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PLGA nanoparticles of a new cinnamic acid derivative inhibits cellular proliferation on breast cancer cell line MCF-7 in a PPARγ dependent way

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Currently, cancer treatments are highly invasive, and they have been associated with a lot of adverse effects that put patient integrity at risk. Therefore, research of novel molecules and delivery systems capable of achieving a therapeutic effect that modifies inhibits and reduces the proliferative activity in cancer cells and, at the same time, reduce adverse effects associated with conventional therapies is imperative. In this study, we analyzed the biological effect of a novel cinnamic acid derivative, 3,4-dichlorobencil-*p*-phenoxylcilamide, in polymeric nanoparticles over MCF–7 breast cancer cells. The nanoparticulated system showed an inhibitory influence over cellular metabolism at equal or higher concentrations than 25 µM of 3,4-dichlorobencil-*p*-phenoxylcilamide, which is associated with PPARγ transcriptional activity, in addition to the decrease in the proliferation antigen Ki-67 basal levels. Those results position this kind of nanoscale system as an alternative on breast cancer treatment and lay the basis for research on the action mechanism associated with its cellular metabolism modulation and relationship with another hallmark on breast cancer cellular models.

1. Introduction

Breast cancer, or mammary gland adenocarcinoma, is a type of cancer characterized by an uncontrolled growth of mammary gland tissue, mainly with a ductal or lobular origin; primarly affecting the female population of Asia (43.6%), Europe (25%) and North America (12.6%) regions and although the current detection program and breast cancer therapy have got a great success reducing mortality incidence associated (MacDonald 2009; The Global Cancer Observatory 2019).

Unfortunately, the highly invasive and adverse effect presented on breast cancer treatment protocols is still an issue, wherein 5% of the cases, the adverse effect associated with Breast cancer chemotherapy, could put patient integrity at risk (Brannon-Peppas and Blanchette 2012; MacDonald 2009; Shapiro and Recht 2001). Hence, in the quest to reduce adverse chemotherapy effects and improve their effectiveness against cancer, researchers have been working in drug delivery systems that could help to this purpose. One of these novel medicines with selective cytotoxicity over cancer cells are nanoscale drug delivery systems (NS-DDS) that work together with cinnamic acid derivatives (CAD) in order to achieve this goal (Beauregard et al. 2015; Brannon-Peppas and Blanchette 2012; De et al. 2011).

The NS-DDS or nanoparticles (NP), are solid or spherical structures with a length from 1 to 1000 nm on at least two dimensions made of organic and inorganic molecules (Aggarwal et al. 2011; ASTM International 2012; Yezhelyev et al. 2006) that will enhance chemotherapeutics efficiency by allowing it to act more selectively and its controlled release inside its target cell (Tabatabaei Mirakabad et al. 2014; Yezhelyev et al. 2006). One of the most used molecules in nanoparticle design is the biopolymer polyglycolic-polylactic acid (PLGA) because of its high biocompatibility, low cytotoxicity due to its degradation through tricarboxylic acid cycle (TCA cycle), and its drug compatibility (Locatelli and Comes Franchini 2012; Tabatabaei Mirakabad et al. 2014), also with the cinnamic acid (CA) and its derivatives (Gomes et al. 2011).

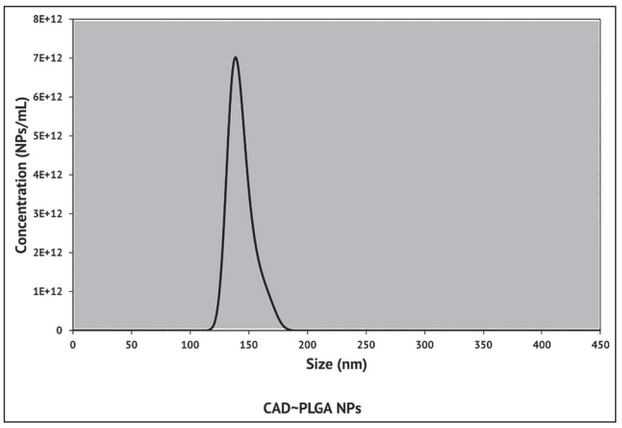

Fig. 1: CAD~PLGA NPs distribution, size and concentration by NTS. CAD~PLGA NPs distribution, showing a peak of concentration near to a size of 151 nm.

