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Highlights

- PaDef defensin from avocado shows cytoxicity on K562 chronic myeloid leukemia cells.
- PaDef defensin did not affect human normal peripheral blood mononuclear cells.
- K562 membrane potential or calcium flow was not affected by PaDef defensin.
- Cytotoxic effects on K562 cells of PaDef are due to the induction of extrinsic apoptosis.

Abstract

Plant defensins, a group of antimicrobial peptides, show selective cytotoxicity toward cancer cells. However, their mechanisms of action remain poorly understood. Here, we evaluated the cytotoxicity of PaDef defensin from avocado (*Persea americana var. drymifolia*) on K562 chronic myeloid leukemia cells and analyzed the pathway involved in the induction of cell death. The defensin PaDef was not cytotoxic against human PBMCs; however, it was cytotoxic for K562 cell line (IC_{50}=97.3 µg/ml) activating apoptosis at 12 h. PaDef did not affect the mitochondrial membrane potential (ΔΨm), neither the transmembranal potential or the release of intracellular calcium. Also, PaDef induced gene expression of caspase 8 (~2 fold), TNF-α (~4 fold) and TNFR1 (~10 fold). In addition, the activation of caspase 8 was detected at 24 h, whereas caspase 9 activity was not

modified, suggesting that the extrinsic apoptosis pathway could be activated. In conclusion, PaDef induces apoptosis on K562 cells, which is related to the activation of caspase 8 and involves the participation of TNF-α, which is a novel property for a plant defensin.

Keywords: Leukemia; antimicrobial peptide; apoptosis; PaDef defensin

1. Introduction

Cancer is a major cause of death worldwide. The leukemias are including in the ten most important types of cancers for their incidence and mortality (Ferlay et al., 2013). The American Cancer Society estimates about 8,950 new cases of chronic myeloid leukemia (CML) in the United States for 2017. Recent treatments to CML (e.g. tyrosine kinase inhibitors) provide a notable therapeutic progress; however, these approaches shown side effects and their long-term safety profile is unknown (Pasic and Lipton, 2017). These limitations have led to the search for new therapeutic alternatives to CML. An attractive alternative is the use of antimicrobial peptides (AMP), which shown cytotoxic properties (Al-Benna et al., 2011; Pushpanathan et al., 2013; Riedl et al., 2011).

The AMP are small peptides (12 to 100 aa), generally cationic and amphipathic that are produced by many organisms as part of their defense mechanisms against pathogens (Cederlund et al., 2011; Peters et al., 2010). Today, more of 2900 AMP have been described (http://aps.unmc.edu/AP/main.php), and ~7% of them are cytotoxic to cancer cells. This cytotoxicity has been associated to the peptide interaction with cancer cells through the negative charged membrane phospholipids, that mainly leads to necrosis (e.g., Dermaseptin B2, D-K_6L_9, MPI-1) (Papo et al., 2006; van Zoggel et al., 2012; Zhang et al., 2010), or apoptosis (e.g., BMAP-28) (Risso et al., 2002). Additionally, other cytotoxic mechanisms have been reported including tumor growth inhibition and angiogenesis (Koskimaki et al., 2009), DNA synthesis/replication inhibition (Kuriyama et al., 2013; Ourth, 2011), reactive oxygen species (ROS) generation (Gao et al., 2013), DNA damage and

autophagy induction (Ren et al., 2013) and changes in the metabolic profile (Kuroda et al., 2015). These mechanisms essentially have been described for AMP from animals; however, the cytotoxic mechanisms for plant AMP are poorly understood.

Plants AMP are small peptides of 40-50 aa (3 -10 kDa) rich in cysteine, which are grouped in twelve families that shown mainly bactericidal and fungicidal activity (Lay and Anderson, 2005; Thomma et al., 2002). However, thionins, defensins and cyclotides families contain AMP cytotoxic for cancer cells (Guzmán-Rodríguez et al., 2015). In particular, plant defensins have drawn attention due to their selectivity towards cancer cells. Nevertheless, there are few studies that describe in detail the mechanisms of induced cell death for plant defensins. Recently, a non-apoptotic cytotoxic mechanism for the plant defensins NaD1 (*Nicotiana alata*) and TPP3 (*Lycopersicon esculentum*) was reported, in both cases the binding of the AMP to the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate was involved (Baxter et al., 2015; Poon et al., 2014).

In a previous study, we isolated the cDNA of PaDef defensin from avocado fruit (*Persea americana* var. drymifolia) and demonstrated its antimicrobial activity (Guzmán-Rodríguez et al., 2013). Further, we showed that this defensin induces intrinsic apoptosis pathway in breast cancer cell line MCF-7 (Guzmán-Rodríguez et al., 2016). In this work, we show evidence that PaDef defensin is also cytotoxic to K562 chronic myeloid leukemia cells through the activation of the extrinsic apoptosis pathway, which is a novel property for this AMP.

2. Material and methods

2.1 Peptide

In this study, we used the mature region of the PaDef defensin (ATCETPSKHFNGLCIRSSNCASVCHGEHFTDGRCQGVRRRCMCLKPC, 47 aa). This AMP was chemically synthesized (BIOMATIK) and the disulfide bond formation was completed as described (Guzmán-Rodríguez et al., 2016). The AMP was dissolved in DMSO 20% and the final concentration of vehicle for all of the experiments was 0.39%, which was used as control. The cytotoxicity of PaDef defensin was evaluated at a range of concentration of 10-200 µg/ml, according to previous report (Mader et al., 2005).

2.2 K562 cell culture

The human K562 chronic myeloid leukemia cells was obtained from American Type Culture Collection. Cells were routinely cultured in RPMI-1640 medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, Corning) and 100 U/ml penicillin and streptomycin (Gibco) and grown in an atmosphere of 5% CO_2 at 37°C.

