

Arachidonyl ethanolamide induces apoptosis of uterine cervix cancer cells via aberrantly expressed vanilloid receptor-1

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Abstract

Objective. Δ^9 -Tetrahydrocannabinol, the active agent of *Cannabis sativa*, exhibits well-documented antitumor properties, but little is known about the possible effects mediated by endogenous cannabinoids on human tumors. In the present study, we analyzed the effect of arachidonyl ethanolamide (AEA) on cervical carcinoma (CxCa) cell lines.

Methods. To assess the sensitivity of CxCa cells to AEA, we selected three cell lines that were exposed to increasing doses of AEA with or without antagonists to receptors to AEA. DNA fragmentation and caspase-7 activity were used as apoptosis markers. The expression of receptors to AEA were analyzed in CxCa cell lines as well as CxCa biopsies.

Results. The major finding was that AEA induced apoptosis of CxCa cell lines via aberrantly expressed vanilloid receptor-1, whereas AEA binding to the classical CB1 and CB2 cannabinoid receptors mediated a protective effect. Furthermore, unexpectedly, a strong expression of the three forms of AEA receptors was observed in ex vivo CxCa biopsies.

Conclusion. Overall, these data suggest that the specific targeting of VR1 by endogenous cannabinoids or synthetic molecules offers attractive opportunities for the development of novel potent anticancer drugs.

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Keywords: Arachidonyl ethanolamide; Cervical cancer; Vanilloid receptor; Apoptosis

Introduction

The discovery of Δ^9 -tetrahydrocannabinol (THC), the main psychotropic constituent of the plant called *Cannabis sativa*, and the subsequent identification of cannabinoid CB1 and CB2 receptors in mammals [1,2] have led to the search of endogenous molecules (i.e., endocannabinoids) that can mediate effects similar to THC. The first identified endocannabinoid was arachidonyl ethanolamide (AEA, also named *anandamide*) [3], which is a highly potent endogenous agonist of the cannabinoid CB1 and CB2 receptors [4,5]. CB1 receptors are predominantly found in the central nervous system (CNS) [1] where they mainly mediate the psychotropic effects of THC and

endocannabinoids, whereas the expression of the CB2 receptor is thought to be restricted to cells of the immune system [6].

The antitumoral properties of THC have recently been pointed out in an elegant series of experiments showing that THC and synthetic analogues inhibited the growth of C6 glioma in rat by inducing apoptosis via CB1 and CB2 receptors, sustained ceramide accumulation and extracellular signal-regulated kinase activity [7]. Furthermore, the same authors showed that the selective activation of CB2 receptor led to growth inhibition of a human glioma subcutaneously implanted in nude mice [8]. In contrast, the possible antitumor effects of AEA have only been investigated on a few selected cell lines of different histological origins [9–12]. It was suggested that AEA might inhibit tumor cell proliferation [9–11] or induce apoptosis [12] independently of CB1 and CB2 receptors, via interaction with the type 1 vanilloid receptor (VR1) [13]. VR1 is an ion channel expressed almost exclusively by sensory neurons, activated by pH, noxious heat (>48 °C)

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and plant toxins and is thought to play an important role in nociception. However, to our knowledge, the direct demonstration of VR1 expression by tumor cells is still missing.

It ensues from these recent data that there is no universal mechanism by which plant-derived and endoge-

nous cannabinoids affect cell viability and induce apoptosis. Indeed, different receptors (e.g. CB1, CB2, VR1) may be involved on the tumor cell surface, and the cellular consequences following their triggering are various, from inhibition of growth factor receptor expression (e.g., nerve growth factor receptor) [10] to direct induction of apoptosis

