]AEA uptake, thus underlying the similarity between the AMT and hVR1 ligand recognition properties. However, by intro-

ducing either a methyl or a hydroxyl group in the *ortho* position of AM404 we synthesized two compounds, VDM11 and VDM12, that although still very active as inhibitors of the AMT (Table 1), were either entirely devoid of agonist activity at hVR1 or much less potent (Fig. 1b). VDM11, the *o*-methyl derivative of AM404, up to 40 μM , only produced a hardly noticeable effect on Ca^{2+} influx into hVR1-transfected cells and yet it exhibited an IC_{50} for AMT inhibition of about 10–11 μM . VDM12, the *o*-hydroxyl derivative of AM404, induced an efficacious agonist response in hVR1-transfected cells only at 40 μM , whereas at the concentrations exerting a half-maximal inhibition of [^{14}C]AEA transport into cells (Table 1), produced a very weak agonist effect on hVR1. VDM13 (arachidonoyl-5-methoxytryptamine) was completely inactive at VR1 even at very high doses, while it was equipotent to VDM11 and VDM12 on the AMT. Under the same experimental conditions AM404 was equipotent to VDM11, VDM12 and VDM13 as an AMT inhibitor (Table 1). Finally, none of the three new compounds antagonized capsaicin action at hVR1 receptors (at 1 μM and with a 30 min pre-incubation, data not shown). Therefore, VDM11, VDM12 and VDM13 are much more selective on AEA facilitated transport vs. vanilloid receptors than the most widely used AMT inhibitor, AM404. We also wanted to assess the affinity of these new compounds for FAAH and the CB_1/CB_2 cannabinoid receptors. VDM11, VDM12 and VDM13 weakly inhibited [^{14}C]AEA hydrolysis by N18TG2 cell membranes (IC_{50} > 50 or = 25 ± 1.6 and 27 ± 0.9 μM , respectively). Under the same conditions AM404 was slightly more potent, with an IC_{50} of 22 ± 0.7 μM . Finally, when tested in cannabinoid receptor binding assays carried out with rat brain or spleen membranes, VDM11, VDM12 and VDM13 produced a ligand displacement lower than 50% up to 5–10 μM concentrations (K_i > 5–10 μM in all cases). Therefore, these compounds, apart from being inactive on CB_2 receptors, are weaker CB_1 ligands than AM404, arvanil or linvanil, whose K_i values for CB_1 receptors are 1.76, 0.5–2.6 and 3.4 μM , respectively [13,14,35]. In summary, by being more selective than previously described AMT inhibitors in all the assays performed here, these novel compounds, and VDM11 in particular, can be proposed as useful pharmacological tools for the study of the role of the AMT in the physiological termination of AEA action. In agreement with the high selectivity of these compounds, we found that, unlike AM404 [17], VDM11 and VDM12 do not relax the rat mesenteric artery (a process mediated by VR1 and cannabinoid receptors [17,36]), nor do they inhibit human breast cancer cell proliferation (a CB_1 -mediated effect [37]) if not at very high doses (V. Di Marzo, D. Melck, Z. J  rai, T. Bisogno and G. Kunos, unpublished observations).

In conclusion, we provided evidence for partially overlapping ligand recognition properties of hVR1 and the AMT. Based on these studies, we have identified possible chemical modifications that may allow AEA analogues to distinguish between hVR1 and the AMT, and have developed novel and selective inhibitors of the latter protein that promise to be fundamental tools for the study of AEA inactivation in vivo.

Acknowledgements: The authors are grateful to P. Hayes and W. Cairns for the cloning and expression of human VR1, Dr. S. Burstein, University of Massachusetts, and Biomol (Plymouth Meeting, PA) for the gift of arachidonoyl-glycine, Mr. A. Schiano Moriello and Ms.

Ines Brandi for assistance. This work was partly funded by the MURST (3933 to V.D.M.).