2.3 MTT viability assay

The cytotoxicity of PaDef defensin on K562 was assessed by MTT assay as described (Guzmán-Rodríguez et al., 2016). Briefly, K562 cells were synchronized in RPMI-1640 medium without serum (12 h). $2x10^4$ cells/well cultured in 96-well flat-bottom plates were treated with PaDef (10-200 µg/ml) or vehicle and incubated during 12-24 h. Further, 10 µl of MTT solution (5 mg/ml, Sigma) in phosphate

buffer saline (PBS) was added to each well and incubated during 4 h at 37ºC. Finally, formazan crystals were dissolved with acid isopropanol (100 μl, 95% isopropanol and 5% of 1 N HCl). The absorbance lectures were performed in a microplate reader (Bio Rad) at 595 nm. Actinomycin D was used as cell death positive control. Cell viability results are reported as the percentage of viable cells with respect to cells treated with the vehicle (DMSO 0.39%). The half maximal inhibitory concentration (IC_{50}) was calculated by regression analysis using Excel (Microsoft) and corroborated by flow cytometry with SYTO® 9 green-fluorescent nucleic acid stain and propidium iodide in a BD Accuri™ C6 flow cytometer (BD Biosciences) according to the manufacturer's instructions. For the rest of the experiments we used the IC_{50}.

2.3 Calcium efflux testing

Calcium efflux was assessed in a BD Accuri™ C6 flow cytometer (BD Biosciences) using the Calcium Assay Kit (BD Biosciences) according to the manufacturer's instructions. For this, K562 cells ($1X10^5$ cells/ml) cultured in RPMI-1640 medium were incubated with the indicator dye for 1 h. Previous to add the treatments (PaDef IC_{50} or vehicle), we established a baseline fluorescence during 3 min. After treatments, the fluorescence intensity was monitored by flow cytometry for other 3 min. Phorbol myristate acetate (3 μM; PMA, Sigma) was used as a positive control.

2.4 Measurement of the transmembrane potential

Changes in the transmembrane potential of K562 cells were evaluated using the DiSC3(5) dye (3,3′-dipropylthiadicarbocyanine iodide, Sigma) as described (Guzmán-Rodríguez et al., 2016). Briefly, K562 cells ($1X10^5$ cells/ well) seeded in 96-well black-wall plates were incubated with DiSC3(5) 0.2 mM (dissolved in Hanks' HEPES buffer) for 30 min in a CO_2 incubator. Then, the treatments (PaDef IC_{50} and vehicle) were added and the fluorescence intensity was monitored for 2 h using a Varioskan spectrophotometer (Thermo Scientific). Valinomycin (0.2 mM, Sigma) was used as a positive control.

2.5 Apoptosis and caspase activation

The apoptosis rate was determined using Annexin V and 7AAD according to the manufacturer's instructions (Annexin V, Alexa Fluor 488 conjugate, Invitrogen) in a BD Accuri™ C6 flow cytometer (BD Biosciences). The data were analyzed with the FlowJo v10.4 software (TreeStar, Inc.). A total of 10,000 events were collected. Actinomycin D (Sigma, 80 µg/ml) was used as a positive control for apoptosis.

The activation of caspases 8 and 9 was determined using the Caspase-Glo 8 and 9 kit (Promega) according to manufacturer's instructions. For this, K562 cells ($6x10^4$ cells/ well) seeded in white 96-well plates were incubated with PaDef IC_{50} or vehicle in serum-free medium during 12-24 h. Further, the caspase substrates were added and the luminescence was registered using a Varioskan spectrophotometer. Also, K562 cells were incubated with the caspase 8 inhibitor Ac-IETD-CHO (Sigma, 100 µM) for 1 h and then treated with PaDef IC_{50} by 24 h and apoptosis rate was determined as described above. Actinomycin D (80 µg/ml) was used as positive control.

2.6 Assessment of mitochondrial membrane potential (ΔΨm)

The effect of PaDef defensin on the ΔΨm of K562 cells (1X10^5 cells/well) was measured using the JC-1 dye (BD Biosciences) in a BD Accuri™ C6 flow cytometer (BD Biosciences) as described (Guzmán-Rodríguez et al., 2016). The dye indicator allows to differentiate between healthy mitochondria (red fluorescence) from those with damage (green fluorescence).

2.7 Determination of reactive oxygen species (ROS)

ROS were measured by flow cytometry in a BD Accuri™ C6 flow cytometer (BD Biosciences) using 2′,7′-dichlorofluorescin diacetate (H2DCFDA) (Thermo Scientific). K562 cells were cultured in RPMI-1640 medium without serum by 12 h. Then, the medium was replaced and the cells (2x10^4 cells/well) were cultured in 96-well plates with 100 µl of fresh medium containing PaDef IC_{50} or vehicle and incubated by 24 h. Next, the cells were collected and incubated with H2DCFDA 25 mM for 2h/37ºC in darkness. Ethanol 12% was used as positive control. A total of 10,000 events were analyzed.

2.8 RNA isolation and gene expression analysis

Total RNA was extracted with Trizol reagent (Invitrogen) from K562 cells (1X10^5 cells/well) cultured in 24-well plates and incubated with PaDef IC_{50} or vehicle for 6, 12, and 24 h, according to the manufacturer's instructions and then used to synthesize cDNA as described (Alva-Murillo et al., 2012). The qPCR analysis was carried out by the comparative Ct method (ΔΔCt) in a StepOne Plus Real-Time

PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The genes analyzed and oligonucleotides employed are shown in Table (1). β-actin was used as endogenous gene.

2.9 Quantification of TNF-α secretion

For the measurement of TNF-α we used the conditioned media from K562 cells treated with PaDef IC_{50} or vehicle. TNF-α concentrations were determined using a Becton Dickinson cytometric bead array (CBA) in a BD Accuri™ C6 flow cytometer (BD Biosciences) according to the manufacturer's instructions.

2.10 Statistical analysis

The data were obtained from three independent experiments performed by triplicate. The significance of the differences was assessed using Student's t-test. A p value < 0.05 was considered significant. In qPCR analysis, fold-change values > 2 or < 0.5 were considered as significant differentially expressed mRNAs (Morey et al., 2006).

3. Results

3.1 PaDef defensin is cytotoxic to K562 cells

PaDef cytotoxicity (10-200 µg/ml) on K562 cells was analyzed by MTT. In Fig. 1A we showed that PaDef was cytotoxic to K562 cells in a concentration-dependent manner at 24 h (Fig. 1A), showing IC_{50} value of 97.3 µg/ml (18.65 µM) (Fig. 1B and

C). A microscopic analysis indicated that K562 cells did not show morphological alterations, which suggests that PaDef does not alter the membrane of these cells (Fig.1D). Also, PaDef did not affect human peripheral blood mononuclear cells evaluated by MTT (Fig. 1E) and flow cytometry (data not shown). For the rest of the experiments we used the IC_{50}.