Various reports have been showing antitumoral effect of cinnamic acid and its derivatives based on regulation of cellular growth and differentiation (De et al. 2011). *Cis* and *trans* isoforms of CAD display antitumoral activity over invasive carcinomas by disruption on DNA synthesis, NF-Kβ activation, histones deacetylation, or by IL-8 production, but without affecting other non-transformed cells as peripheric blood mononuclear cells (PBMCs) (Hunke et al. 2018; Prijatelj et al. 2013). This CAD antitumoral activity can also be related to its cellular antioxidant an anti-inflammatory activities, interacting with PPARγ, ROS production, Nrf2 antioxidant response or by LOX-5 inhibition, which could lead to growth cellular signaling reduction, apoptosis induction or even cellular arrest (Abduljabbar et al. 2015; Beauregard et al. 2015; Hunke et al. 2018; Pontiki and Hadjipavlou-Litina 2018; Prijatelj et al. 2013).

Table: CAD~PLGA NPs characterization (NP=Nanoparticle; PdI = Polydispersity Index)

	Mean	Standard Error
Concentration (NPs/mL)	6.3 x 10^{12}	2.91 x 10^{11}
Size (nm)	151.6667	10.8286
Capture efficiency (%)	81.1808	0.4103
Z Potential	-6.8733	0.8273

However, important limitations of CAD are their poor stability and water insolubility, regardless of the lack of information about their biological mechanisms on transformed cells (Gomes et al. 2011; Subramanian and Ponnuchamy, 2018).

In this paper, we propose a PLGA based NS-DDS of a new cinnamic acid derivative (3,4-dichlorobencil-*p*-phenoxylcilamide) that eliminates CADs limitations, and the evaluation of its cytotoxic effect, a glance of its relationship with PPARγ activity and anti-proliferative effects over MCF-7 breast cancer cell line, as a first step in the understanding of its induced biological effects and to dilucidated its action mechanism.

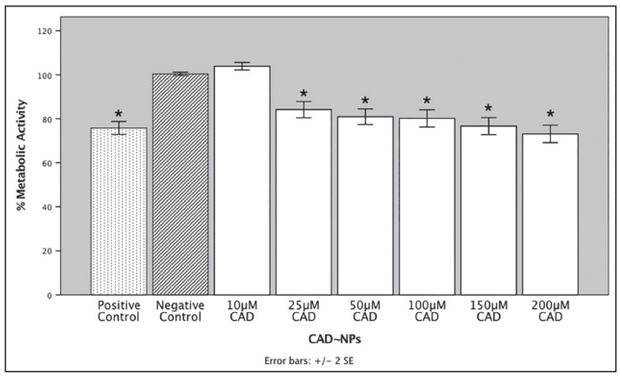

Fig. 2: CAD~PLGA NPs cytotoxicity effect after 24 h exposition over MCF-7 cell line by resazurin reduction assay. Data from CAD~PLGA NPs concentration of 10 µM (2 x 10^9 NP/mL) to 200 µM (4x 10^{10} NP/mL) of loaded CAD are shown. Cytotoxic effect is shown as the percentage of metabolic activity reduction. A dose-dependent decrease in metabolic activity, near 20%, is observed from concentration higher than and equal to 25 µM of CAD (5 x 10^9 NP/mL), being statistically significant compared to the negative control (unexposed cells). As metabolic activity reduction positive control, MCF-7 cells were exposed to 1 mM of hydrogen peroxide. Data shown as mean ± 2SE of three independent assays. * indicates no significant difference between groups. p<0.05.