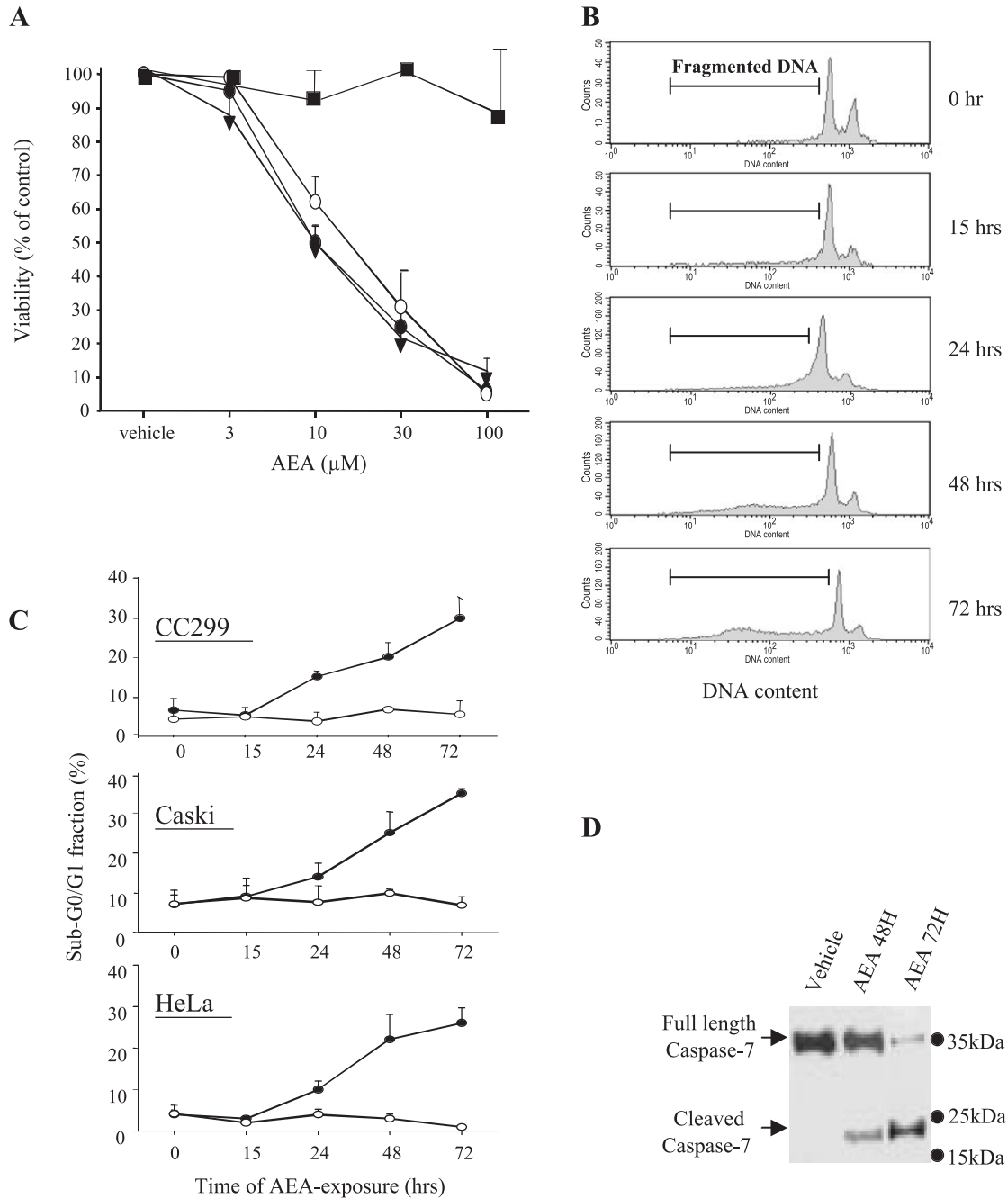


Fig. 1. AEA-induced apoptosis of CxCa cell lines. (A) The viability of healthy donor PBLs (black square), CC299 (black triangle), Caski (white circle) and HeLa cells (black circle) was assessed in a MTT assay after 5 days of AEA exposure. AEA was added daily in culture medium because of its high instability. Vehicle consisted in ethanol and never exceeded 1% of the final culture medium volume. Results are expressed as mean \pm SD of triplicates. (B) Flow cytometry analysis of DNA fragmentation of CC299 cell line exposed to 30 μM AEA for 0, 15, 24, 48 and 72 h. Similar DNA content patterns were obtained with Caski and HeLa cells (percentages of cells in sub-G0/G1 reported in C for each cell line). (C) Kinetics analysis of the sub-G0/G1 cell fraction (fragmented DNA) in CxCa cells treated with 30 μM AEA (black circle) or vehicle (white circle). Results are expressed as mean \pm SD of duplicates. (D) Western blot analysis of caspase-7 activation in CC299 cells. Cleaved form of caspase-7 (20 kDa) was observed 48 h after addition of 30 μM AEA.

cascade or inhibition of the ras pathway [14–16]. To assess whether some common characteristics might be found in tumor cells derived from a given cell lineage—and thus facilitate the development of novel anticancer therapies—we focused on cervical cancer. Despite some decrease in its incidence usually attributed to screening and the recent hope that HPV vaccine could prevent the development of malignant lesions [17], cervical cancer is the second leading cause of cancer in women and remains a therapeutic challenge for the future. We show here that cervical cancer cells are sensitive to AEA-induced apoptosis via VR1 that is aberrantly expressed in vitro and in vivo while CB1 and CB2 receptors play a protective role. This data opens new research roads for selective targeting of cervical cancer cells.