3.2 PaDef defensin does not affect the cell membrane of K562 cells

To determine if cytotoxicity of PaDef defensin on K562 cells involves cell membrane damage, we evaluated the intracellular calcium efflux and membrane electrical potential. The data indicated that cytotoxicity of PaDef was not related to the cell membrane damage due that the efflux of intracellular calcium was not affected (Fig. 2A) nor the membrane electrical potential (Fig. 2B).

3.3 PaDef defensin cytotoxicity on K562 cells involve extrinsic apoptosis

In agreement with the results, PaDef defensin cytotoxicity on K562 cells is not related to cell membrane damage. Thus, we evaluated if PaDef induces apoptosis in K562 cells. As shown in Fig. 3, PaDef induced apoptosis from 12 h (> 30%) in K562 cells, which was similar to the effect showed by actinomycin D.

To determine the apoptosis pathway involved in PaDef cytotoxicity, first we evaluated the mitochondrial membrane potential ($\Delta\Psi m$) using the dye JC-1. K562 cells treated with PaDef IC_{50} at 6, 12 and 24 h did not show modifications in the $\Delta\Psi m$ (Fig. 4). On the other hand, we detected that PaDef up-regulates the gene expression of caspase 8 (~2 fold), but not the expression of caspase 9 gene (Fig. 5). Accordingly, the PaDef treatment induced the activation of caspase 8 but not

caspase 9 (Fig. 5). In addition, when caspase 8 activity was blocked with the specific inhibitor Ac-IETD-CHO, the apoptosis rate of K562 cells was reduced (~30%) (Fig. 6). Also, we did not detect modifications in the ROS production in K562 cells treated with PaDef (data not shown). These results suggest that PaDef activates extrinsic apoptosis in K562 cells.

3.4 PaDef induces TNF-α and TNF receptor 1 (TNFR1) gene expression and TNF-α protein secretion

The extrinsic apoptosis may be activated by TNFα/TNFR1 interaction. For this reason, we analyzed the effects of PaDef IC_{50} on TNF-α and TNFR1 gene expression. qPCR analysis demonstrated that PaDef treatment (24 h) significantly induced the TNF-α gene expression (~4 fold), as well as the expression of TNFR1 since 6 h (~10 fold). Additionally, PaDef treatment favors the TNF-α protein secretion at 24 h (~5 fold) (Fig. 7). These results support the fact that PaDef induces extrinsic apoptosis in K562 cells.

4. Discussion

Cytotoxicity and specificity of antimicrobial peptides have been considered as an therapeutic alternative in cancer treatment (Al-Benna et al., 2011; Pushpanathan et al., 2013; Riedl et al., 2011). Plants are an important source of antimicrobial peptides; however, most of the studies carried out with these AMP essentially describe their cytotoxic effects, but the underlying mechanisms are poorly understood (Guzmán-Rodríguez et al., 2015). Additionally, these studies have

been mainly focused to solid tumor cells. In this work, we showed that PaDef defensin is cytotoxic to K562 chronic myeloid leukemia cells trough extrinsic apoptosis pathway, thereby expanding the activities described for this peptide. Cytotoxicity of PaDef defensin on K562 cells was in a concentration-dependent manner with a IC_{50} of 97.3 µg/ml (18.65 µM) (Fig. 1). Interestingly, the PaDef IC_{50} was lower than that reported for others plant defensins on leukemia cells such as coccinin (30 µM) (Ngai and Ng, 2004) and phaseococcin (40 µM) from *Phaseolus coccineus* (Ngai and Ng, 2005), gymnin (50 µM) from *Gymnocladus chinensis* (Wong and Ng, 2003), but was greater to the effect reported for NaD1 from *Nicotiana alata* (10 µM) (Baxter et al., 2017). In addition, in a previous study we reported that PaDef is cytotoxic to human breast cancer cell line MCF-7 at IC_{50} of 141.62 µg/ml (27.23 µM) (Guzmán-Rodríguez et al., 2016). These results suggest that PaDef defensin is more active to leukemia cells than to cells derived from solid tumors; however, it is necessary to evaluate its effect against a wider number of cell lines from different origins. Also, PaDef lacks toxicity toward human peripheral blood mononuclear cells as reported previously (Guzmán-Rodríguez et al., 2016). These results support the fact that this avocado defensin is an attractive peptide to evaluate its effects on cancer cells.

There are few studies where the cytotoxic mechanisms of plant defensins have been described in detail. Respect to plant defensins their cytotoxicity on leukemia cells involves essentially membrane damage that leads to cell lysis, as reported for plant defensins NaD1 (*N. alata*) and TPP3 (*L. esculentum*) (Baxter et al., 2015; Poon et al. 2014). Remarkably, we demonstrated that PaDef does not affect the membrane integrity of K562 cells, because the calcium intracellular efflux and

membrane potential were not modified (Fig. 2). These data suggest that cytotoxicity of plant defensin PaDef on K562 cells involves other mechanism.

The principal cytotoxic non-membranolytic mechanism described in plant defensins is apoptosis and essentially it has been evaluated in the fungus *Candida albicans*, as reported for the radish defensin RsAFP2, which induces apoptosis by caspase activation, or HsAFP1 defensin, which induces apoptosis in this fungus through ROS production (Aerts et al., 2009; Aerts et al., 2011). Caspase-dependent apoptosis can be activated by the extrinsic pathway (mediated by transmembrane receptors) or by the intrinsic pathway (mitochondria-mediated). Previously, we demonstrated that PaDef activates the intrinsic apoptosis pathway in MCF-7 cells (Guzmán-Rodríguez et al., 2016). Noteworthy, in this work we showed evidences supporting that PaDef cytotoxicity on K562 cells occurs through extrinsic apoptosis pathway due that this defensin favors the expression and activation of the typical initiator caspase 8 (Fig. 5 and 6). These data were supported by the fact that we did not detect modifications in ROS production neither in the mitochondrial membrane potential in K562 cells treated with PaDef. Besides, this peptide did not change the expression nor the activity of caspase 9 (initiating caspase of the intrinsic pathway). To our knowledge, this is the first report indicating that a plant defensin induces extrinsic apoptosis on human leukemia cells.

