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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Involvement of reactive oxygen species in the oleoylethanolamide effects and its pyrazonilic analogue in melanoma cells

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Abstract The search for more substances that effectively fight melanoma is extremely important, because of its aggressive nature. In this sense, the molecular hybridization is a promising strategy. The aim of this work is to evaluate whether the antiproliferative effect of the endocannabinoid oleoylethanolamide can be improved with the addition of a trifluoromethylated pyrazolinic nucleus on its structure in B16F10 cell line. The pyrazolinic analog was named oleoyl pyrazoline. We also compared the effects of oleoylethanolamide and that of the classic endocannabinoid anandamide (AEA). The cell viability was evaluated by MTT assay, the intracellular reactive oxygen species generation by fluorimetry, and apoptosis/necrosis by fluorescent microscopy. Also, α-tocopherol antioxidant was used to evaluate the involvement of reactive oxygen species in the cellular response. Although the effects of AEA occur in smaller concentrations, the results show that the effects of AEA and oleoylethanolamide were similar. The results showed a decrease in cell viability, induction of apoptosis and necrosis, and increased generation of reactive oxygen species by the oleoylethanolamide, while the oleoyl pyrazoline increased cell proliferation and decreased reactive oxygen species. Additionally, the effects of oleoylethanolamide in cell viability were decreased by inhibiting the generation of reactive oxygen species by α-tocopherol. Therefore, it is possible to suggest the involvement of reactive oxygen species in the effect of oleoylethanolamide in the B16F10 cells. Considering the great need to find substances that can fight melanoma and the lack of greater elucidation in the action mechanisms of cannabinoids and their analogs, this work provides important new information that could serve as reference to other studies.

Keywords Cell viability · Apoptosis · B16F10 cell line · Oleoylpyrazoline · Oxidative stress

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Introduction

The search for more effective substances for treatments that prevent or reduce the proliferation of melanoma cells, even in advanced stage, is extremely important due to the aggressive nature of this pathology. It was estimated that 76,380 new cases of melanoma would be diagnosed in 2016 (American Cancer Society 2016). Even though melanoma accounts for only 1% of all skin cancer cases worldwide, it is responsible for the vast majority of skin cancer related deaths (American Cancer Society 2016).

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Cannabinoid substances are highlighted among the several substances studied for use as chemotherapeutic (Velasco et al. 2012). These are lipid chemical substances naturally occurring in the plant *Cannabis sativa*, being ∆(9)-tetrahydrocannabinol, considered the most representative of the group (Pertwee 2008). As well as the natural cannabinoids, the synthetic cannabinoids such as WIN-55,512-2, JWH-133, and (*R*)-methanandamide, also bind to CB_1 and CB_2 cannabinoid receptors to exert their biological effects. In addition, according to Nikan et al., CB receptors are over-expressed in some tumor cells, and cannabinoid receptor ligands affect these tumoral cells selectively (Nikan et al. 2016). The discovery of two cannabinoid substances endogenously produced, *N*-arachidonoylethanolamide (anandamide, AEA) and sn-2-arachidonoyl glycerol (2-AG), led to new scientific research that contributed to identifying another endogenous substance derived from fatty acid as the oleoylethanolamide (OEA), an *N*-acyl ethanolamide (Pertwee 2010; Mechoulam et al. 1995; Mechoulam et al. 2002). However the effects of OEA against melanoma have not yet been studied.

The possibility that certain cannabinoid substances can be used as antitumoral drugs has been investigated for a long time (White et al. 1976). Since the identification of these substances in the 1960s and 70 s, several similar substances have been synthesized and used as therapeutic drugs with various medicinal effects (Honório et al. 2006). According to Bifulco et al. (2006) and Sarfaraz et al. (2008), cannabinoid substances have a potential application as antitumor drugs that can cause a decrease in cell survival and proliferation. It has also recently been suggested that increased levels of endocannabinoids and the blocking of its degradation leads to a decreased viability of tumor cells (Hamtiaux et al. 2012). According to Cudaback et al. (2010), the increased synthesis of endogenous cannabinoid, the catabolism of several ligands inside cells and tissues, and the increased expression of different receptors appear to be important in signaling cascades by different kinases. These signaling cascades result in pro-autophagic, proapoptotic, antiproliferative or anti-apoptotic effects and such effects have been observed in some tissues and cancer cells. According to Blázquez et al. (2006), melanoma cells actively express cannabinoid receptors (CB_1 and CB_2) and the activation of these receptors inhibits the growth of melanoma cells in vivo and in vitro.