Materials and methods

Materials

Arachidonyl ethanolamide (AEA) and the VR1 selective antagonist capsaizepine (CZ) [18] were purchased from Sigma (St Louis, MO). Selective antagonists to CB1 and CB2, namely SR141716A [19] and SR144528 [20], respectively, were kindly provided by Sanofi-Synthelabo (Montpellier, France).

Cells and cervical biopsies

Fresh CxCa biopsies ($n = 8$) were provided by the Gynecology Department of the University Hospital of Geneva after informed consent of patients. Tumor material was obtained during colposcopy or surgery. After enzymatic digestion with 0.1% collagenase type IA (Sigma), 0.05% protease type I (Sigma) and 0.002% DNase type II (Sigma), tumor cells were cultured in Dulbecco's modified essential medium (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Life Technologies) in a 37%, 5% CO₂ humidified incubator. Cells were detached with versene (Life Technologies) once a week and used for experiments after at least 10 and up to 20 passages. Caski (squamous carcinoma of the uterine cervix, ATCC number CRL-1550) and HeLa (adenocarcinoma of the uterine cervix, ATCC number CCL-2) cell lines were obtained from American Type Culture Collection (Manassas, VA). Peripheral blood lymphocytes were obtained from healthy donors by Ficoll gradient separation.

Reverse transcription and polymerase chain reaction (PCR)

Total RNA was extracted from up to 5×10^6 cells or small tissue biopsies with the Qiagen RNA extraction MiniKit

(Qiagen, Hilden, Germany) and converted into cDNA using reverse transcriptase and oligo(dT) primer (Life Technologies). cDNA was amplified in nonsaturating conditions (30–35 cycles) with the following primers (Life Technologies): CB1 forward 5'CGCAAAGATAGCCCCAACGTGT3', CB1 reverse 5'CAGATTGCAGTTTCTCGCAGTT3'; CB2 forward 5'TTTCCCACTGATCCCCAATG3', CB2 reverse 5'AGTTGATGAGGCACAGCATG3'; VR1 forward 5'CTCACGAGGAAGGTGAGCTG3', VR1 reverse 5'TCGATGGCGATGTGCAGTGC3'. Amplification products were visualized on a 2% agarose gel. Specificity was assessed by enzymatic restriction.

Protein extraction and Western blotting

Dry pellets of detached CxCa cell lines were extensively washed in saline and suspended in protease inhibitors-containing buffer (Boehringer Mannheim, Rotkreuz, Switzerland). Cell lysates were centrifuged and the supernatant containing the cytosolic fraction was kept at -20°C for further analysis. Before running on a 10% acrylamide gel (Bio-Rad, Hercules, CA), samples were heated at 95°C for 4 min. After running, proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and stained overnight at 4°C with CB1 (rabbit polyclonal antibody, 1/2500, Biosource, Cammarillo, CA), CB2 (rabbit polyclonal antibody, 1/2500, Sanofi-Synthelabo) or VR1 antibody (rabbit polyclonal antibody, 1/500, Chemicon, Temecula, CA). After extensive washing, the nitrocellulose membrane was incubated in buffer containing 1:2000 donkey antirabbit HRP-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) at room temperature for 45 min. Immunocomplexes were visualized using an enhanced chemiluminescence detection system (ECL, Amersham, Germany).

