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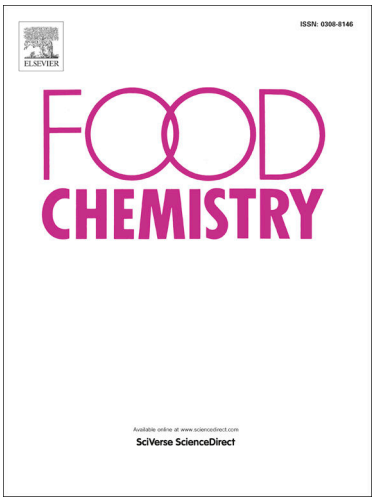

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A comparative study of the antiangiogenic activity of hydroxytyrosyl alkyl ethers

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ABSTRACT

The phenolic compound hydroxytyrosol and its derivatives are responsible for some of the health benefits of the intake of virgin olive oil, having shown antiangiogenic properties. In this study, we explored the antiangiogenic potential of six synthetic hydroxytyrosyl alkyl ethers (HT C1, C2, C4, C6, C8 and C12). Our results showed that all compounds affected endothelial cell viability in vitro at low micromolar doses. In addition, compounds HT C1, C2, C4 and C6 inhibited endothelial cell migration and formation of tubular-like structures. In these assays, hydroxytyrosyl hexyl ether (HT C6) exhibited the most potent inhibitory activity in vitro, activating as well apoptosis in endothelial cells. Furthermore, the antiangiogenic activity of HT C6 was confirmed in vivo in the chick chorioallantoic membrane assay. Hence, we present hydroxytyrosol synthetic derivative HT C6 as a new antiangiogenic compound and as a good candidate for an antiangiogenic drug in the treatment of angiogenesis-dependent diseases.

Keywords: Angiogenesis; bovine aorta endothelial cells (BAEC); chorioallantoic membrane (CAM) assay; hydroxytyrosyl alkyl ethers; apoptosis

1. Introduction

In recent years, scientific evidence of the beneficial effects of compounds present in different foods has suggested their possible use as drugs (Rishton 2008), in addition to their preventive effects with respect to some diseases, particularly cancer. In this context, the concept of "angioprevention" arises, consisting either on the use of foods rich in molecules inhibiting angiogenesis, or of these molecules in the form of drugs, in order to prevent, delay or combat the growth of possible tumors (Albini, Tosetti, Li, Noonan & Li, 2012).

In this sense, Mediterranean diet is one of the most popular dietary patterns, with strong evidence on its health beneficial effects (Dinu et al., 2018). The adherence to Mediterranean diet is related to a decreased incidence of serious illnesses such as cancer and cardiovascular diseases, and it also contributes to greater longevity compared to other dietary patterns (Estruch et al., 2018; Toledo et al., 2015). Mediterranean dietary patterns deliver a high content of desirable nutrients, beneficial to health and low content of undesirable nutrients, being proposed as well as a source of angiopreventive compounds (Martínez-Poveda, Torres-Vargas, Ocaña, García-Caballero, Medina & Quesada, 2019). Some studies suggest that the lower incidence of cancer detected in some areas of the Mediterranean basin compared with populations living in Northern Europe or the US may be related to an abundant consumption of olives and olive oil, which would provide a continuous supply of polyphenols that among other effects could reduce oxidative stress by inhibiting lipid peroxidation (Covas 2007; Owen, et al, 2000). Olive oils contain a complex mixture of different types of compounds, the proportion of which varies depending on the type of olive, ripening, growing conditions, storage, extraction method and degree of refining. Virgin olive oil (VOO) has the highest content of phenolic compounds, which are lost throughout the oil refining process (Ghanbari, Anwar, Alkharfy, Gilani & Saari, 2012).

Although the effects of olive oil on health have traditionally been attributed to its high oleic acid content, a large number of scientific studies have now shown that these effects should also be attributed to certain minority compounds in VOO, especially those included in the phenolic fraction (Scoditti, et al., 2012). Among other multiple biological activities, antiangiogenic properties have been reported for some compounds present in VOO such as hydroxytyrosol, oleuropein, squalene, and some triterpenic

acids such as ursolic acid, among others (Ahmad Farooqi, et al., 2017; Lamy, Ouanouki, Béliveau & Desrosiers, 2014; Cárdenas, Quesada & Medina, 2004).