The relation between cannabinoid receptors and oxidative stress has already been reported. Bifulco et al. (2008) observed that the cannabinoid interaction with transient receptor potential vanilloid promotes the activation of more than one cell mechanism, such as mitochondrial apoptosis pathway activation, that occurs at the same time as the reactive oxygen species (ROS) generation increase, and consequently increased oxidative stress. Carracedo et al. (2006) showed that treatment on pancreatic tumor cells using a specific cannabinoid increased the activation of ATF-4 and TRB3 genes, which are related to oxidative stress (among other pathways) leading to apoptosis. Furthermore, several studies have shown that cannabinoids can realize its antiproliferative effects and induce cell death dependent of ROS. For instance, anandamide leads to an increased ROS generation in tumor cells (Massi et al. 2003; Sarker et al. 2003). Jacobsson et al. (2001) demonstrated that anandamide and 2-AG antiproliferative effects in glioma cells can be completely inhibited by the antioxidant α-tocopherol. However, according to Ambrosini et al. (2006), OEA also presents antioxidant properties resulting in beneficial effects on in vitro capacitation of human sperm. Thus the role of cannabinoids on regulation of oxidative stress remains controversial. Although the cell death induced by ROS seems to be supported by cannabinoids, a protective effect by cannabinoids against induced cell death by ROS can also be observed. The role of CB receptors on prooxidant and antioxidant effects is unclear and both receptor-dependent and receptor-independent effects were related, varying according to different cannabinoids (Zolese et al. 2005).

Previous studies have shown that trihalomethylated pyrazolines may have remarkable biological effects, and because they present a wide range of applications in the Medicinal Chemistry, they may be considered privileged structures. According to Hassan et al. (2011), pyrazole compounds may induce oxidative stress and sometimes the antitumor activity may be followed by a considerable increase in superoxide dismutase activity, decrease in catalase and glutathione peroxidase activity, in addition to decreased levels of glutathione, high production of H_2O_2, NO and free radicals, leading to death of tumor cells.

These compounds were reported to have a wide range of biological applications, by its antimicrobial, antinociceptive, analgesic, intestinal stimulatory secretion, antidepressant, anticonvulsivant, antipyretic, anti-inflammatory and hypophagic properties (Nenajdenko and Balenkova 2011; Sauzem et al. 2008). According to Kumar et al. (2013), molecular modeling and docking studies have been discussed for the development of novel anticancer agents bearing pyrazole moiety. Pyrazole analogs have been found to inhibit the active site of kinase family enzymes and various growth factors like vascular endothelial growth factor, fibroblast growth factor, tumor necrosis factor, tumor growth factor (TGF-α and TGF-β) and BRAF gene.

The development of synthetic biologically active compounds is of great interest to the biochemistry and pharmaceutical communities; many heterocyclic compounds present a great structural diversity and biological activity,

which justify the interest in the development of new compounds with therapeutic potential. Among the molecular design strategies, the molecular hybridization is a very promising strategy working from the union of two distinct structural features of bioactive molecules in a new molecule. This strategy can generate new molecules that exhibit the combination of both activities of the original molecules in a single molecule, change in selectivity, and reduction in undesirable side effects (Junior et al. 2007).

With this in mind, and coupled with the excellent results that we have obtained with regard to the cannabinoid analogs fatty amides (Santos et al. 2015) and fatty heterocycles as antiproliferative compounds (Treptow et al. 2015; Moraes et al. 2017) – including activity against melanoma–this study proposes a structural modification of the OEA by coupling it with an heterocyclic nucleus–oleoyl trifluoromethylated pyrazoline (OPZ) (Fig. 1)–in order to verify if this association is capable to improve the antiproliferative effect of endocannabinoids, AEA and OEA (Fig. 1). In addition, we verified the involvement of ROS production in the effects of the endocannabinoid OEA and its analog OPZ in melanoma cells.

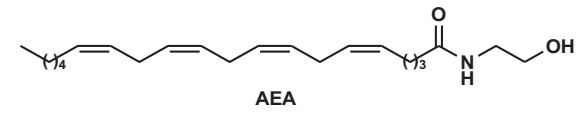
Materials and methods

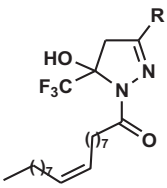
Cell culture

The murine melanoma cell line, B16F10, was obtained from the Rio de Janeiro Cell Bank. B16F10 cell line was maintained in DMEM medium, supplemented with sodium bicarbonate (0.2 g/L), L-glutamine (0.3 g/L), Hepes (3 g/L), 10% fetal bovine serum, 1% antibiotic and antimycotic, in cell culture flasks at 37 °C.