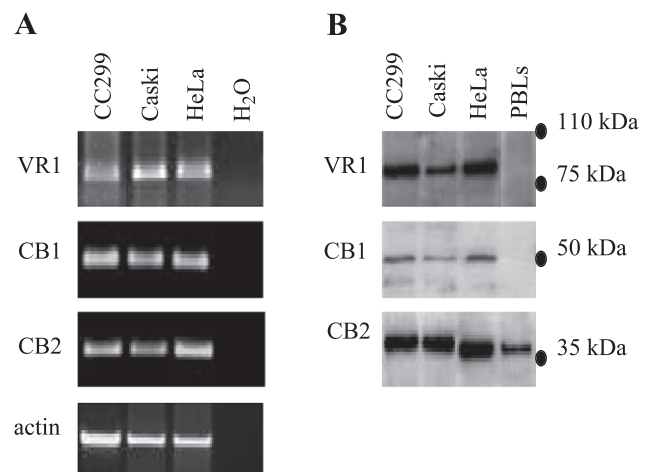


Fig. 2. CB1, CB2 and VR1 are expressed by CxCa cell lines. Expression of cannabinoid receptors was assessed by RT-PCR (A) and Western blot analysis (B). Expected protein size was 84 kDa for VR1, 52 kDa for CB1 and 38 kDa for CB2.

DNA fragmentation

After the indicated time of AEA exposure, cells were detached and fixed in 70% ice-cold ethanol overnight. After washing, cells were treated with RNase (Sigma) and labeled with 50 µg/ml propidium iodide (PI, Sigma). After 30 min at 37°C, DNA content was analyzed by flow cytometry (Facsan, Becton-Dickinson).

Caspase-7 analysis

After the indicated time of AEA exposure, cells were detached and Western blots were performed as described

above. Full-length as well as cleaved caspase-7 were detected using a rabbit anti-caspase-7 antibody (Cell Signaling Technology, Beverly, MA).

Viability test

Tumor cells were seeded in six-well plates and treated with indicated drugs or adequate vehicle. After 3–5 days, floating and adherent cells were collected and suspended in 200 µl PBS and 20 µl thiazolyl blue tetrazolium bromide at 5 mg/ml (MTT thereafter, Sigma). After 1 h at 37°C with 5% CO₂, samples were centrifuged and pellets were dissolved in isopropanol (Sigma). Optical