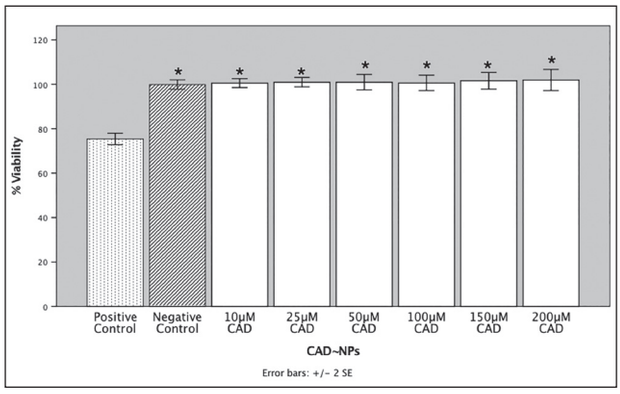

Fig. 3: CAD~PLGA NPs Cytotoxicity effect after 24 h exposition over MCF-7 cell line by crystal violet inclusion assay. Data from CAD~PLGA NPs concentration of 10 µM (2 x 10^9 NP/mL) to 200 µM (4 x 10^{10} NP/mL) of loaded CAD are shown. Cytotoxic effect is shown as the percentage of viability reduction. There is not statistically significant viability reduction after 24 h CAD~PLGA NPs exposition compared to the negative control (unexposed cells). As viability reduction positive control, MCF-7 cells were exposed to 1 mM of hydrogen peroxide. Data shown as mean ± 2SE of three independent assays. * indicates no significant difference between groups. p<0.05.

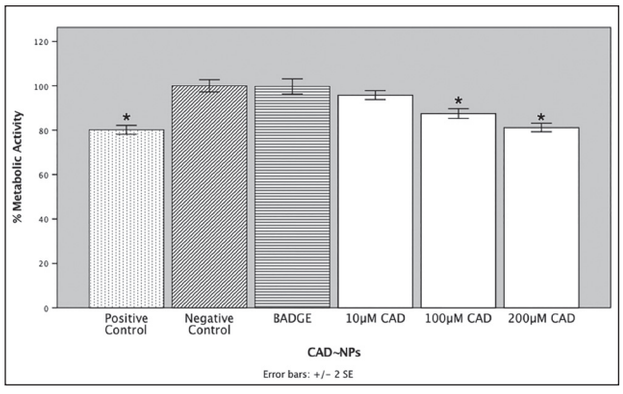

Fig. 4A: Effect of PPARγ receptor over CAD~PLGA NPs cytotoxic effect after 24h exposition over MCF-7 cell line. A. CAD~PLGA NPs cytotoxic effect over MCF-7 cell line with intact PPARγ receptor activity. B. CAD~PLGA NPs cytotoxic effect over MCF-7 cell line with inhibited PPARγ receptor activity by BADGE. A metabolic activity reduction, above 20%, is shown at concentrations of 100 µM (2 x 10^{10} NP/mL) and 200 µM (4 x 10^{10} NP/mL) of loaded CAD on MCF-7 cell line with intact PPARγ receptor activity (A); on the other hand, on those cell with PPARγ receptor activity inhibited by BADGE (B), the exposition to CAD~PLGA NPs did not show a statistically significant reduction in metabolic activity. Data shown as mean ± 2SE of three independent assays. * indicates no significant difference between groups. p<0.05.

2. Investigations and results

This work aims to evaluate the impact over cellular viability and proliferation due to MCF-7 exposition to a new cinnamic acid derivative (3,4-dichlorobencil-*p*-phenoxylcilamide) nanoparticles; to achieve this goal, we loaded CAD into polylactic-glycolic acid nanoparticles as described above, getting a system monodisperse with an average size of 152±11 nm, a concentration of 6.3 x 10^{12}± 0,3 NPs/mL (Fig. 1), and a capture efficiency of 81±0.4% (Table).