Synthesis of compounds

OEA e OPZ were obtained by the synthetic pathway shown in Fig. 2 from the same precursor, the methyl oleate, obtained from the oleic acid. OEA was synthesized according to a published method (D'Oca et al. 2010; Santos et al. 2015), from aminolysis reaction of methyl oleate (0.3 mmol) in the presence of ethanolamine (1.8 mmol) in acetonitrile for 24 h (Fig. 2). The progress of the reactions was monitored by silica gel thin-layer chromatography. The raw products were purified via column chromatography





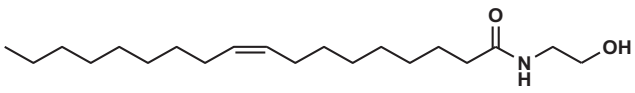


Fig. 1 Evaluated compounds: anandamide (AEA), oleoylethanolamide (OEA), oleoyl pyrazoline (OPZ)

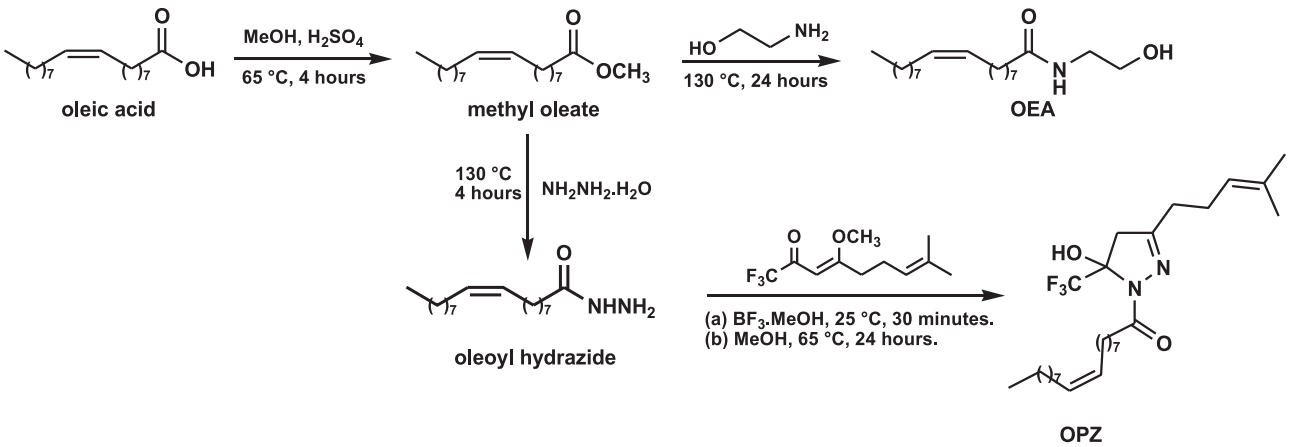


Fig. 2 Synthesis of oleoyl ethanolamide (OEA) and oleoyl pyrazoline (OPZ) from oleic acid

on silica gel (n-hexane/ethyl acetate, 7:3) and analyzed by ^{1}H NMR, and the data are in agreement with the literature. The synthesis of OPZ was performed according to Beck et al., (Beck et al. 2012). In a first step, the methyl oleate hydrazinolysis was realized in the presence of the hydrazine to obtain the oleic hydrazide, which was then subjected to reaction cyclocondensation with (*Z*)-1,1,1-trifluoro-4-methoxy-8-metilnone-3,7-dien-2-one (enone). After the reaction was complete, the precipitate that formed was filtered and dried under vacuum to obtain the new analog OPZ, (Fig. 2). The compound was analyzed by ^{1}H NMR, and GC/MS, and the data are in agreement with the literature. Anandamide (AEA) was synthesized according to a published method (D'Oca et al. 2010; Santos et al. 2015), by reacting the respective arachidonic acid (0.3 mmol) with ethanolamine (0.3 mmol), triethylamine (0.3 mmol), and a catalytic amount of (dimethylamino) pyridine, and dicyclohexylcarbodiimide (0.3 mmol) in CH_2Cl_2 was added dropwise to the above reaction mixture, which was then stirred at room temperature for 24 h. The solid dicyclohexylurea formed was removed by filtration and the solvent was evaporated under reduced pressure (Fig. 3). AEA was analyzed by ^{1}H NMR, and the data are in agreement with the literature.

Oleoylethanolamide

N-(2-hydroxyethyl) oleamide: $C_{20}H_{39}NO_2$. MW 325.53 g mol^{-1}. Yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.10 (sl, 1H), 5.34 (m, 2H), 3.72 (m, 2H), 3.41 (m, 3H), 2.23 (t, $J = 7{,}2$ Hz, 2H), 2.01 (m, 4H), 1.63 (m, 2H), 1.30 (m, 20H), 0,88 (t, $J = 6{,}9$ Hz, 3H).

Oleoyl pyrazoline

(*Z*)-1-(5-hydroxy-3-(4-methylpent-3-enyl)-5-(trifluoromethyl)-4,5-dihydropyrazol-1-yl)octadec-9-en-1-one. $C_{28}H_{47}F_3N_2O_2$. MW 500.68 g mol^{-1}. Yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) 6.36 (s, 2H, 2H–C=C), 5.12 (d, 1H, H–C=C $(CH_3)_2$, 3.44 (d, 1H, *J* 18.5 Hz, H4a), 3.12 (d, 1H, *J* 18.5 Hz, H4b), 2.67 (m, 6H, $3CH_2$–CH=C), 2.40 (m, 2H, CH_2), 2.31 (m, 2H, CH_2), 1.71 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.56 (m, 2H, $2CH_2$), 1.27 (m, 20H, $12CH_2$), 0.90 (t, 3H, CH_3). Obtained Mass: $[M^+]$: 500.0.