The apoptosis extrinsic pathway involves transmembrane receptor-mediated interactions, such as the tumor necrosis factor receptor (Locksley et al., 2001). Also, TNF-α can induce apoptosis through the activation of caspase 8 in the RIPK1- FADD- caspase- 8 complex (Tummers and Green, 2017). In this work,

PaDef treatment induced the expression of both TNF-α and TNFR1 genes, which is in agreement with the activation of extrinsic apoptosis in K562 cells. Accordingly, PaDef treatment induced the TNF-α protein secretion ~5 fold (Fig. 7). The data from this study indicate that PaDef defensin induces cell death in K562 cells by the extrinsic apoptosis pathway, which is a novel property for this AMP.

5. Conclusions

PaDef activates apoptosis in K562 cells by extrinsic apoptosis, which could involve the participation of TNF-α. This activity suggests that this defensin could be a potential molecule in the treatment of leukemia.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. PaDef defensin from avocado is cytotoxic to K562 chronic myeloid leukemia cells. (A) Cytotoxicity of PaDef on K562 cells. Viability of cells treated with PaDef defensin (10, 20, 50, 100 and 200 µg/ml) was assessed by MTT assay at 24 h. Data are shown as the percentage of viable cells with respect to cells treated with vehicle (DMSO 0.74%). *Indicates statistically significant differences with respect to vehicle (*P<0.05*). **(B)** The half maximal inhibitory concentration (IC_{50}) of PaDef on K562 cells was calculated by regression analysis; IC_{50} = 97.3 µg/ml; R^2 = 0.9094. **(C)** *In vitro* assessment of PaDef IC_{50} by flow cytometry at 24 h. Representative plots of the different conditions are shown. Act-D (1.5 mM) was used as a positive control. **(D)** K562 cell morphology after PaDef treatment. Representative photographs taken by light field microscopy are shown. Scale bars: 10 µm. **(E)** Effect of PaDef on the viability of human peripheral blood mononuclear cells. Cells were treated with PaDef (100, 200 and 300 µg/ml) and viability was evaluated by MTT (24 h). Data represent the media of three independent experiments performed in triplicate. *Indicates statistically significant differences with respect to vehicle (*P<0.05*).

Fig. 2. PaDef defensin does not cause damage to the cell membrane of K562 cells. Calcium efflux was assessed by flow cytometer using the Calcium Assay Kit (BD Biosciences). The panel shows representative plots **(A)** and relative fluorescence intensities for intracellular calcium release **(B)**. Measurements were performed for 6 min. PMA (3 µM) was used as a positive control. **(C)** The

modification in the membrane potential was assessed using a membrane potential-sensitive dye. Cells were previously incubated with the dye DiSC3(5) (200 μM) for 30 min and then treated with PaDef IC_{50}. DMSO (5%) was used as a positive control. The addition of treatments is indicated by an arrow.

Fig. 3. PaDef defensin induces apoptosis in K562 cells. (A) Cells treated with PaDef IC_{50} (12 and 24 h) were analyzed by flow cytometry using Annexin V/7AAD staining to establish the apoptotic rate. The quadrants indicate viable cells (lower left quadrant), early apoptosis (lower right quadrant), late apoptosis (upper right quadrant) and necrotic cells (upper left quadrant). **(B)** The graphics show the relative fluorescence for each time of treatment (relative units). Each bar shows the mean of triplicates ±SE of three independent experiments. *Indicates statistically significant differences with respect to vehicle (*$P<0.05$*).

Fig. 4. PaDef defensin did not modify the mitochondrial membrane potential (ΔΨm) in K562 cells. Cells were analyzed by flow cytometry using the JC-1 dye after the add the treatments: vehicle (DMSO 0.74%), Act-D, and PaDef IC_{50} for 6, 12 and 24 h.

Fig. 5. PaDef defensin induces the mRNA expression and activity of caspase 8 in K562 cells. (A) mRNA expression of caspase 8 (left graph) and caspase 9 (right graph) was analyzed by RT-qPCR. Cells were treated with PaDef IC_{50} for 6, 12 and 24 h. mRNA expression of control cells (vehicle and Act-D) was analyzed at

24 h. Fold-change values > 2 or < 0.5 were considered significant expressed mRNAs. **(B)** Activity of caspase 8 (left graph) and caspase 9 (right graph) was measured in cells treated with PaDef IC_{50} at 12 and 24 h by luminescence. Each bar shows the mean of triplicates ±SE of three independent experiments. *Indicates statistically significant differences with respect to vehicle (*P<0.05*).

Figure 6. Involvement of caspase 8 in the apoptosis induced by defensin PaDef in K562 cells. Cells were treated 1 h with the caspase 8 specific inhibitor Ac-IETD-CHO (100 μM), and then the cells were treated 24 h with PaDef IC_{50}, and apoptosis was assessed by flow cytometry measuring the relative fluorescence of Annexin V. *Indicates statistically significant differences with respect to PaDef treatment (*P<0.05*).

Figure 7. PaDef induces the TNF-α and TNF receptor 1 (TNFR1) mRNA expression and the TNF-α protein secretion. Cells treated with PaDef IC_{50} for 6, 12 and 24 h were analyzed by qPCR to analyze mRNA expression of TNF-α **(A)** and TNFR1 **(B)**. mRNA expression of control cells (vehicle and Act-D) were analyzed at 24 h. Fold-change values > 2 or < 0.5 were considered significant expressed mRNAs. **(C)** Secretion of TNF-α was analyzed using a cytometric bead array (CBA). Cells were treated with PaDef IC_{50} for 24 h. A total of 350 events were analyzed.