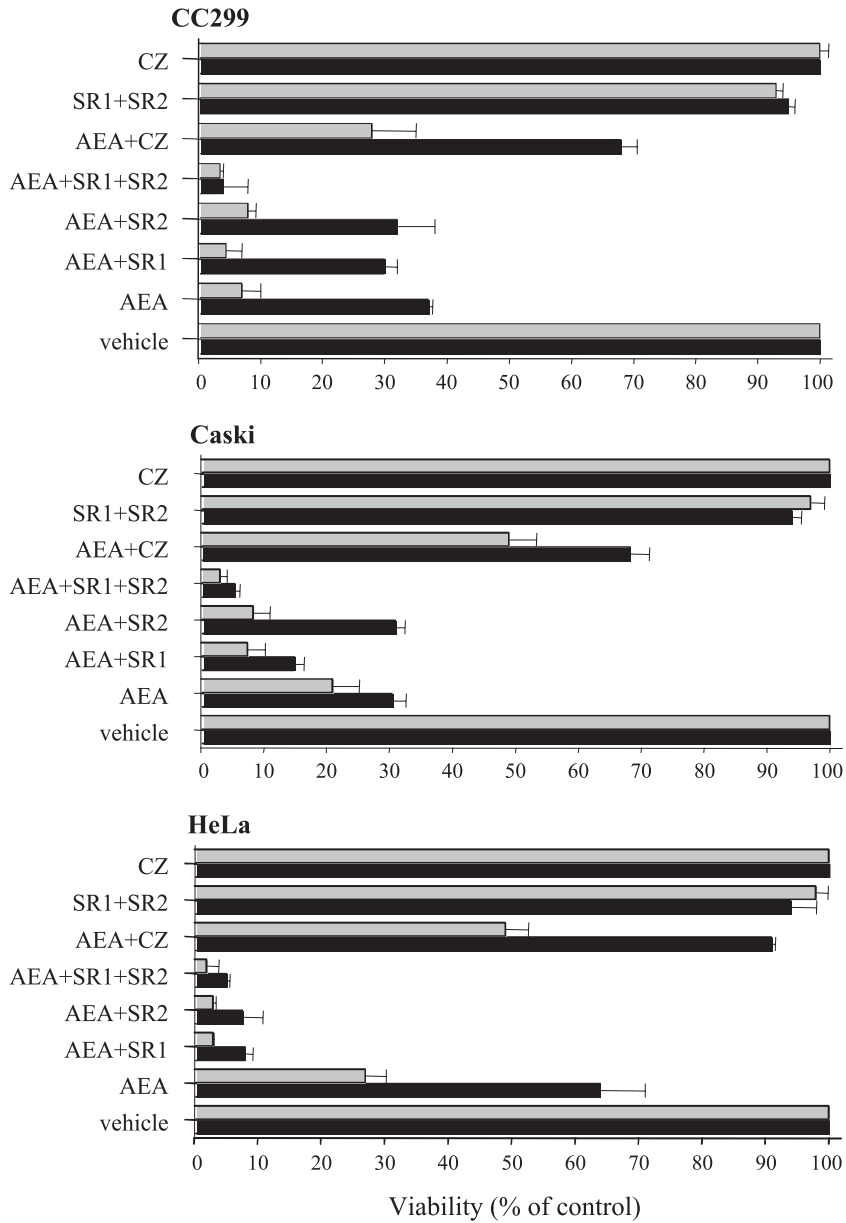


Fig. 3. VR1 is involved in AEA-induced death of cervical cancer cells, whereas CB1 and CB2 are protective. Viability of CC299, Caski and HeLa cell lines was assessed by MTT assay after 3 days (black histogram) or 5 days (gray histogram) of AEA exposure. Antagonists to CB1 (SR1, 0.2 µM), CB2 (SR2, 0.2 µM) and VR1 (capsazepine, CZ, 0.5 µM) were added 15 min before addition of 30 µM AEA. Results are expressed as mean ± SD of triplicates.

density was read at 550 nm and viability was calculated as follows:

$$\text{Viability (\%)} = \frac{[\text{O.D (AEA or vehicle - treated cells)}]}{[\text{O.D (untreated cells)}]} \times 100.$$

Statistical analysis

MTT assays were performed in triplicates at least three times each. Results of proliferation tests are presented as mean of triplicates \pm standard deviation. Viability differences were assessed with the use of unpaired Student's *t* test and were considered as significant for $P < 0.01$.

Results

The effects mediated by AEA were investigated on three different CxCa cell lines. Caski and HeLa cell lines are long-term and well-characterized cell lines, while C299 has been generated in our laboratory and was used between 10 and 20 passages. Cell lines were exposed to increasing doses of AEA for 5 days and their viability was assessed in a classical MTT assay. The three cell lines exhibited similar dose-dependent sensitivity to AEA resulting in dramatic cell death (Fig. 1A) whereas the viability of PHA-stimulated PBLs was not significantly affected. The mechanism of cell death was then studied and we detected AEA-induced DNA fragmentation of CxCa cells starting 24 h after addition of AEA (Fig. 1B), with an increasing proportion of cells in sub-G0/G1 (Fig. 1C). No DNA fragmentation was observed in cells treated with vehicle only. DNA fragmentation being one hallmark of apoptosis, we then studied whether AEA might activate effector caspase-7. As shown in Fig. 1D, 30 μM AEA induced the cleavage of caspase-7 in CC299 cell line, with similar results obtained in Caski and HeLa cell lines (not shown).

AEA is likely to exert its effects through receptors expressed on the surface of target cells. As previously suggested, there are several theoretical candidates (i.e., CB1, CB2, VR1), but their expression on CxCa cells is unknown. Using RT-PCR and Western blot analysis, we found evidence for mRNA (Fig. 2A) and protein (Fig. 2B) expression of CB1, CB2 and VR1 by the Caski, HeLa and C299 cell lines.

The respective contribution of CB1, CB2 and VR1 in AEA-induced apoptosis was then explored using selective receptor antagonists. Cell viability was analyzed after exposure of each cell line to AEA at 30 μM for 3 or 5 days. Addition of SR141716A (SR1, antagonist of CB1) and/or SR144528 (SR2, antagonist of CB2) at 0.2 μM not only did not protect cells from AEA-induced death, but rather appeared to act in synergy with AEA to decrease cell

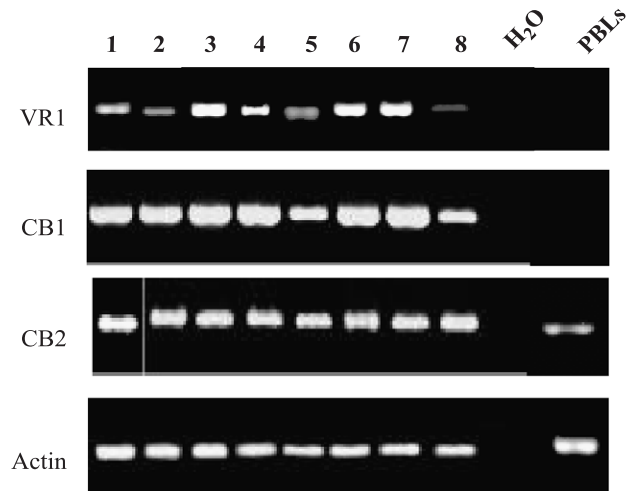


Fig. 4. Expression of receptors to AEA in cervix malignancies. RT-PCR analysis using specific primers for VR1, CB1 and CB2 was performed on stage Ib–II CxCa biopsies.