Hydroxytyrosol, 2-(3,4-dihydroxyphenyl) ethanol, (HT) is a phenolic component present in the olives and is the principal antioxidant compound contributing to the high stability of VOO. In recent years, HT has been shown to have numerous beneficial effects, among others antidiabetic, cardioprotective, neuroprotective and antitumor effects (Reyes, et al., 2017; Catalán, et al., 2016; Zubair, et al., 2017).

Our group characterized for the first time the antiangiogenic activity of HT (Fortes, García-Vilas, Quesada & Medina, 2012; García Vilas, Quesada & Medina, 2017). The effects observed in the *in vitro* and *in vivo* tests were obtained with doses of HT similar to those absorbed after a moderate consumption of VOO, similar to those which would correspond to its daily intake in the Mediterranean diet (Miró-Casas, Covas, Fitó, Farré-Albadalejo, Marrugar & De la Torre, 2003). Other groups have confirmed the effect of the compound on angiogenesis (Lamy, Ouanouki, Béliveau & Desrosiers, 2014) reinforcing the interest in this polyphenol, or its structural derivatives, which could be applied to the therapy of angiogenesis-dependent diseases.

Based on our previous results with HT, we proposed the idea that the structural modification of HT might improve its antiangiogenic activity, giving rise to new drug candidates for the treatment of diseases characterized by a deregulated angiogenesis. As part of a search program for HT derivatives with improved profiles, the antiangiogenic potential of five HT derivatives was recently evaluated by us (López-Jiménez, Gallardo, Espartero, Madrona, Quesada & Medina, 2018). Results showed that introduction of a nitro group in the HT ring was detrimental for its antiangiogenic activity. Conversely, acetylation and, to a greater extent, alkylation of the alcoholic hydroxyl group led to an improvement in this activity, both in *in vitro* and *in vivo* tests. Based on these results, hydroxytyrosyl alkyl ether derivatives were selected for further studies.

Following previous research of our group, in this work we go forward in the identification of new antiangiogenic HT derivatives, exploring the potential antiangiogenic activity of a series of hydroxytyrosyl alkyl ether derivatives with variable length of alkyl chains, previously synthesized by us (Madrona, et al., 2009; Pereira-Caro, et al., 2011). We propose the working hypothesis that the length of the aliphatic chain in these HT derivatives could be determinant in their overall potential antiangiogenic activities. The obtained results have led to select hydroxytyrosyl hexyl

ether (HT C6) as the most active compound, and its antiangiogenic potential has been characterized in more detail.

2. Material and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) 1g/L glucose, penicillin/streptomycin, amphotericin B, L-glutamine and bovine foetal serum (FBS) were supplied by Biowhittaker-Lonza (Walkersville, MD, USA).

The general reagents used for the different assays were obtained from Sigma-Aldrich (Merck; Darmstadt, Germany). Caspase-Glo® 3/7 Assay kit was purchased from Promega Biotech Ibérica (Madrid, Spain) and Matrigel was from Corning (New York, NY, USA).

Rabbit anti-PARP antibody was from Cell Signaling Techonology (Denver, MA, USA) and detects the 116 kDa full length from PARP and the 89 kDa cleaved fragment; rabbit anti-cleaved-lamin A antibody was from Cell Signaling Technology and detects the small cleavage fragment of lamin A (28 kDa). Rabbit anti-GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The HRP-linked goat anti-rabbit IgG antibody was purchased from Sigma-Aldrich (Merck, Darmstadt, Germany).

The hydroxytyrosylalkylether derivatives HT C1, C2, C4, C6, C8 and C12 (Figure 1A) tested in this study were synthesized as previously described (Madrona et al., 2009; Pereira-Caro et al., 2011). The compounds were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 200 mM and stored at -20ºC until use.

2.2. In vitro culture of endothelial cells

Bovine aortic endothelial cells (BAEC) were isolated by the Gospodarowicz method (Gospodarowicz, Moran, Braun & Birdwell, 1976) and cultured in DMEM (1g/L glucose), supplemented with 1% penicillin/streptomycin solution, 0.5% amphotericin B, 2mM L-glutamine and 10% FBS.