Fig 1

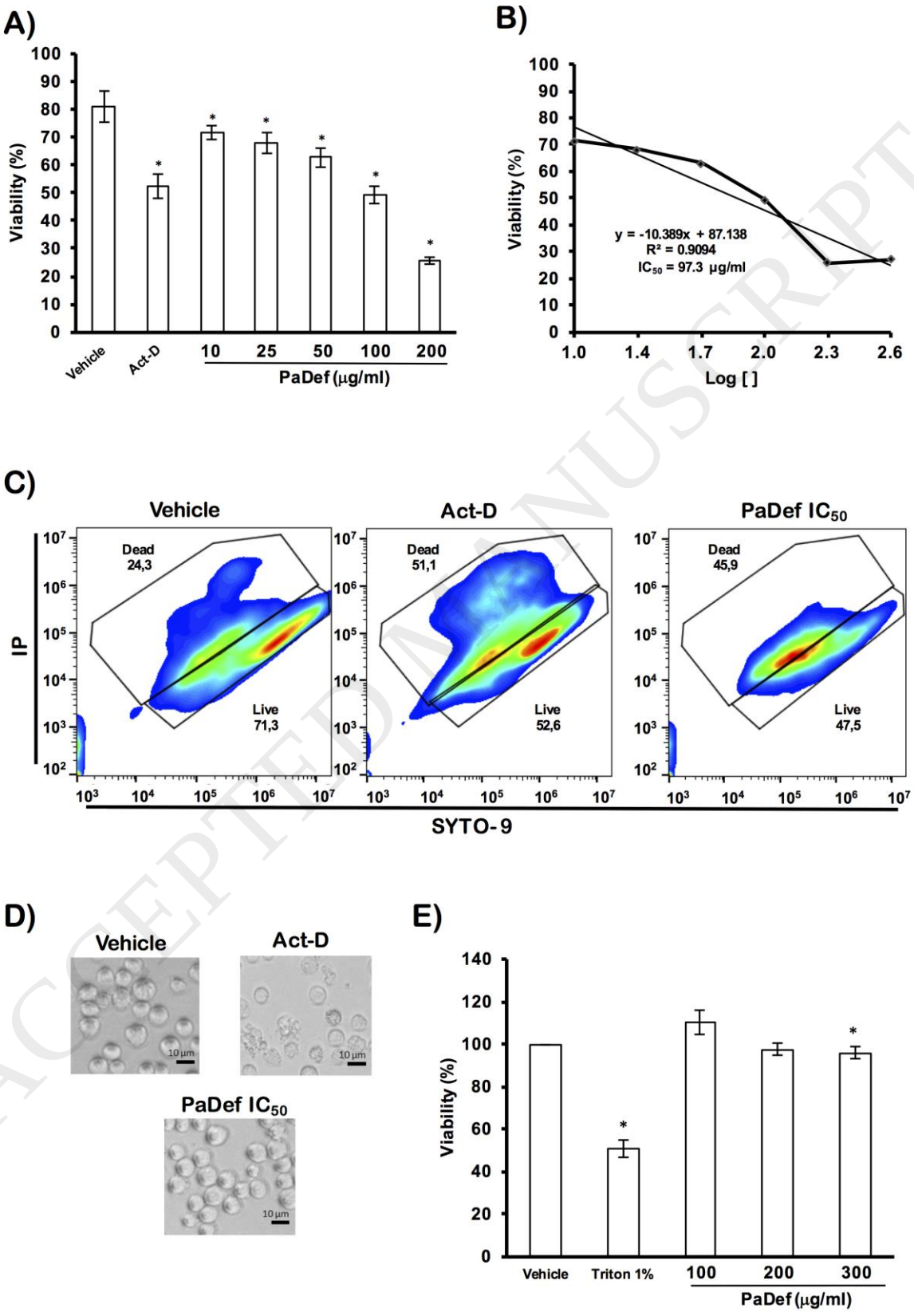

Fig 2

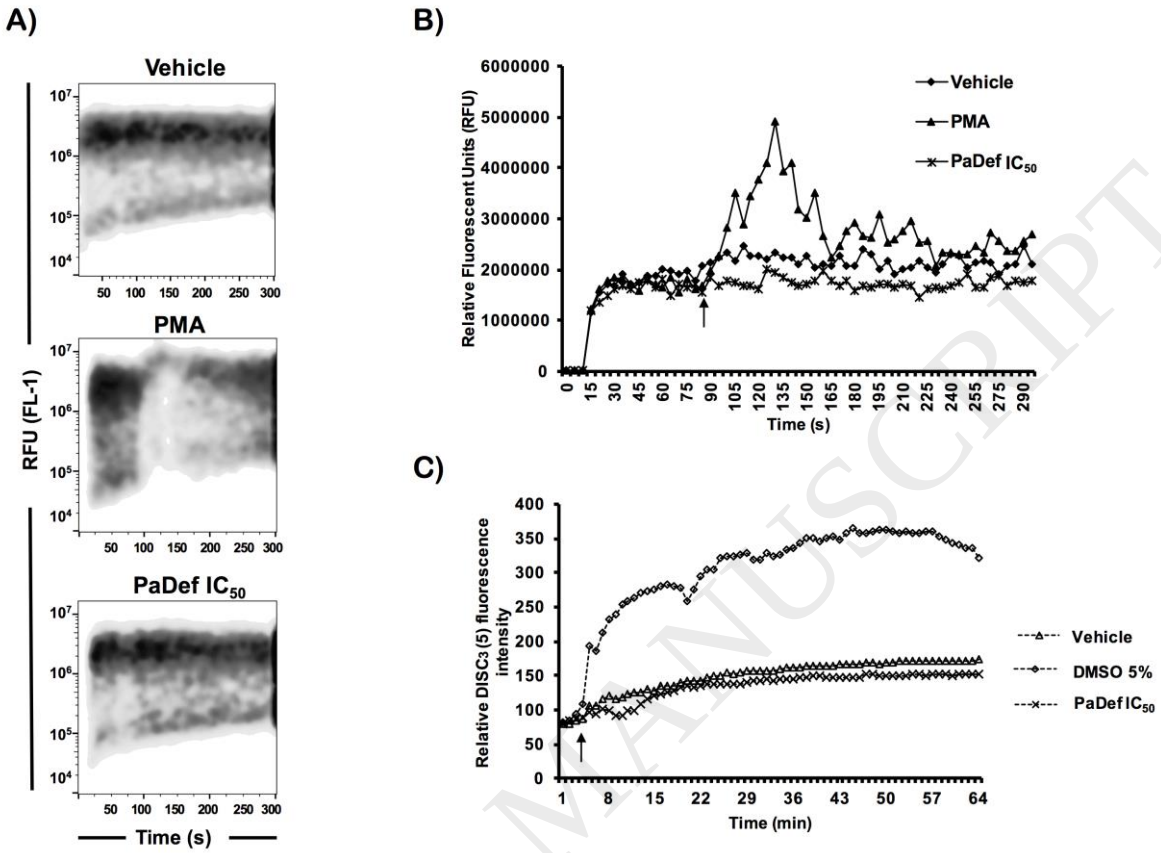

Fig 3

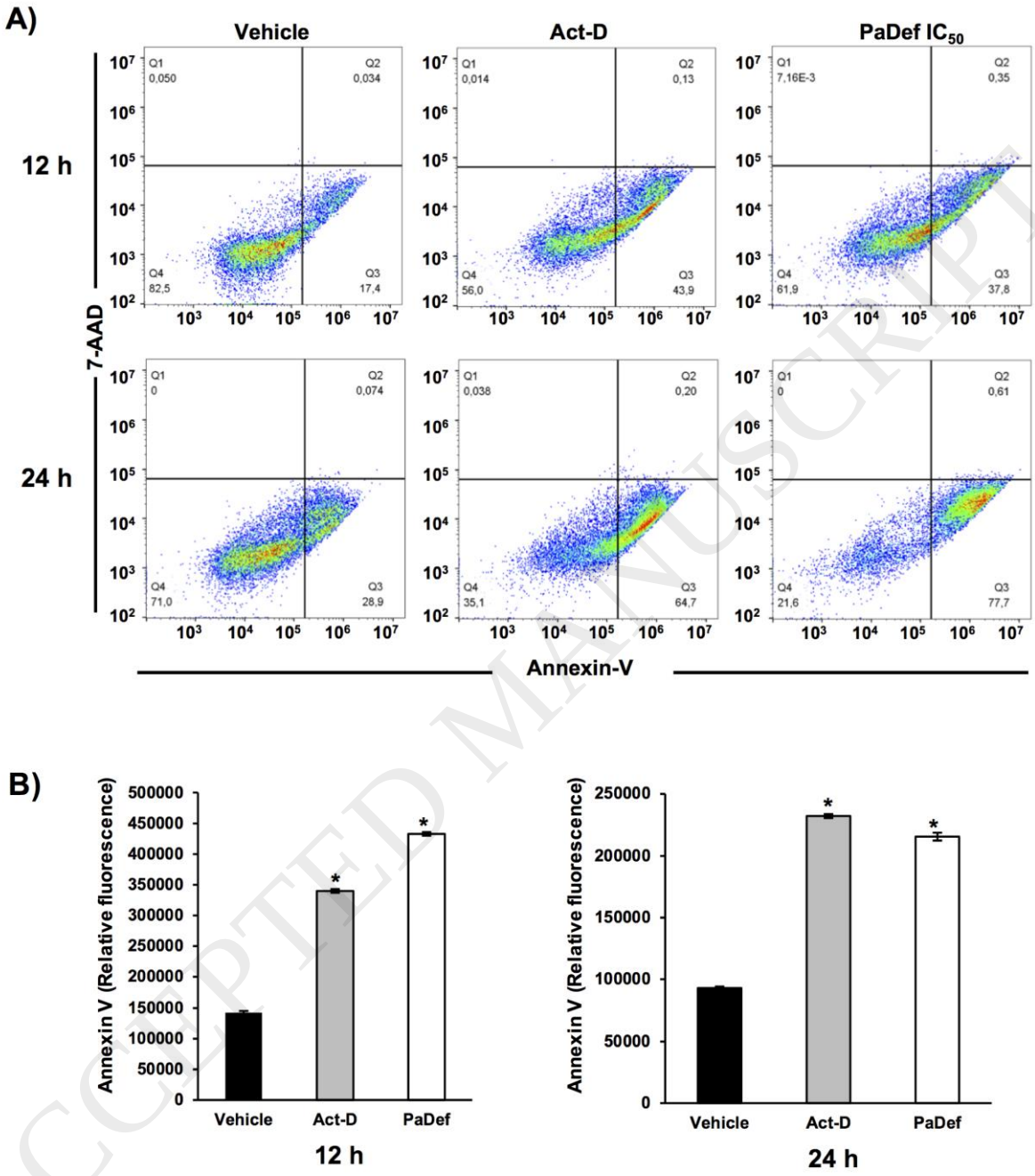

Fig 4

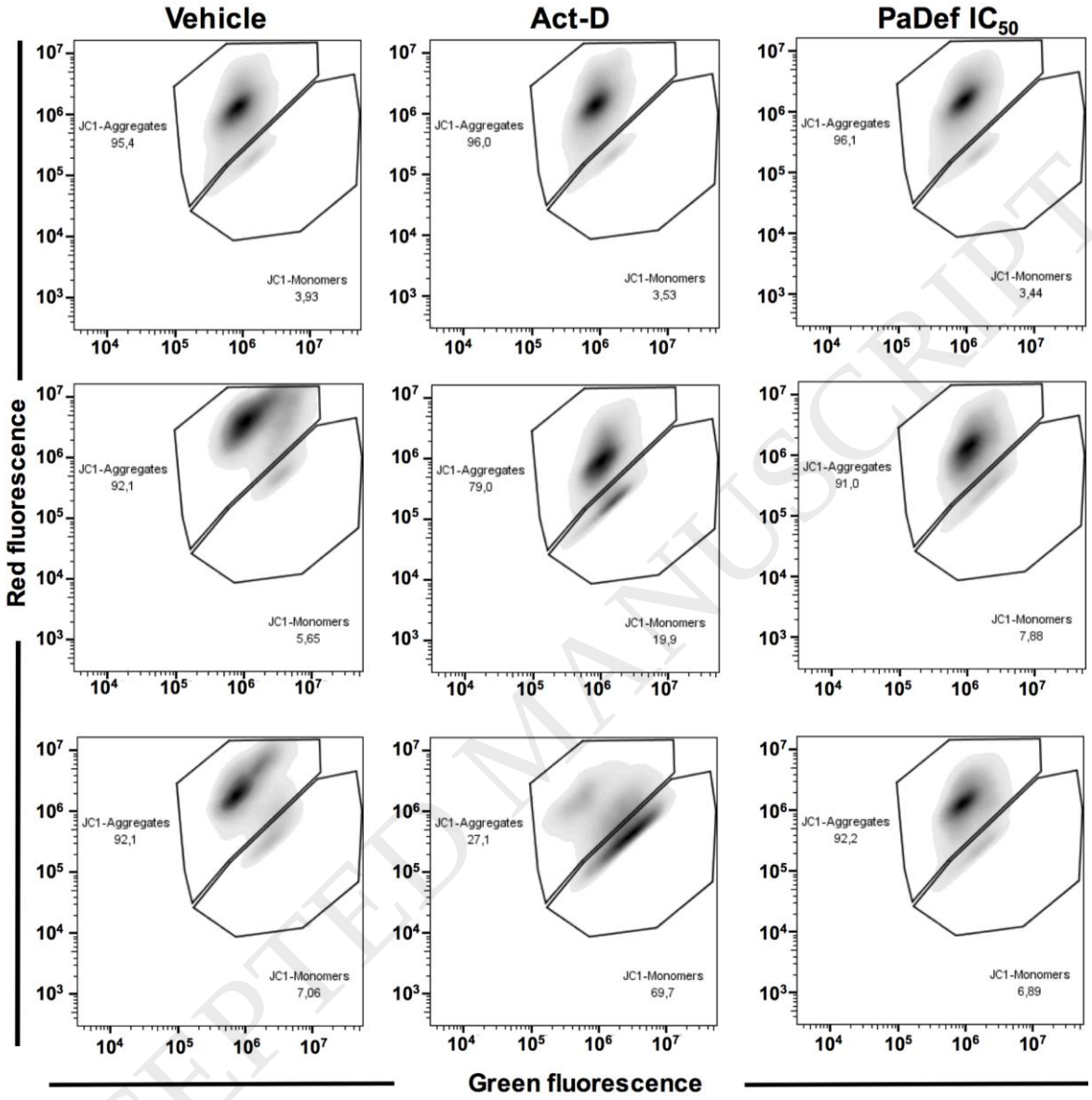

Fig 5

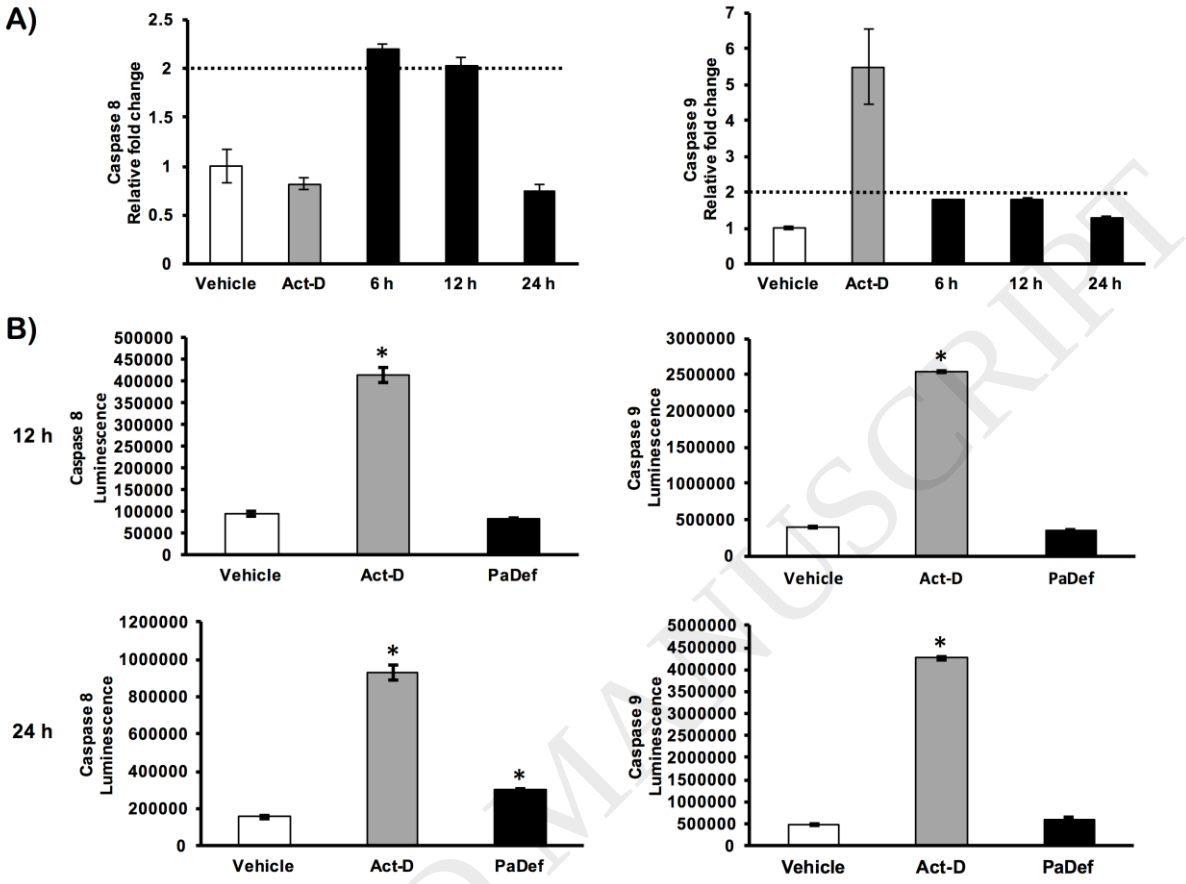