To get a better understanding of CAD~PLGA NPs effects over the MCF-7 cell line, we performed the resazurin reduction and crystal violet inclusion assay to determine the cytotoxic effect of different concentrations of CAD~PLGA NPs; using as cytotoxicity positive control 1 mM of H_2O_2. As shown in Fig. 2, we found that at concentrations equal to or higher than 25 µM of loaded NPs there is a significative cellular metabolism inhibition associated to viability reduction (p<0,05) in the resazurin reduction assay, with a decline similar to H_2O_2 positive control (near 30%) at concentrations ≥ 50µM, getting a calculated IC_{50} of 404µM±9,5. However, at the crystal violet inclusion assay, none of the inhibitory NPs concentrations showed an effect (Fig. 3).

To determine the influence of PPARγ transcriptional activity over the nanoparticles metabolism inhibitory effect, its activity was inhibited on breast cancer model MCF-7 by its exposition to bisphenol A diglycidyl ether (BADGE), a highly affine antagonist to PPARγ that has been demonstrated inhibits its transcriptional activity without reduction of cellular viability (Kim et al. 2006; Kota et al. 2005; Wright et al. 2000). For this assay, we evaluated nanoparticle cytotoxic effects at concentrations of 10 µM, 100 µM, and 200 µM of CAD into two groups of MCF-7 cells, one of them with PPARγ activity inhibited. Our experiments showed an inhibition of the cytotoxic effect associated to nanoparticles exposition in the group were PPARγ transcriptional activity was inhibited (Fig. 4A); on the other hand, we found a significative inhibition of metabolic activity at 100 µM and 200 µM concentrations of CAD in the group where PPARγ activity remained intact.

MCF-7 cells were exposed to BADGE (Fig. 4B), and we found a lack of cytotoxic effect, even at concentrations of 200 µM of CAD, in the groups where PPARγ transcriptional activity was inhibited by BADGE; furthermore, in groups with PPARγ regular activity the nanoparticles cytotoxic effect was present.

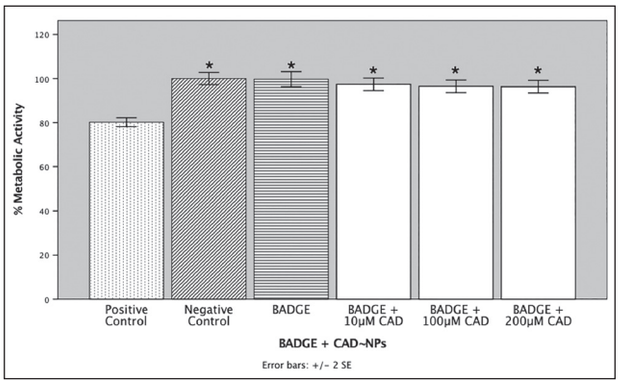

Fig. 4B: Effect of PPARγ receptor over CAD~PLGA NPs cytotoxic effect after 24 h exposition over MCF-7 cell line. A. CAD~PLGA NPs cytotoxic effect over MCF-7 cell line with intact PPARγ receptor activity. B. CAD~PLGA NPs cytotoxic effect over MCF-7 cell line with inhibited PPARγ receptor activity by BADGE. A metabolic activity reduction, above 20%, is shown at concentrations of 100 μM (2 x 10^{10} NP/mL) and 200 μM (4 x 10^{10} NP/mL) of loaded CAD on MCF-7 cell line with intact PPARγ receptor activity (A); on the other hand, on those cell with PPARγ receptor activity inhibited by BADGE (B), the exposition to CAD~PLGA NPs did not show a statistically significant reduction in metabolic activity. Data shown as mean ± 2SE of three independent assays. * indicates no significant difference between groups. p<0.05.

To elucidate the effect of CAD~PLGA NPs over cellular proliferation, we determined the levels of Ki-67 proliferation antigen, a nuclear protein highly expressed on M phase and high rate mitosis cells like cancer cell lines (Kaszak et al. 2018; Russo, 2016), on MCF-7 cell exposed to 10 μM, 100 μM and 200 μM of encapsulated CAD for 24 h. We found a significative reduction of more than 60% on the basal level of Ki-67 antigen at 100 μM and 200 μM concentrations, but not in our NPs control group (Fig. 5), associating this finding to the activity of our CAD over MCF-7.