2.3. MTT cell survival assay

Cell survival in presence of the different compounds was determined by the MTT dye reduction assay as described in (Rodríguez-Nieto et al., 2002; Martínez-Poveda et al., 2013). Briefly, 2.5 x 10^3 BAEC per well were growth in 96-well plates in presence of serial dilutions (½) of the tested compounds in quadruplicate (starting dose 200 μM). After 72 h incubation, 10 μL of MTT solution (5mg/mL) were added per well, allowing incubation for additional 4h. The resulting formazan crystals were dissolved in 0.04 N 2-propanol-HCl and absorbance in wells was measured at 550 nm with a plate reader spectrophotometer (BioTek Eon). IC_{50}values were calculated as the concentration of the compound that allows 50% of cell survival after 3 days of treatment, considering as 100% the absorbance value of control condition (DMSO). At least three independent replicates were performed of this assay. Concentrations in the range of the IC_{50} value for each HT derivative in BAEC were used as the reference concentration for the different in vitro assays.

2.4. Cell cycle analysis by flow cytometry

BAEC in 6-well plates at 80% confluence were incubated in the presence or absence of the different compounds tested at concentrations in the range of their calculated IC_{50} values, in presence of DMSO (negative control) and 10 μM 2-methoxyestradiol (2-ME, positive control of cell cycle impairment). After overnight incubation, cells were collected, washed with PBS and permeabilized with ice-cold 70% ethanol, during 1 h. Permeabilized BAEC were then incubated with RNAse (100 μg/mL) and propidium iodide (40 μg/mL) for 1h at 37ºC protected from light. The percentages of cells in the G1, S and G2/M phases of the cycle, and the population in sub-G1 (fragmented DNA), were determined using a BD Biosciences FACS VERSETM flow cytometer (Becton Dickinson). Resulting data were analysed with the BD FACSuite program (Becton Dickinson). Three independent replicates were performed of this assay.

2.5. Cell migration assay (wound-healing)

The migratory activity of BAEC was determined by the wound-healing assay as previously described (Cárdenas, Quesada & Medina, 2004). Briefly, confluent BAEC monolayers in 6-well plates were wounded with a pipette tip. After PBS washing, cells were incubated in the presence or absence of the HT derivatives at doses in the range of their calculated IC_{50} values. The wounded area in each condition was photographed at

different time points (time 0 and 7 h) with a Nikon DS-Ri2 camera attached to a Nikon Eclipse Ti microscope. The cell-free area was quantified at 7h of incubation with the software ImageJ (NIH, National Institutes of Health of the United States), normalizing it with respect to the values of the time zero. Three independent replicates were performed of this assay.

2.6. Endothelial tubular-like structures formation on Matrigel

Cell suspensions of 5 x 10^4 BAEC in serum-free DMEM were seeded on 96-well plates coated with 50 µL of Matrigel (10.5 mg/mL) in presence of HT derivatives and minimum inhibitory concentrations (MIC) were determined in each case. Controls including the corresponding DMSO amount (negative control of inhibition) or staurosporine 2 µM (positive control of inhibition) were also included. In the assay, each experimental condition was tested in duplicate. After incubating at 37ºC and 5% CO2 for 5h, photographs were taken using a Nikon DS-Ri2 camera attached to a Nikon Eclipse Ti microscope. Those concentrations in which closed tubular-like structures were not observed were considered positive in terms of complete inhibition of the process. Three independent replicates were performed of this assay.

2.7. Vascular disruption assay on Matrigel

For the vascular disruption assay, BAEC were seeded on Matrigel in the same conditions described for the tubulogenesis assay but in absence of the compounds. When tubular-like structures were formed on Matrigel, HT C6 10 µM was added to wells, and DMSO (vehicle) or Combretastatin A-4 phosphate (CA4P) at 0.2 µM were used as negative and positive control of vascular disruption, respectively (Nagaiah & Remick, 2010). , After 2h of incubation at 37ºC and 5% CO_2, photographs were taken using a Nikon DS-Ri2 camera attached to a Nikon Eclipse Ti microscope and the presence of intact or disrupted tubular-like structures was analysed in each condition.

2.8. Nuclei staining with Hoechst dye 33342

Fluorescent dye Hoechst 33342 staining was used to assess the presence of condensed chromatin in cell nuclei, as previously described (Martínez-Poveda B. et al., 2007). Briefly, BAEC were cultured on gelatine-coated coverslips until reach 80 % of confluence, and then cells were incubated in presence of HT C6 at different

concentrations (1, 5 or 10 μM). DMSO was used as negative control (vehicle) and 10 μM 2-methoxyestradiol was used as a positive control of apoptosis induction in this assay. After overnight incubation, cells were fixed in 10% formalin solution and stained with Hoechst 33342 dye (1 mg/mL). Stained nuclei were photographed under fluorescence microscope (Leica, TCS-NT, Heidelberg) and nuclei with were quantified. Results were expressed as percentage of nuclei with condensed chromatin (overstained nuclei) relative to total nuclei present in the picture. Three independent replicates were performed of this assay.