Fig 6

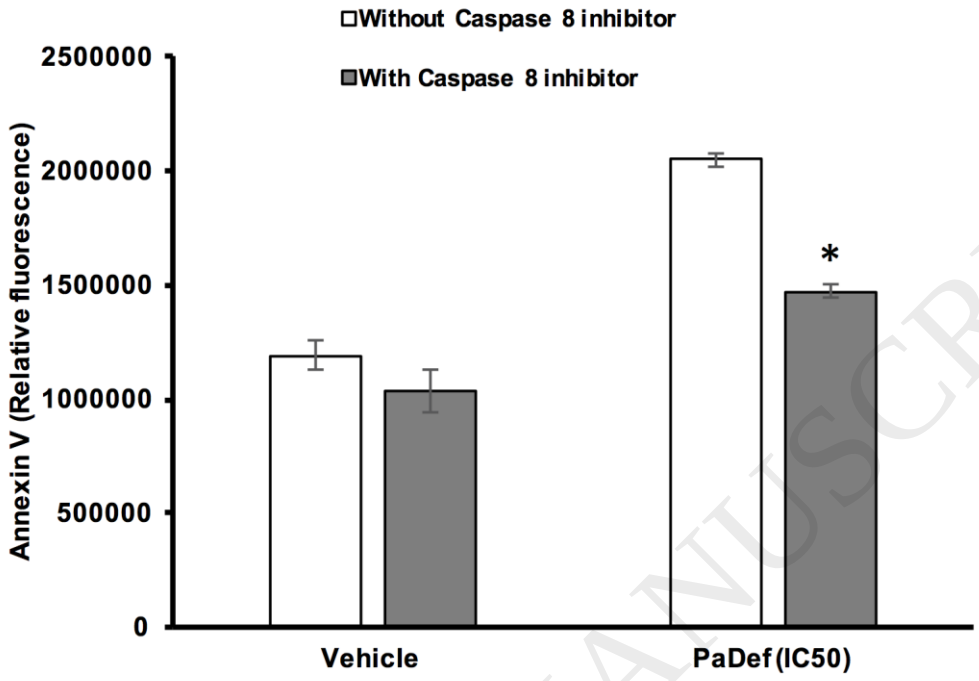

Fig 7

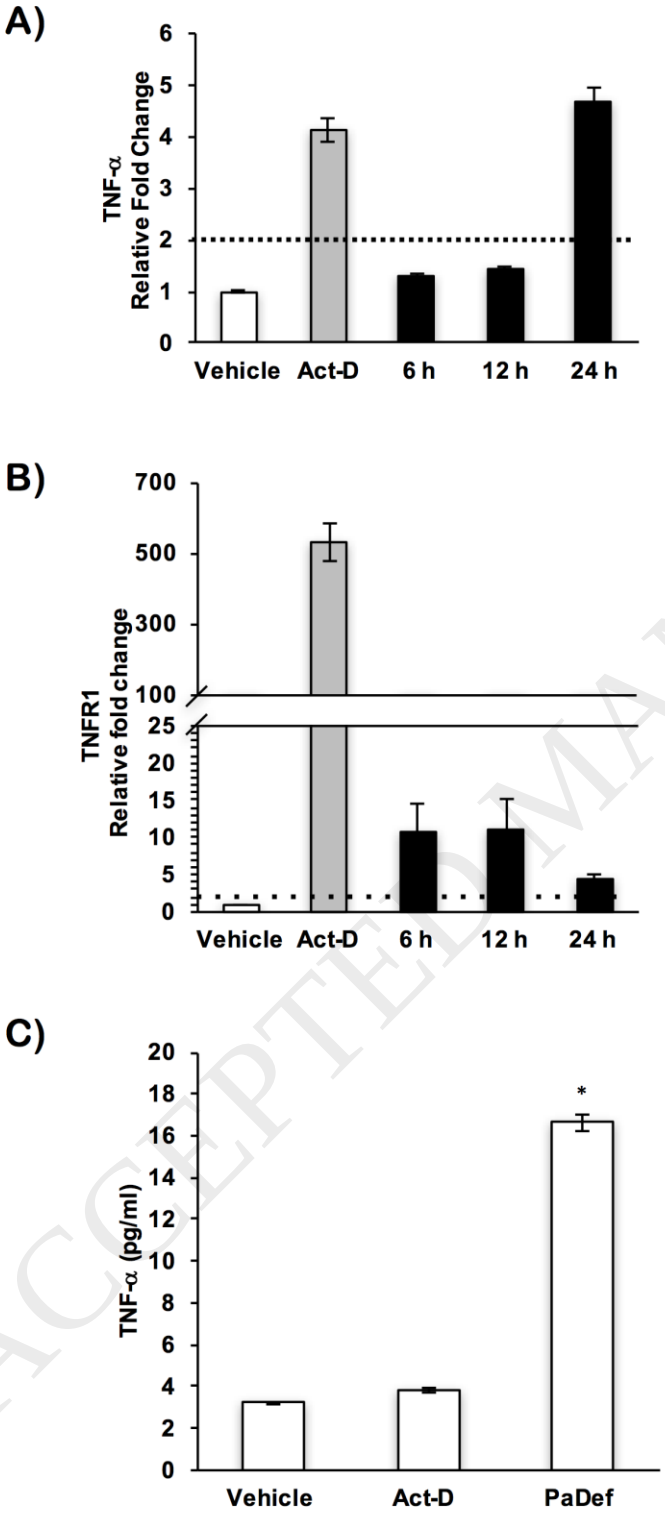

Table 1. Sequence of primers used in this study

Gene	Sequence	Tm(ºC)	Product size (pb)	Reference
TNF-α	5' CCCCTGGAGATAACCTCCCA 3' 5' CAGACGGGAGACAGGAGAGC 3'	55.5	101	Mookherjee et al., 2006
TNFR1	5' CGCTACCAACGGTGGAAGTC 3' 5' CAAGCTCCCCCTCTTTTTCAG 3'			Cubillas et al., 2010
Caspase 8	5`AGATCTGGCCTCCCTCAAGTTCCT 3` 5`AAATTTGAGCCCTGCCTGGTGTCT 3`	66	244	Guzmán-Rodríguez et al., 2016
Caspase 9	5`AGGACATGCTGGCTTCGTTTCTG 3` 5`CCAAATCCTCCAGAACCAATGTCC 3`	66	257	Guzmán-Rodríguez et al., 2016
β-actin	5`AAAACCTAACTTGCGCAGAAAACA 3` 5`TGTCACCTTCACCGTTCCACTTT 3`	57	317	Guzmán-Rodríguez et al., 2016