Anandamide

N-arachidonoylethanolamine. $C_{22}H_{37}NO_2$. MW 347.53 g·mol^{-1}. Colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.90 (s, 1H), 5.36 (m, 8H), 3.72 (s, 2H), 3.42 (m, 2H), 2.82 (m, 6H), 2.62 (s, 1H), 2.21 (m, 2H), 2.12 (q, *J* 6.8 Hz, 2H), 2.05 (q, *J* 6.9 Hz, 2H), 1.73 (m, 2H), 1.32 (m, 6H), 0.89 (t, *J* 6.9 Hz, 3H).

Cells and treatments with the compounds

The B16F10 cells were centrifuged, suspense in DMEM medium (2×10^5 cells/mL) and incubated for 24 h to adhere in cell culture plates at 37 °C. The compounds oleoyl ethanolamide (OEA) and oleoyl pyrazoline (OPZ) were diluted in ethanol and Tween 20 (Synth) and added in the cells in the maximum volume of 0.05% (0.04% ethanol and 0.01% Tween) which was not cytotoxic (Fig. 4a). The anandamide (AEA) was diluted in DMSO and added in the cells in the maximum volume of 1% which also was not cytotoxic (Fig. 4b). The control cells received the same volume of solvent of the highest concentrations of compounds. The concentrations of OEA or OPZ tested were 50, 125, 250, 375, and 500 µM until 72 h. Already the concentrations of AEA were 0,1, 1, 10, 50 and 100 µM until 72 h.

Cellular viability

The viability of B16F10 cells exposed to AEA, OEA and OPZ was measured by method of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) 0, 24, 48, and 72 h after incubation at 37 °C, according to Trindade et al. (Trindade et al. 1999). Briefly, the cells after incubation were washed with PBS and 200 µl DMEM medium β-mercaptoethanol free and 20 µl of MTT (5 mg/ml) was added to each well. The plates were incubated for 3 h at 37 °C. The medium was removed and formazan crystals were dissolved in 200 µl of dimethylsulfoxide (DMSO, Sigma) with gentle shaking. The absorbance values at 490 nm were determined on a multiwell plate reader (ELX 800 Universal Microplate Reader, Bio-TEK).