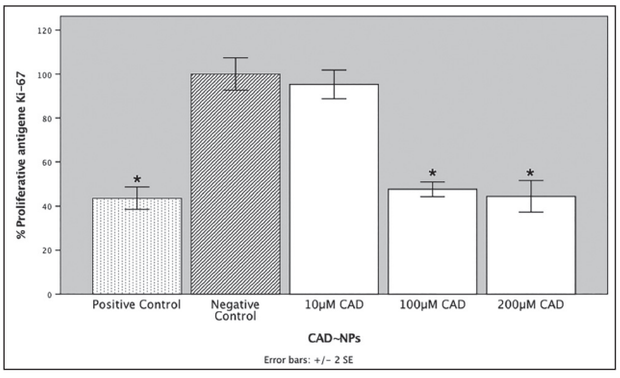

Fig. 5: CAD~PLGA NPs effect over Ki-67 antigen concentration on MCF-7 cell line at 24 h NPs exposition. Concentration of Ki-67 antigen is shown as percentage. There is a statistically significant reduction of Ki-67 antigen, near 60%, on those cells exposed to 100 μM (2 x 10^{10} NP/mL) and 200 μM (4 x 10^{10} NP/mL) of loaded CAD, compared to the negative control (unexposed cells). Data shown as mean ± 2SE of three independent assays. * indicates no significant difference between groups. p<0.05.

3. Discussion

In this study, we found a metabolic inhibitory effect on MCF-7 cells exposed at concentrations equal to or higher than 25 μM of encapsulated CAD with an IC_{50} of 404±9 μM. This represents a remarkable reduction, compared with other IC_{50} of cinnamic acid derivatives reported by Hunke et al. (2018) on MCF-7, who found IC_{50} values of 0.6 – 2.9 mM for several CAD tested. We associate this IC_{50} reduction to CAD encapsulation into PLGA nanoparticles. Furthermore, this could be an advantage for the use of CAD, because this nanoparticulate system allows to suspend CAD in aqueous solutions under physiological conditions without its dissolution in organic solvents as dimethyl sulfoxide, and avoiding cytotoxic interaction due to its association with PLGA, due to its hydrolysis under TCA cycle (Tabatabaei Mirakabad et al. 2014). However, loaded-CAD metallic nanoparticles have proved to be a more efficient vector in terms of IC_{50} reduction, as in Subramanian and Ponnuchamy's work, whose gold CAD nanoparticles had needed smaller concentration of CAD to exert its cytotoxic effect due to the synergetic effect between their CAD and gold nanoparticles cytotoxicity (Subramanian and Ponnuchamy 2018). However, the vectorization of drugs into metallic nanosystems as gold nanoparticles has been associated with the appearance of adverse effects on *in vivo* models at concentrations of 2.2 mg/kg. These adverse effects could include bodyweight loss, anemia, and hyperreactivity of white blood cells (Zhang et al. 2010); the situation does not show up when PLGA nanoparticles are used as pharmacological vehicles due to their high biocompatibility and low toxicity (Tabatabaei Mirakabad et al. 2014).

To get a glance on the implications of our nanoparticulated system with PPARγ, we evaluated the relationship between nanoparticles and PPARγ activity, a nuclear factor that plays a vital role on energetic metabolism modulation, by inhibiting its transcriptional activity with BADGE. We found a lack of cytotoxic effect, even at concentrations of 200 μM of CAD, in the groups where PPARγ transcriptional activity was inhibited by BADGE; furthermore, in groups with PPARγ of regular activity cytotoxic effect of the nanoparticles was present. This findings suggest a CAD~PLGA NPs dependence to PPARγ functional transcriptional activity to exert its cytotoxic effect over MCF-7 breast cancer cells, a property usually associated to PPARγ agonists like thiazolidinediones (TZD) (Pseftogas et al. 2017; Wright et al. 2000; Yan et al. 2009), but rarely seen in CAD (Houpis et al. 2005; Liu et al. 1995), leading to the possibility that this CAD acts as a PPARγ agonist on MCF-7 cells or as an intermediary in the PPARγ signalling pathway.