2.9. Measurement of caspases 3 and 7 activity

A total amount of 1.3 x 10^4 BAEC were seeded per well in 96-well luminometry plates and incubated overnight in presence or absence of different concentrations of HT C6 (1, 5 or 10 μM). DMSO was used as negative control (vehicle) and 10 μM 2-methoxyestradiol was used as a positive control of caspase 3 and 7 activity inductor in this assay. In the assay, each experimental condition was tested in duplicate or in triplicate. Then, the Caspase-Glo® 3/7 reagent was added to the wells according to the manufacturer's instructions, and luminescence was detected after 30 minutes with a GLOMAX 96 microplate luminometer (Promega Biotech Ibérica, Madrid, Spain). Three independent replicates were performed of this assay.

2.10. Analysis of protein expression by Western blot

BAEC were grown until reach 70-80% of confluence, and cells were then incubated overnight in the presence of different concentrations of HT C6 (1, 5 or 10 μM). DMSO was used as negative control (vehicle) and 10 μM 2-methoxiestradiol was used as positive control. Proteins were extracted in Laemmli buffer (0.125 M Tris-HCl pH 6.8, 20 % glycerol, 4% SDS, 0.004% bromophenol blue, 10% 2-mercaptoethanol) and 30 μg of total protein were subjected to SDS-PAGE denaturing electrophoresis. After electrophoresis, gels were electrotransferred to a nitrocellulose membrane. Membranes were blocked in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20) containing 5% semi-skimmed milk and then incubated overnight with anti-PARP1 or anti-cleaved lamin A antibodies diluted 1:500 in TBS-T with 5% BSA. After incubation with the anti-rabbit secondary antibody diluted 1:5000 in blocking buffer, signal was detected using the SuperSignal West Picochemiluminescence system (Pierce, IL, USA)

and an imaging system Chemidoc XRS (Bio-Rad, Hercules, CA, USA). The same membranes were incubated with anti-GAPDH antibody at a dilution of 1:1000. Blots were quantified by densitometry with the software ImageJ. The experiments were performed in duplicate or triplicate.

2.11. In vivo chicken chorioallantoic membrane (CAM) assay

Chick fertilized eggs were purchased from Granja Santa Isabel (Córdoba, Spain). Eggs were incubated at 38ºC in a humidified incubator with tilting tray (Mesalles 25 L-HS, Barcelona, Spain) and windowed after 3 days. At day 8 methylcellulose discs containing different amounts of HT C6 were implanted onto the CAM and sealed eggs were then incubated for additional 48 h. For the negative control condition, DMSO (vehicle) was added to the discs, and the compound aeroplysinin-1 (5 nmol/disc) was used as positive control of angiogenesis inhibitor (Rodríguez-Nieto et al., 2002). After incubation, CAM were observed under a scope (Leica) and photographs were taken with a Nikon DS-Ri2 camera. The CAM was scored positive for angiogenesis inhibition when abnormalities in the development of the vasculature were detected, such as reduced density of vessels in the area under the disc, disorganization of the vasculature and/or centrifugal growth of the vessels in the periphery of the disc. Different conditions were assayed in at least 5 CAM. Results were blind analysed by two independent observers.

2.12. Statistical analysis

The results are shown as the mean value of at least three independent replicates and their corresponding standard deviation (SD) values. Statistical significance was determined by one-way ANOVA (Dunnett's multiple comparisons test); values of $P < 0.05$ were considered statistically significant. Significance was indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Statistical analysis of the data was performed using Prism-GraphPad software.

3. Results

3.1. Hydroxytyrosyl alkyl ethers inhibit endothelial cell growth in vitro

During the formation of a new vessel by the angiogenic process, quiescent endothelial cells are activated in response to angiogenic signals and they start to proliferate. In order to determine a possible inhibitory effect on endothelial cell growth in vitro, we performed MTT assay to obtain dose-response curves in BAEC of the different compounds assayed. As shown in Figure 1B, all the tested hydroxytyrosyl alkyl ethers inhibited BAEC growth in a dose-dependent manner. IC_{50} values calculated from the survival curves are shown in Figure 1C. Interestingly, IC_{50} values decreased with the length of the ether chain in the HT C1 to C12 compounds. However, we observed problems of solubility for HT C8 and HT C12 compounds, suggesting that the actual IC_{50} values could be even lower than those estimated by us. For this reason, we discarded HT C8 and HT C12 from the study and only used HT C1 to C6 in the following experiments.