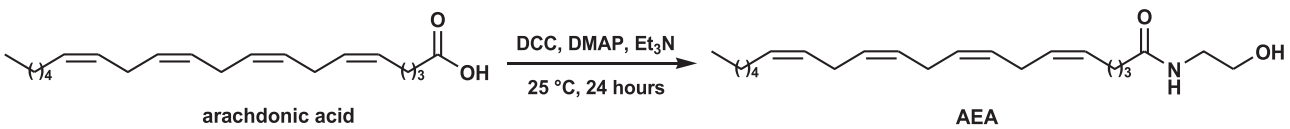


Fig. 3 Synthesis of anandamide (AEA) from arachdonic acid

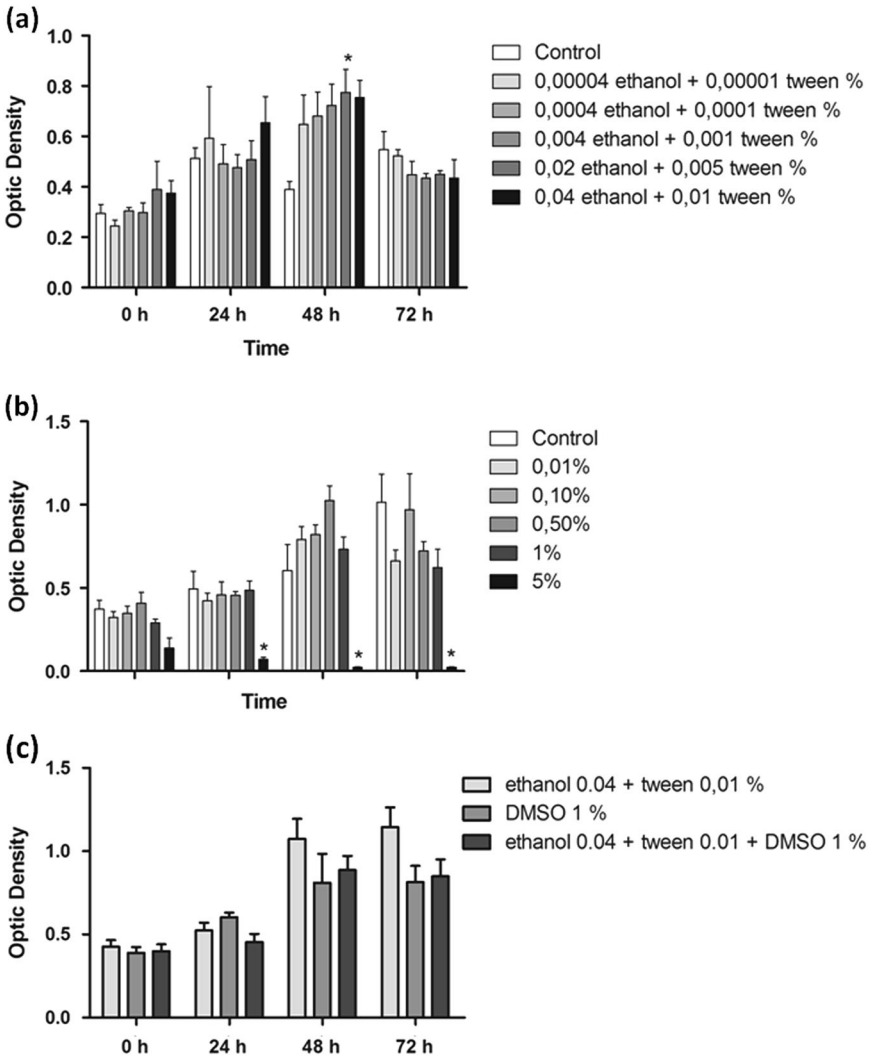


Fig. 4 Cells B16F10 treated with **a** ethanol and tween; **b** DMSO; and **c** ethanol and tween, DMSO or both solvents at the same concentration. Data are shown as mean ± standart error. *Asterisk* indicates significant difference from the respective control at each exposure time ($p < 0.05$)

Quantitative analysis of apoptosis and necrosis

The evaluation of apoptosis and necrosis was realized according to Ribble et al. modified, with the addition of 2 µL of work solution composed by PBS, with 100 µg/mL of acridine orange and 100 µg/mL of ethidium bromide (Ribble et al. 2005). The analysis was performed from areas captured from the well plate (20X) with epifluorescence microscope (Olympus IX81). All cells were analyzed and the data were expressed in percentage related to total cells number visualized on the captured area. The cells were classified according Kosmider et al. modified, as following: those evincing green or yellow-orange fluorescence cytoplasm and green nucleus were considered viable cells; those presenting orange nucleus with fragmented chromatin were considered apoptotic. Those cells with uniformly orange-stained nucleus were considered necrotic (Kosmider et al. 2004).

Assessment of intracellular ROS formation

The B16F10 cells were centrifuged, suspense in DMEM medium (2×10^5 cells/mL) and incubated for 24 h to adhere in cell culture plates with DMEM medium at 37 °C. After this, they were treated in medium with 50, 125, and 250 µM of OEA or OPZ and incubated at 37 °C during 24 h. Then the cells were washed with PBS (two times) and incubated for 30 min at 37 °C with the fluorogenic compound 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) at a final concentration of 40 µM. After the loading with H_2DCF-DA, the cells were washed with PBS two times and then suspended in fresh PBS. Aliquots of 160 µl of each sample (five replicates) were placed into an ELISA plate and the fluorescence intensity was determined during 90 min at 37 °C, using a fluorometer (Victor 2, Perkin Elmer), with an excitation and emission wavelength of 485 and 520 nm, respectively. ROS levels were expressed in terms of fluorescence area, after fitting fluorescence data to a second

order polynomial and integrating between 0 and 90 min in order to obtain its area.

α-tocopherol

To verify the involvement of ROS generation in the effects of OEA was used an antioxidant (α-tocopherol-Vitamin E) at the concentration of de 25 μg/ml dissolved with DMSO. The cells were treated with α-tocoferol, 250 μM of OEA, and α-tocoferol and 250 μM of OEA during 24 h. The control cells received the same volume of solvent used in each condition (Ethanol and Tween or DMSO, or DMSO plus Ethanol and Tween) (Figs. 4a–c, respectively). It was made assessment of intracellular ROS formation and cell viability assay by MTT. In this case, the data are presented as percentage of viable cells in relation to respective controls.

Statistical analysis

Each experiment was repeated on three independent times with, at minimum triplicate samples. The results were expressed as the means ± SEs. Analysis of variance (ANOVA) was used to determine significant differences among groups. Tukey's significant difference post hoc test was used for pair wise comparisons after analysis of variance. Statistical significance was accepted at $p < 0.05$.

Results

AEA

When exposed to AEA the B16F10 cell line showed lower percentage of viable cells from 24 h after treatment with concentrations of 50 and 100 μM when compared to the control (Fig. 5a).

OEA

When exposed to OEA the B16F10 cell line showed lower percentage of viable cells 24 h after treatment with concentrations of 250 and 375 μM compared to control. After 48 h, it was observed a lower percentage of viable cells compared to control in cells treated with a concentration of 375 μM of OEA. At 72 h, this effect was no longer observed, except for the concentration of 500 μM, which was cytotoxic from 24 h exposure (Fig. 5b).

OPZ

On OPZ exposure, there were not any significant differences immediately, 24 and 48 h after treatment with different concentrations. However 72 h after treatment it was observed a significant increase in the percentage of viable cells treated with 50, 125, and 375 μM of OPZ (Fig. 5c).

