European Journal of Pharmacology 718 (2013) 154–159

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Anticancer activity of anandamide in human cutaneous melanoma cells

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ARTICLE INFO

Article history:
Received 23 May 2013
Received in revised form
26 July 2013
Accepted 27 August 2013
Available online 13 September 2013

Keywords:
Anandamide
Endocannabinoid system
Melanoma
Cytotoxicity
Apoptosis

ABSTRACT

Cannabinoids are implicated in the control of cell proliferation, but little is known about the role of the endocannabinoid system in human malignant melanoma. This study was aimed at characterizing the *in vitro* antitumor activity of anandamide (AEA) in A375 melanoma cells. The mRNA expression of genes that code for proteins involved in the metabolism and in the mechanism of AEA action was assessed by RT-PCR. Cell viability was tested using WST-1 assay and the apoptotic cell death was determined by measuring caspase 3/7 activities. A375 cells express high levels of fatty acid amide hydrolase (FAAH), cyclooxygenase (COX)-2, cannabinoid receptor 1 (CB_1), transient receptor potential cation channel subfamily V member 1 (TRPV1) and G-protein-coupled receptor 55 (GPR55) genes. AEA induced a concentration-dependent cytotoxicity with an IC_{50} of 5.8 ± 0.7 μM and such an effect was associated to a caspase-dependent apoptotic pathway. AEA cytotoxicity was potentiated by FAAH inhibition (2-fold increase, $p < 0.05$) and mitigated by COX-2 or lipoxygenase (LOX) inhibition (5- and 3-fold decrease, respectively; $p < 0.01$). Blocking CB_1 receptors partially decreased AEA cytotoxicity, whereas selective antagonism on the TRPV1 barely affected the mechanism of AEA action. Finally, methyl-β-cyclodextrin, a membrane cholesterol depletory, completely reversed the cytotoxicity induced by the selective GPR55 agonist, O-1602, and AEA. Overall, these findings demonstrate that AEA induces cytotoxicity against human melanoma cells in the micromolar range of concentrations through a complex mechanism, which involves COX-2 and LOX-derived product synthesis and CB_1 activation. Lipid raft modulation, probably linked to GPR55 activation, might also have a role.

1. Introduction

The endocannabinoid system consists of CB_1 and CB_2 receptors, their endogenous ligands (endocannabinoids), among which anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most prominent ones, and proteins involved in biosynthesis, intracellular trafficking, cellular re-uptake and degradation (Di Marzo, 2009; Hermanson and Marnett, 2011; Chicca et al., 2012).

Endocannabinoids are synthesized on demand and released outside the cell where they activate CB receptors. Then, they are rapidly taken up by the cells most likely *via* a putative transmembrane transporter-mediated process (Fowler, 2012; Chicca et al., 2012) and very rapidly and fully hydrolyzed by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (Hermanson and Marnett, 2011). Endocannabinoids can also undergo oxygenation mainly by cycloxygenase (COX)-2 and lipoxygenases (LOXs) leading to the formation of prostaglandin-ethanolamides and -glyceryl esters, which retain some biological activities (Battista et al., 2012). Thus, endocannabinoid degradation is a crucial step in the termination of the cannabinoid-mediated signaling and in the synthesis of other bioactive molecules.

Endocannabinoids regulate both core and emerging hallmarks of cancer (Guindon and Hohmann, 2011) and higher endocannabinoid levels were found in cancers and pre-malignant lesions rather than the normal tissue (Flygare and Sander, 2008; Petersen et al., 2005) suggesting a role of the endocannabinoid system in the progression of the neoplastic phenotype. *In vitro* and *in vivo* studies have shown that natural and synthetic cannabinoids are efficacious in reducing cancer progression (Guindon and Hohmann, 2011), although the observed effects are complex and sometimes contradictory. For example, both receptor-dependent and independent mechanisms have been reported (Guindon and

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http://dx.doi.org/10.1016/j.ejphar.2013.08.039

Hohmann, 2011) and several interesting targets may account for the anticancer effect of cannabinoids, including transient receptor potential cation channel subfamily V member 1 (TRPV1), G-protein-coupled receptor 55 (GPR55) and peroxisome proliferator-activated receptors (PPARs) (Grimaldi and Capasso, 2011; Thomas et al., 2007; O'Sullivan, 2007).

Cutaneous melanoma is one of the most aggressive human cancers (Blázquez et al., 2006). Metastatic patients have poor prognosis and they are generally refractory to conventional chemotherapy (Wolchok and Saenger, 2007). While the occurrence of a dysregulation in the endocannabinoid system was observed in various human skin cancers (Casanova et al., 2003), there are only few data about the role of cannabinoids in human malignant melanoma. Blázquez and co-workers (2006) reported that cannabinoids inhibit the *in vivo* growth of melanomas expressing CB_1 and CB_2 receptors by decreasing proliferation, angiogenesis and metastasis formation, while increasing apoptosis. Notwithstanding the participation of several ligands (including AEA) in the anticancer action of cannabinoids in melanoma has been already demonstrated (Hamtiaux et al., 2012; Kenessey et al., 2012), the specific molecular mechanism of action involved remains unclear.

Overall, this notion prompted us to characterize the *in vitro* antitumor activity of AEA on human A375 melanoma cells to provide further insights into the molecular mechanism of AEA action in this specific type of cancer.