3.2. Effects of hydroxytyrosyl alkyl ethers on endothelial cell cycle

In order to study the effect of the compounds on the cell cycle of endothelial cells, BAEC were incubated overnight with the different HT derivatives at doses that were in the range of their calculated IC_{50} values (50, 30, 16, 10 μM for HT C1, C2, C4 and C6 respectively) and the distribution of cells in the different phases of the cycle was determined by flow cytometry (Figure 1D). According to the histograms, only the treatment with HT C6 seemed to exert a significant effect on the cell cycle of BAEC (Figure 1D), as compared to negative control (DMSO). Quantification of the percentage of BAEC population in each phase of the cycle revealed that HT C6 significantly increased Sub-G1 population, with a concomitant decrease of cells in G0/G1 and S/G2/M phases (Figure 1E). This suggests a possible induction of apoptosis in BAEC in response to the compound that deserves to be further explored.

3.3. Hydroxytyrosyl alkyl ethers inhibit endothelial cell migration

For the formation of new vessels, activated endothelial cells must migrate to the angiogenic stimulus, being cell migration an essential step of the angiogenic process. In order to evaluate the effects of the compounds on the migratory capacity of the cells *in vitro* we performed wound-healing assays in presence of FBS. As shown in Figure 2A, compounds HT C1 to C6 tested at doses in the range of their calculated IC_{50} values (50, 30, 16, 10 μM for HT C1, C2, C4 and C6 respectively) were able to reduce the

migration potential of BAEC after 7 h, as compared to control (DMSO). Once again, the most evident effect was observed for HT C6. For this reason, we carried out new wound assays testing different concentrations of HT C6. Figure 2B shows that HT C6 significantly inhibits BAEC migration in a dose-response manner.

3.4. Hydroxytyrosyl alkyl ethers inhibit the formation of endothelial tubular-like structures on Matrigel

Endothelial cells, when deposited on Matrigel, align spontaneously forming tubular structures that resemble capillaries. This "tubulogenesis" assay on Matrigel is one of the most widely used for the study of angiogenesis *in vitro*, and is accepted as a model for the study of the later stages of angiogenesis, in which the monolayer of endothelial cells leads to the formation of a new capillary (Thaloor, Singh, Sindhu, Prasad, Kleinman & Maheshwari, 1998). In order to check if HT derivatives were able to interfere in this process, we tested them in this assay at concentrations in the range of their IC_{50} values, obtaining a 100% inhibition of the tubular-like structures formation in all the cases (data not shown). We stablished then the minimum inhibitory concentration (MIC) for each derivative, showed in Figure 3A. From here on, we decided to focus our attention on a deeper characterization of the hydroxytyrosyl hexyl ether HT C6, the tested compound that showed inhibitory effects at lower doses in the performed assays.

3.5. Hydroxytyrosyl hexyl ether (HT C6) does not disrupt already formed endothelial tubular-like structures on Matrigel

In view of the inhibitory effect showed by HT C6 in the formation of endothelial tubular-like structures, we investigated the possibility of a vascular disruptive effect of this compound on already formed structures. Figure 3B shows that the compound HT C6 did not produce any effect on the existing structures, (even at a dose higher than that needed to completely inhibits the formation of those tube-like structures) compared with the strong effect observed in the positive control (CA4P, a well-known vascular disruptor compound (Nagaiah&Remick, 2010)).

3.6. Hydroxytyrosyl hexyl ether (HT C6) induces endothelial cell apoptosis

The significant increase of the Sub-G1 population of BAEC treated with HT C6 shown in Figure 1E suggested that this compound could induce apoptosis in BAEC. To

confirm the apoptogenic activity of HT C6 compound in these cells, nuclei were stained with Hoechst dye 33342. In cells treated with 2-methoxyestradiol (positive control), nuclear shrinkage (an apoptosis-related morphological change) was induced (Figure 4A). The same effect in nuclei of endothelial cells was observed in the presence of 10 μM HT C6 derivative, supporting the pro-apoptotic effect of this compound (Figure 4A and 4B).