ROS

After 24 h of treatment with OEA, B16F10 cells showed an increase in ROS generation in concentration of 250 μM compared to control (Fig. 6a). For OPZ was observed a reduction in the generation of ROS in concentration of 50 μM, suggesting an antioxidant effect of this molecule (Fig. 6b).

Apoptosis and necrosis

It was observed a decrease in cell viability while the concentration of OEA increased. In the concentrations of 125 and 250 μM, there was a decrease in number of viable cells and an increase in cell apoptosis. At highest concentrations (375 and 500 μM) all cells suffered necrosis (Fig. 7a–c).

α-tocoferol

In the treatment of OEA with α–tocopherol, the ROS generation was significantly lower than treatment with only OEA and statistically equal to the treatment with only α-tocopherol (Fig. 8a). Compared to its control, only OEA showed significant difference in the percentage of viable cells (Fig. 8b).

Discussion

According to Bifulco et al. (2006), the potential application of cannabinoid substances as antitumor drugs is supported by the evidence of decreased cell proliferation and survival, among others. In this study, two endocannabinoid substances were used: a classic endocannabinoid, the anandamide (AEA), and the OEA, that was proposed as a member of the endocannabinoid family later (Galve-Roperh et al. 2013). According to Adinolfi et al. (2013), AEA induces cytotoxicity against human melanoma cells (A375) in micromolar concentrations through a complex mechanism which includes, among other mechanisms, the CB1 activation.

Although the effects of AEA occur in smaller concentrations, showing that the classic endocannabinoid (AEA) has a more potent effect than OEA, the results of our experiment show that the effects of the two cannabinoids (AEA and OEA) are similar.

In the present study, there was a decrease in viable cell number and an increase in apoptotic cells in concentrations