CAD has proved to have a cytotoxic effect on cancer cell lines like K562, Hela, Fem-X, and MCF-7 in which they exert a cellular viability reduction associated with disruption on cell cycle progression and induction of apoptosic (Beauregard et al. 2015; Hunke et al. 2018; Prijatelj et al. 2013). Hunke et al. (2018) also reported that this cytotoxic effect on MCF-7 was not related to a loss on cellular viability; moreover, the effects are related with the disruption on cell cycle, due to an arrest on G0/G1 state associated to a reduction or disruption on cellular metabolism. These findings could be related to the lack of cytotoxic effect in the crystal violet inclusion assay, in which CAD~PLGA NPs exposition does not show a reduction of cellular viability. Moreover, there is a reduction of Ki-67 antigen, a protein related with cellular proliferation that has acquired importance due to its high correlation with the malignancy degree and clinical outcome in breast cancer cases (Petrelli et al. 2015). This finding is similar to that obtained with CAD~PLGA NPs exposition and has also been shown by PPARγ agonists like rosiglitazone. Exposure to this kind of TZD reduces Ki-67 levels in a range of 77% to 96%. Therefore, we suggest that CAD effects could be related to the cellular arrest in G0/G1 state (Kim et al. 2018; Nojima et al. 2016) due to a disruption on cellular metabolism without cellular viability loss and this effect could be associated with the activity of PPARγ.

Some authors have associated the cellular arrest exhibited by PPARγ agonists to a variety of mechanisms, one of them related to a decrease in Rb phosphorylation levels, which leads to a reduction in CDK2/4 activity or increasing p27 levels (Kim et al. 2015; Nojima et al. 2016). Another mechanism could be associated with the antioxidant property of CAD, activating Nrf2 through PKC, AMPK, CKII or ROS pathway (Hseu et al. 2018; Ma, 2013; Pontiki et al. 2014; Pontiki and Hadjipavlou-Litina, 2018) that hints at PPARγ expression and execution of its antiproliferative effect (Cho et al. 2010; Lee 2017). Even due to a reduction of cell grown signals mediated by LOX-5 inhibition (Pontiki et al. 2014; Pontiki and Hadjipavlou-Litina 2018), PTEN overexpression (Kim et al. 2015; Suzuki et al. 2006) or by inhibition of NF-κβ pathway leading to apoptosis (Kim et al. 2015; Wahli and Michalik 2012),

but if our CAD~PLGA NPs exhibits one of those mechanisms is still unclear and warrants further studies.

In conclusion, we developed a PLGA nanoparticulate system loaded with CAD, which after exposition over MCF-7 breast cancer cell line induces a cellular viability reduction near to 30% at concentrations of 25 µM of CAD. This effect was associated to PPAR gamma transcriptional activity, as shown by the lack of effect after its inhibition by BADGE. This reduction on cellular viability seems to be associated with a decrease in cellular proliferation as indicated by Ki67 basal level reduction at 100 µM and 200 µM concentrations of CAD, which could be related to an interface arrest on cells exposed to our nanoparticulate system. These findings lay the foundations for investigations of the association between cellular metabolism modulation and CAD, as well as its relationship with other hallmarks on breast cancer cellular models, positioning this kind of nanoscale system as a future alternative on breast cancer treatment.

4. Experimental

4.1. Materials

Polylactic-co-glycolic acid (Sigma-Aldrich, USA), cinnamic acid derivative (CAD) 3,4-dichlorobencil-*p*-phenoxylcilamide (Medical Chemistry Laboratory-UNAM, MEX), acetone (Sigma-Aldrich, USA), polyvinyl alcohol (Sigma-Aldrich, USA), resazurin (Sigma-Aldrich, USA), crystal violet (Sigma-Aldrich, USA), methanol (Sigma-Aldrich, USA), bisphenol A diglycidyl ether (Sigma-Aldrich, USA).