2. Material and methods

2.1. Chemicals

AEA (CB_1/CB_2 receptor agonist, TRPV1 agonist, putative GPR55 agonist, PPARs agonist), ACEA (selective CB_1 receptor agonist), JWH-133 (selective CB_2 receptor agonist), and AM251 (selective CB_1 receptor antagonist) were obtained from Tocris Bioscience (Northpoint, UK). O1602 (selective GPR55 agonist) and capsazepine (CPZ, selective TRPV1 antagonist) were obtained from Ascent Scientific (Cambridge, UK). Caffeic acid (selective LOX inhibitor), URB597 (selective FAAH inhibitor), methyl-β-cyclodextrin (MCD, lipid raft disruptor), and capsaicin (TRPV1 agonist), were obtained from Sigma Aldrich (Milan, Italy). Rofecoxib (selective COX-2 inhibitor) was kindly gifted by Dr. V. Calderone (Department of Pharmacy, University of Pisa). Compounds were dissolved in their specific solvents and further diluted in sterile culture medium immediately before their use. DMSO did not exceed 0.5% v/v in the culture medium.

2.2. Cell culture

The human cutaneous melanoma cancer cell line A375 was obtained from the American Type Culture Collection (ATCC, Rockville, MD), cultured in DMEM medium supplemented with L-glutamine (2 mM), 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich, Milan, Italy) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Low cell passages (between 5 and 20) were used in the present study.

2.3. RT-PCR characterization of AEA targets

Total RNA from A375 cells was extracted by using the RNeasy® Mini kit (following manufacturer's instructions), dissolved in RNase-Free water, UV-quantified by NanoDrop™ Lite (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and stored at −80 °C until use. 1 μg of RNA was reverse-transcribed by the QuantiTect® Reverse Transcription kit (Qiagen, Valencia, *CA*, USA). PCR reactions were performed by the HotStartTaq Master Mix kit (Qiagen, Valencia, *CA*, USA). Primer sequences were: 5′-GTGAAGGTCGGAGTCAACG (F) and 5′-GGTGAAGACGGCCAGTGGACTC (R) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH); 5′-CCACTCCCGCAGCCTCCG (F) and 5′-ATCAGGCAAAACGCCACCAC (R) for CB_1 receptor; 5′-GGTGACAGAGATAGCCAATG (F) and 5′-GCCAATGAACAGGTATGAGG (R) for CB_2 receptor; 5′-GACTTCAAGGCTGTCTTCATCATCC (F) and 5′-CAGGGAGAAGCTCAGGGTGCGC (R) for TRPV1; 5′-CTGGAAGCTTTGGCTTTACG (F) and 5′-GTTGTGTGACATCCCGACAG (R) for PPARα; 5′-TTCAGAAATGCCTTGCAGTG (F) and 5′-CACCTCTTTGCTCTGCTCCT (R) for PPARγ; 5′–ACAGTTTGCAGTCCACATCC (F) and 5′-ACGCTTCCGTACATGCTGAC (R) for GPR55; 5′-GAACTGCAGCACGAGATCGA (F) and 5′-CCCTCCACTGGCAATCA (R) for hFAAH; 5′-TGAAACCCACTCCAAACACA (F) and 5′-AACTGATGCGTGAAGTGCTG (R) for COX-2.

cDNA samples were incubated for 15 min at 95 °C to activate the polymerase, then amplified for the suitable number of cycles (30 cycles for GAPDH; 35 cycles for human CB_1, CB_2, TRPV1, GPR55 and FAAH and 36 cycles for PPARα/γ and COX-2) for 1 min at 95 °C, 1 min at the specific annealing temperature for each AEA target (55 °C for GAPDH, CB_2, GPR55, and COX-2; 56 °C for PPARα/γ and FAAH; 59.7 °C for CB_1; and 69 °C for TRPV1), 1 min at 72 °C, plus elongation step at 72 °C for 10 min. GAPDH was used as housekeeping gene. Primers were designed by Oligo-Primer Analysis Software 5.0, synthesized by Sigma-Aldrich (Milan, Italy) and their specificity was verified by Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The identity of the PCR products was confirmed by sequencing.

2.4. Cell viability assay

Cell viability was measured using a method based on the cleavage of the 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1) to formazan by mitochondrial dehydrogenase activity following manufacturer's instructions (cell proliferation reagent WST-1; Roche, Mannheim, Germany). Briefly, cells (5×10^3/well) were seeded in 96-well plate in 10% FBS medium; after 24 h, the complete medium was replaced with compound-containing 1% FBS medium. To this aim, each compound was dissolved in its specific solvent and then diluted in low serum medium up to obtaining the concentrations to be tested. Freshly stock solutions were prepared the day of the experiment. All compounds were tested in a concentration range of 0.1–100 μM and their effects evaluated after 48 h, in the presence or absence of the following specific inhibitors/antagonists: URB597, rofecoxib, caffeic acid, AM251, and CPZ. Each compound was added 2 h before the AEA treatment. Following drug exposure, WST-1 was added and the absorbance was measured at 450 nm using a microplate readerVictor^{2TM} (Wallac, PerkinElmer, Waltham, USA). Optical density values from vehicle-treated cells were considered as 100% of cell viability.

2.5. Caspase activity assay

Caspase 3/7 activities were assayed by the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, Milan, Italy), according to the manufacturer's instructions. Briefly, cells were plated onto 96-well plates (5×10^3/well) in the presence of AEA at 10 μM for 24 h. After cell lysis, the caspase 3/7-assay substrate (Z-DEVD) 2-Rhodamine 110 was added and the fluorescence measured at excitation and emission wavelengths of 485 and 530 nm, respectively. Values were expressed as the ratio between fluorescent signals generated in treated cells and those produced in controls. Each value was obtained from three independent experiments carried out in triplicate.