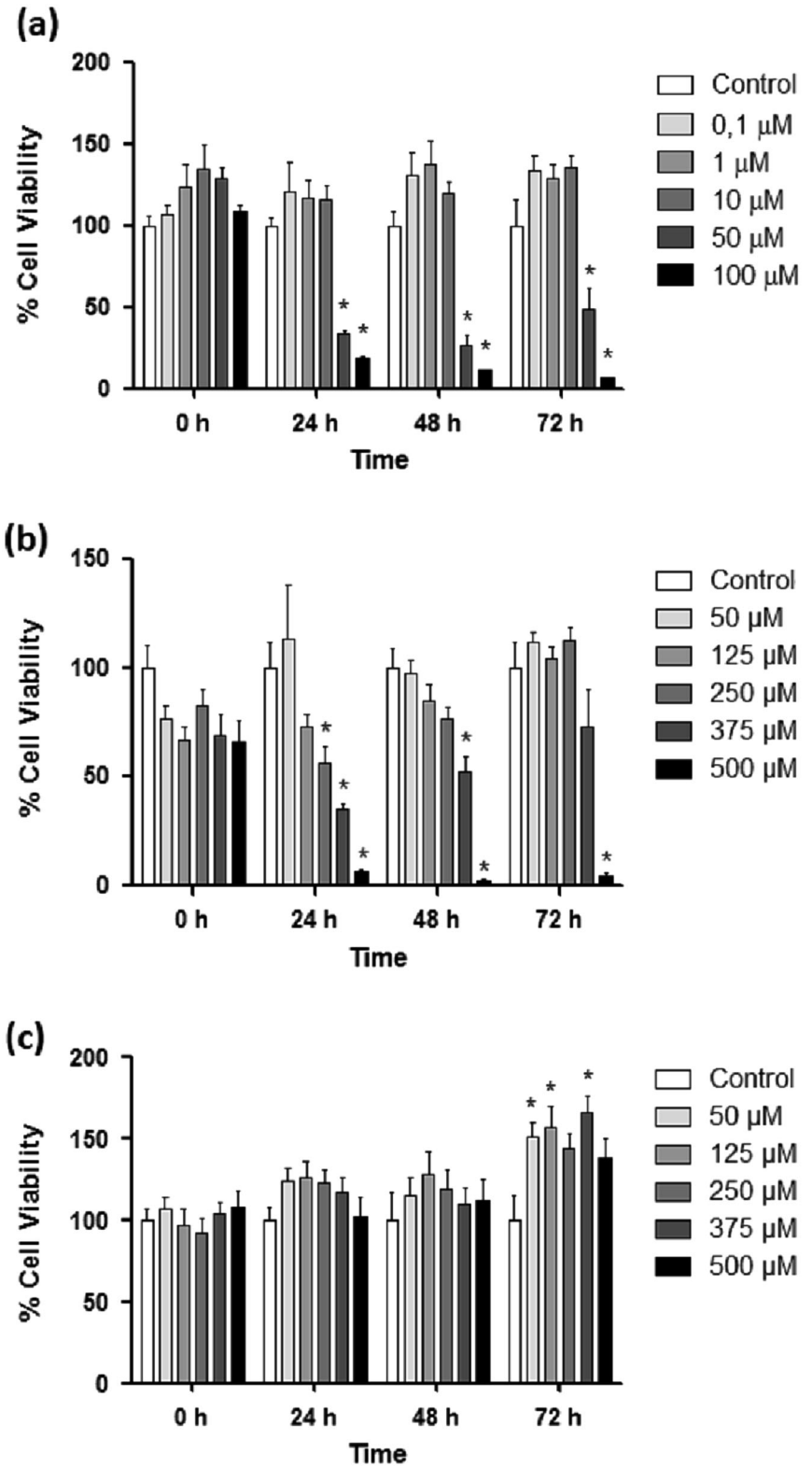


Fig. 5 **a** Percentage of viable B16F10 cells treated with different concentrations of AEA, **b** OEA and **c** OPZ immediately, 24, 48, and 72 h after exposure. Data shown are the mean ± standard error. *Asterisk* indicates significant difference from the respective control at each exposure time ($p < 0.05$)

of 125 and 250 µM of OEA, after 24 h. Higher concentrations (375 and 500 µM) led to cell death by necrosis. According to these results, we suppose that the intermediate concentrations might allow an initiation to a cellular response, leading cells to apoptosis, whereas higher concentrations do not allow any attempt to cell response. Supporting that view, Giuliano et al. (2009) demonstrated that the utilization of the synthetic cannabinoid WIN

55,212-2 in liver cancer cells was associated with the upregulation of proapoptotic factors such as bax, bid, and bcl-xs, and the corresponding down-regulation of anti-apoptotic, cell protective factors such as bcl-2.

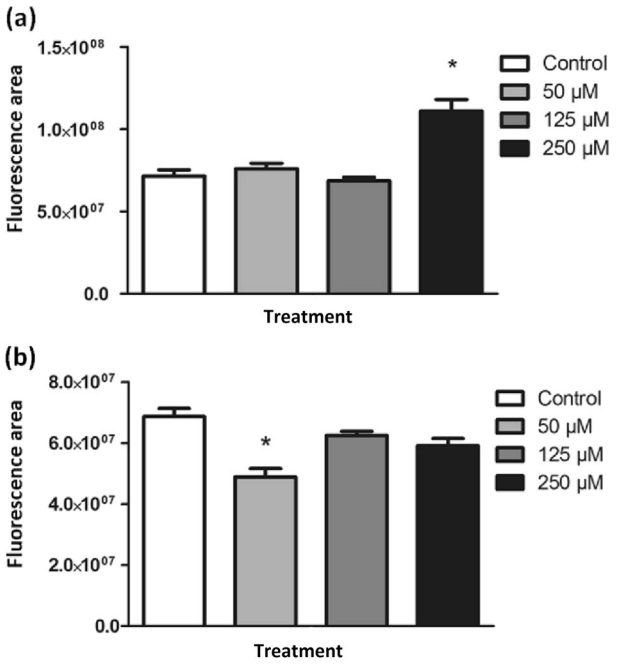


Fig. 6 **a** ROS generation in B16F10 cells treated with 50, 125, and 250 µM of OEA and **b** OPZ for 24 h. Data shown are the mean ± standart error. *Asterisk* indicates significant difference from the respective control at each exposure time ($p < 0.05$)

However, according to Ma et al. (2015) previous treatment with OEA on human umbilical vein endothelial cells shows a protective effect against cytotoxic H_2O_2-induced effects, decreases the intracellular ROS level and caspase-3 activation, and reverses H_2O_2-induced lipid peroxidation and cellular antioxidant potential descent on the cell line.

Nevertheless, when the endocannabinoid was tested in its original form (OEA), a considerable effect of decrease in cell viability was observed. This effect on cell viability was accompanied by an increase of ROS generation in the initial 24 h, for the concentration of 250 µM of OEA. Moreover, when an antioxidant was used in the same exposure condition, it demonstrated a decrease in ROS generation accompanied by a decrease in OEA effect on cell viability. Additionally, according to the results obtained for OPZ, it is possible to demonstrate that the structural change in OEA led to loss of oxidant capacity, as well as the loss of ability to reduce cell viability. Thus, these results suggest the involvement of ROS generation in the effect of OEA in the cell line B16F10.