4.2. Poly lactic-co-glycolic acid nanoparticles with cinnamic acid derivative (CAD~PLGA NPs)

CAD~PLGA NPs were prepared by the single solvent-emulsion method. For CAD~PLGA NPs, about 56 mg of PLGA and 15 mg of 3,4-dichlorobencil-*p*-phenoxylcilamide (in 10 mL acetone) were added dropwise to an aqueous PVA 1% solution (20 mL) followed by constant stirring at room temperature by 30 min. In order to concentrate the NPs, the system was ultra-centrifugated at 15000 rpm for 1 h at 4 °C temperature. The formation of nanoparticles, concentration, size, and distribution was monitored by Nanoparticle Tracking Analysis (NTA) using Nano Sight NS300 (Malvern, UK). The encapsulation efficiency was determined by supernatant evaluation of 3,4-dichlorobencil-*p*-phenoxylcilamide presence by UV spectrophotometry at λ 325 nm on a spectrophotometer Du-64 (Beckman, USA).

4.3. Cell culture

MCF-7 breast cancer cells were obtained from American Type Cell Culture Collection (ATCC, USA) and grown in Dulbecco's Modified Eagles medium containing 4.5g/L of Glucose, L-glutamine and sodium pyruvate (Corning, USA), 10% fetal bovine serum (Corning, USA) and 100U/µL of penicillin-streptomycin (Corning, USA). The cells were maintained at 5% CO_2 at a controlled temperature of 37 °C.

4.4. Cell viability estimation

The cytotoxic effect of CAD~PLGA NPs over MCF-7 cells was studied by two methodologies, Resazurin reduction assay, as described by Escobar and Aristizábal (2010), and by crystal violet inclusion, as reported by Feoktistova et al. (2016), both of them with slight modifications. In brief, 96 well cell culture plates were seeded at 10^3 cells per well and allowed to adhere for 24 h. The medium was then replaced with a fresh medium containing a different concentration of CAD~PLGA NPs (0.0001 µM to 200 µM of encapsulated cinnamic acid derivative) and incubated for 24 h. After the exposition, to perform the resazurin reduction assay, the medium was replaced with 0.001% resazurin (in fresh medium) for 1 h at 37 °C. The absorbances of every well were read at λ 595 nm by UV spectrophotometry.

For the crystal violet inclusion assay, after nanoparticles exposition, cells were stained on crystal violet 0.5% at 20 rpm for 20 min, four times rinsed with PBS pH 7.4 and air-dried for at least 2 h. Crystal violet elution was performed on methanol for 20 min at 20 rpm. The average absorbances of every sample were prepared determined at 570 nm by UV spectrophotometry.

4.5. PPARγ inhibition

To determine CAD~PLGA NPs and PPARγ relationship over nanoparticles cytotoxic effect induction, we seeded 96 wells cell plates at 10^3 cells per well; seeded wells were divided into two groups, one of them pre-treated with bisphenol A diglycidyl ether (BADGE) 20 µM for 1 h at 37 °C, as suggested by Kim et al. (2006), while the other remained untreated. Both groups were exposed to 10 µM, 100 µM, and 200 µM concentrations of NPs for 24 h at 37 °C and the nanoparticles cytotoxic effect was determined by resazurin reduction assay as described above.

4.6. Proliferation assay: Ki-67 determination

To asses CAD~PLGA NPs impact over cellular proliferation, an Enzyme-linked immunosorbent assay (ELISA) was conducted. Six well cell culture plates were seeded at 10^5 cells per well and exposed to NP concentration equal to 10 µM, 100 µM and 200 µM of the encapsulated cinnamic acid derivative by 24 h. The quantification of intracellular Ki-67 levels was measured by commercial kit Human Antigen Ki-67 ELISA Kit (CUSABIO, USA), following manufacturer instructions.

4.7. Statistical analysis

Statistical analysis was conducted on SPSS software (IBM, USA). Results are showed as media and standard media error of three independent experiments with at least three replicants each one. Differences among groups were detected by the ANOVA test with the Tukey posthoc test, $p<0.05$ was considered as statistical significance.

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Conflict of interest: The authors declare no relevant conflicts of interest.

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