References

- [1] Pertwee, R.G. (1997) *Pharmacol. Ther.* 74, 129–180.
- [2] Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R. (1992) *Science* 258, 1946–1949.
- [3] Deutsch, D.G. and Chin, S.A. (1993) *Biochem. Pharmacol.* 46, 791–796.
- [4] Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C. and Piomelli, D. (1994) *Nature* 372, 686–691.
- [5] Hillard, C.J., Edgemond, W.S., Jarrahian, A. and Campbell, W.B. (1997) *J. Neurochem.* 69, 631–638.
- [6] Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L. and Di Marzo, V. (1997) *J. Biol. Chem.* 272, 3315–3323.
- [7] Ueda, N., Puffenbarger, R.A., Yamamoto, S. and Deutsch, D. (2000) *Chem. Phys. Lipids*, in press.
- [8] Holzer, P. (1991) *Pharmacol. Rev.* 43, 143–201.
- [9] Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. and Julius, D. (1997) *Nature* 389, 816–824.
- [10] Liu, L., Lo, Y., Chen, I. and Simon, S.A. (1997) *J. Neurosci.* 17, 4101–4111.
- [11] Szallasi, A., Blumberg, P.M., Annicelli, L.L., Krause, J.E. and Cortright, D.N. (1999) *Mol. Pharmacol.* 56, 581–587.
- [12] Di Marzo, V., Bisogno, T., Melck, D., Ross, R., Brockie, H., Stevenson, L., Pertwee, R.G. and De Petrocellis, L. (1998) *FEBS Lett.* 436, 449–454.
- [13] Melck, D., Bisogno, T., De Petrocellis, L., Chuang, H., Julius, D., Bifulco, M. and Di Marzo, V. (1999) *Biochem. Biophys. Res. Commun.* 262, 275–284.
- [14] Di Marzo, V., Breivogel, C., Bisogno, T., Melck, D., Patrick, G., Tao, Q., Szallasi, A., Razdan, R.K. and Martin, B.R. (2000) *Eur. J. Pharmacol.*, in press.
- [15] Maccarrone, M., Bari, M., Lorenzon, T., Bisogno, T., Di Marzo, V. and Finazzi-Agro, A. (2000) *J. Biol. Chem.* 275, 13484–13492.
- [16] Beltramo, M., Stella, N., Calignano, A., Lin, S.Y., Makriyannis, A. and Piomelli, D. (1997) *Science* 277, 1094–1097.
- [17] Zygmunt, P.M., Chuang, H., Movahed, P., Julius, D. and Hogestatt, E.D. (2000) *Eur. J. Pharmacol.* 396, 39–42.
- [18] Jerman, J.C., Brough, S.J., Davis, J.B., Middlemiss, D.N. and Smart, D. (2000) *Br. J. Pharmacol. (Proc. Suppl.)* 129, 73P.
- [19] Smart, D. and Jerman, J.C. (2000) *Trends Pharmacol. Sci.* 21, 134.
- [20] Zygmunt, P.M., Petersson, J., Andersson, D.A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D. and Hogestatt, E.D. (1999) *Nature* 400, 452–457.
- [21] Smart, D., Gunthorpe, M.J., Jerman, J.C., Nasir, S., Gray, J., Muir, A.I., Chambers, J.K., Randall, A.D. and Davis, J.B. (2000) *Br. J. Pharmacol.* 129, 227–230.
- [22] Hayes, P., Meadows, H.J., Gunthorpe, M.J., Harries, M.H. et al. (2000) *Pain*, in press.
- [23] Zygmunt, P.M., Julius, D., Di Marzo, V. and Hogestatt, E.D. (2000) *Trends Pharmacol. Sci.* 21, 43–44.
- [24] Szolcsanyi, J. (2000) *Trends Pharmacol. Sci.* 21, 203–204.
- [25] Szallasi, A. and Di Marzo, V. (2000) *Trends Neurosci.* 23, 491–497.
- [26] Herkenham, M., Lynn, A.B., Little, M.D., Johnson, M.R., Melvin, L.S., de Costa, B.R. and Rice, K.C. (1990) *Proc. Natl. Acad. Sci. USA* 87, 1932–1936.
- [27] Sasamura, T., Sasaki, M., Tohda, C. and Kuraishi, Y. (1998) *Neuroreport* 9, 2045–2048.
- [28] Mezey, E., Toth, Z.E., Cortright, D.N., Arzubi, M.K., Krause, J.E., Elde, R., Guo, A., Blumberg, P.M. and Szallasi, A. (2000) *Proc. Natl. Acad. Sci. USA* 97, 3655–3660.
- [29] Bevan, S., Hothi, S., Hughes, G., James, I.F., Rang, H.P., Shah, K., Walpole, C.S.J. and Yeats, J.C. (1992) *Br. J. Pharmacol.* 107, 544–552.
- [30] Dray, A., Forbes, C.A. and Burgess, G.M. (1990) *Neurosci. Lett.* 110, 52–59.
- [31] Janusz, J.M., Buckwalter, B.L., Young, P.A. and LaHann, T.R. et al. (1993) *J. Med. Chem.* 36, 2595–2604.
- [32] Walpole, S.J., Wrigglesworth, R., Bevan, S., Campbell, E.A., Dray, A., James, I.F., Masdin, K.J., Perkins, M.N. and Winter, J. (1993) *J. Med. Chem.* 36, 2381–2389.
- [33] Bisogno, T., Melck, D., Bobrov, Y., De Petrocellis, L., Gretskaya, N.M., Bezuglov, V.V. and Di Marzo, V. (2000) *Biochem. J.*, in press.
- [34] Walpole, S.J., Wrigglesworth, R., Bevan, S., Campbell, E.A., Dray, A., James, I.F., Masdin, K.J., Perkins, M.N. and Winter, J. (1993) *J. Med. Chem.* 36, 2373–2380.
- [35] Khanolkar, A.D. and Makriyannis, A. (1999) *Life Sci.* 65, 607–616.
- [36] Jarai, Z., Wagner, J.A., Varga, K., Lake, K.D., Compton, D.R., Martin, B.R., Zimmer, A.M., Bonner, T.I., Buckley, N.E., Mezey, E., Razdan, R.K., Zimmer, A. and Kunos, G. (1999) *Proc. Natl. Acad. Sci. USA* 96, 14136–14141.
- [37] De Petrocellis, L., Melck, D., Palmisano, A., Bisogno, T., Laezza, C., Bifulco, M. and Di Marzo, V. (1998) *Proc. Natl. Acad. Sci. USA* 95, 8375–8380.