After 72 h of exposure to modified structure (OPZ), the opposite effect was observed, with an increase in cell proliferation. Previous studies also suggest that different pyrazole isoforms can generate different effects on organisms. Balbi et al. (2011) synthetized thirty-six pyrazole-derived isoforms and tested its antiproliferative effects on three tumoral cell lines. Just four of those presented good antiproliferative effects and had capacity to induce apoptosis on

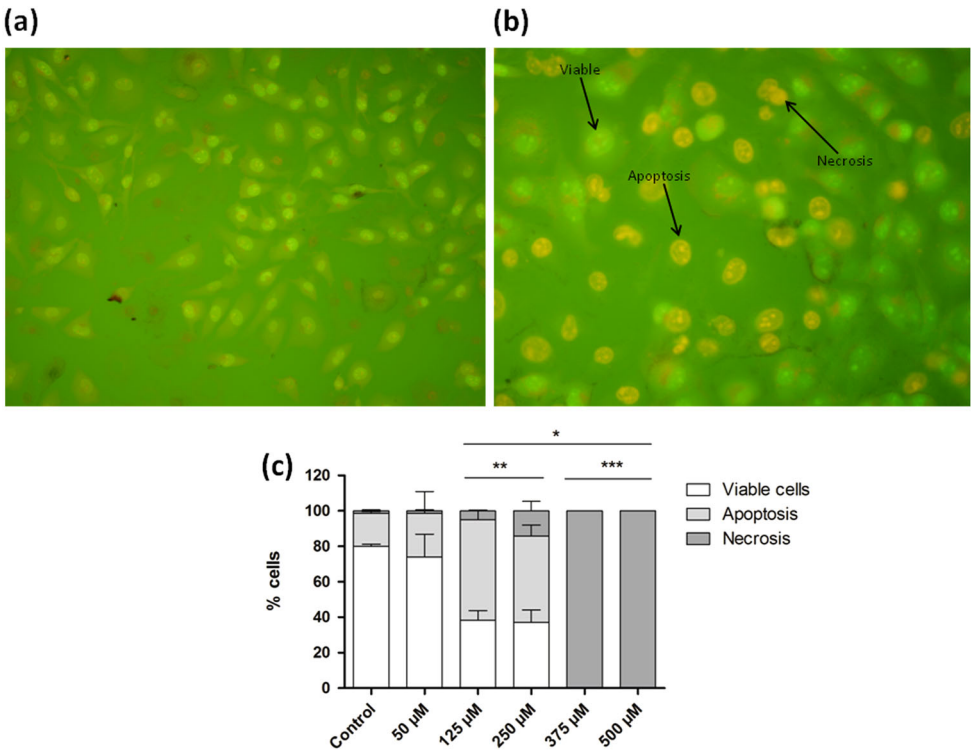


Fig. 7 **a** Visual field of B16F10 cells in the control group demonstrating viable cells and **b** cells treated with 125 µM of OEA showing viable cells, in apoptosis or necrosis captured by fluorescence microscopy. **c** Percentage of viable, apoptotic and necrotic B16F10 cells 24 h after treatment with 50, 125, 250, 375, and 500 µM of OEA from the visual field captured by a fluorescence microscope. Data shown are the mean ± standard error. *Asterisks* (*), (**), and (***) indicate the significant difference in relation to control on viable, apoptotic, and necrotic cells number, respectively ($p < 0.05$)

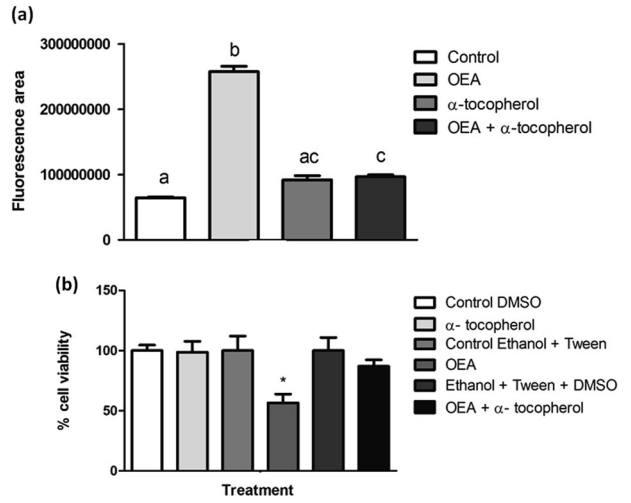


Fig. 8 **a** ROS generation and **b** cell viability in B16F10 cells exposed for 24 h to OEA (250 μM) in the presence and absence of antioxidant (α-tocopherol). Data are shown as mean ± standart error. *Different letters* mean significant differences between treatments ($p < 0.05$) and *asterisk* means significant difference between the treatment and its respective control (which received the same vehicle used as solvent)

the tested lines. Something similar may have occurred in our experiment.

According to Massi et al. (2006), the involvement of ROS in apoptosis mechanisms induced by cannabinoid has been demonstrated in glioma and leukemia cells, and its involvement has been confirmed by using N-acetylcysteine, an antioxidant capable to reduce ROS production, or by the use of NAD(P), an oxidase inhibitor that can attenuate the effects of cannabinoids.

Molecular mechanisms of OEA action are not yet completely elucidated (Fonseca et al. 2013), and more studies may be required to improve the knowledge about this molecule. Furthermore, we have demonstrated that the addition of the pyrazolinic group in the OEA structure caused a significant change in its effect.

Finally, this work intends to make a contribution by showing the dependence of the increase of ROS generation to the cellular death by apoptosis, induced by endocannabinoids in the B16F10 melanoma cells.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no competing interests.

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