viability (AEA + SR1 and/or SR2 vs. AEA: $P < 0.01$, Fig. 3). This data suggests that CB1 and CB2 receptors are likely to play a protective role against AEA-induced death of cervical cancer cells. In contrast, the addition of the VR1 selective antagonist CZ prevented the death of each cell line exposed to AEA (AEA + CZ vs. AEA: $P < 0.01$), although the protective effect of CZ was not complete.

To assess whether the aberrant expression of CB1, CB2 and VR1 observed on cervical cell lines might reflect the in vivo conditions, we performed ex vivo RT-PCR analysis on CxCa biopsies. A strong RT-PCR signal was observed for CB1, CB2 and VR1 in all analyzed tumor samples (Fig. 4).

Discussion

In this study, we showed that CxCa cell lines, either established for a very long time (Caski and HeLa) or recently derived from a tumor biopsy (CC299), are sensitive to the pro-apoptotic effects mediated by the endogenous cannabinoid AEA. We also performed the first expression analysis of cannabinoid receptors by cervical cancer cells and tissues, and observed a strong expression of CB1, CB2 and VR1 both in vitro and in vivo. Finally, we provided evidence that VR1 is involved in the transmission of AEA-induced apoptosis, whereas CB1 and CB2 were protective.

The biological effects of cannabinoids and endocannabinoids are complex and pleiotropic. Unlike what was previously reported with THC in glioma models [1,6], the AEA-induced death of CxCa cells was not mediated by CB1 and CB2 receptors. Indeed, the addition of selective antagonists to CB1 and/or CB2, namely SR141716A and SR144528, respectively, exacerbated the toxic effects of AEA, suggesting that both CB1 and CB2 are actually able to protect CxCa cells from AEA. Although the protective role of CB1

receptor has well been documented in the CNS [21], the present finding, suggesting that both CB1 and CB2 (that can act synergistically) might play a similar role in transformed epithelial cells of the uterine cervix, was unexpected.

Despite some controversies [22], there is now compelling evidence that AEA is an endogenous ligand and full agonist at VR1 [12]. To assess the possible involvement of VR1 in the AEA-induced death of CxCa cells, we used the VR1 antagonist CZ. In contrast to CB1 and CB2 antagonists, the addition of CZ protected cells against AEA, suggesting a key contribution of VR1 in AEA-induced apoptosis of CxCa cell lines. However, the addition of CZ never led to a complete protection. One explanation could be that AEA is able to bind to other CZ-insensitive subtypes of vanilloid receptors. The interest of the functional data presented here with VR1 antagonist are reinforced by the strong VR1 expression we have observed both in vitro and in vivo. Recent functional data by others have suggested that VR1 could be expressed in some tumor cell lines including rat glioma, human neuroblastoma and lymphoma [6,7]. This was more directly assessed in the present study with RT-PCR and Western blot analysis, although the precise topography of expression remains to be established with new generation monoclonal antibodies currently in development.

In the present study, AEA was used as previously reported [9–12] at micromolar concentration to identify the receptor involved in apoptosis. In physiological conditions, AEA is detected in nanomolar concentrations, exerting paracrine effects in the central nervous system (CNS). It is therefore unlikely that AEA could contribute to the host surveillance for tumors located outside the CNS. However, high AEA concentration may theoretically be achieved for therapeutic purpose, although AEA is not the most appropriate choice considering its short half-life. Intense pharmacological research is ongoing to design analogue synthetic drugs with higher stability allowing their clinical use at lower concentration than AEA.

Overall, the present phenotypic and functional data indicate that the selective targeting of VR1 is an attractive new area of anticancer drug development, avoiding possible CB1- and CB2-mediated effects. Our recent observations in a series of human gliomas showing a prominent role of VR1 in AEA-induced apoptosis (manuscript in preparation) suggest that this novel treatment strategy is not restricted to cervical cancers but should be applied to VR1 expressing cancers from other origins.

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