To further characterize the pro-apoptotic effect of HT C6 on BAEC, we analysed if the compound was able to induce the activation of effector caspases 3 and 7. Interestingly, the treatment of endothelial cells with 5 and 10 μM HT C6 strongly increased the activity of these caspases, even more than the positive control (2-methoxyestradiol) did (Figure 5A). This effect was exerted in a dose-dependent manner, and confirmed the pro-apoptotic activity of HT C6 in endothelial cells

As a consequence of the activation of effector caspases during apoptosis, several target proteins are cleaved in the cells, as is the case of poly (ADP-ribose) polimerase-1, PARP-1 (Scovassi & Diedrich, 2004; Tewari et al., 1995) and the nuclear membrane protein lamin-A (Takahashi, Musy, Martins, Poirier, Moyer & Earnshaw, 1996). This is in agreement with the results preented in Figures 5B and 5C, showing that the treatment of endothelial cells with HT C6 induced the cleavage of PARP1 and lamin-A.

3.7. Hydroxytyrosyl hexyl ether (HT C6) inhibits in vivo angiogenesis

One of the most widely used models for the *in vivo* study of angiogenesis is the chorioallantoic membrane (CAM) of the chick embryo. This membrane is formed during the embryonic development of the chicken and is heavily irrigated by vessels formed by activation of the angiogenic process. As shown in Figure 6A, negative control condition (DMSO) allowed the normal neovascularization of the CAM, giving rise to a perfectly oriented and dense network formed by vessels of different sizes. On the other hand, the presence of aeroplysinin-1 in the CAM (positive control) interfered with the vascularization of the membrane, affecting the growth of low-calibre vessels in the area of the disc. When compound HT C6 was placed on the CAM, the vascularization of the membrane was impaired, causing effects that included the presence of rebounds in the peripheral vessels to the methylcellulose disc containing the compound, haemorrhages and/or a lesser vascularization under the disc and in its surroundings. Figure 6B shows the percentage of CAMs in which an inhibition of

angiogenesis was observed, depending on the different amounts of HT C6 included in the methylcellulose disc. An amount of 5 nmol/disc of HT C6 compound produced an inhibitory effect in more than 50% of the individuals, reaching values higher than 80% for 20 nmol/disc.

4. Discussion

Hydroxytyrosol (HT) exhibits a series of activities on targets related to different pathologies. This may account for some of the beneficial effects described for VOO, in addition to explaining the pharmacological interest aroused by this compound, both in the field of therapy and in the prevention of some diseases. Our group was the first to describe the antiangiogenic activity of HT by means of *in vitro* and *in vivo* studies, thus extending the field of application of the compound to the treatment of diseases characterized by a deregulated angiogenesis (Fortes, García-Vilas, Quesada & Medina, 2012; García Vilas, Quesada & Medina, 2017). The therapeutic potential of HT aroused interest in the synthesis of more active or less toxic HT derivatives. Thus, HT derivatives with better neuroprotective (González Correa, Navas, López-Villodres, Trujillo, Espartero & De la Cruz, 2008), anti-inflammatory (Tabernero, et al., 2014; Maloney, et al., 2013) and antitumor (Zubair, et al., 2017) activities have been described, among others. Recently, our group showed that the chemical modification of HT, in particular through acetylation or etherification reactions, can improve its antiangiogenic activity (López-Jiménez, Gallardo, Espartero, Madrona, Quesada & Medina, 2018). In the present work, we wanted to test the hypothesis that the length of the aliphatic chain in hydroxytyrosyl alkyl ethers could be involved in their overall antiangiogenic potential. Our results indicate that this is the case, with an increasing antiangiogenic potential as the lateral chain length increases from 1 to 6 carbons. The results obtained with the compound HT C6 in the tests carried out on endothelial cells *in vitro*, indicate that this HT derivative inhibits several key stages of the angiogenic process and induces endothelial cell apoptosis.

Endothelial cells, normally quiescent in adults, proliferate in response to the "angiogenic switch" connection. In principle, a compound capable of inhibiting the growth of these cells or inducing their death could be considered a potential antiangiogenic drug. Herein, we show that hydroxytyrosyl alkyl ethers inhibit the growth of BAEC at concentrations that are much lower than those previously published

for HT and some of its derivatives (García-Vilas, Quesada & Medina, 2017; López-Jiménez, Gallardo, Espartero, Madrona, Quesada & Medina, 2018). Furthermore, the estimated IC_{50} values decreased with the increasing length of the alkyl chain, thus reinforcing our hypothesis. These IC_{50} values were much lower than those previously reported for these hydroxytyrosyl alkyl ethers on the in vitro growth of human lung fibroblasts (MRC5) and A549 lung cancer cells (Calderón-Montaño, et al., 2013). This observation suggests a certain preference for the growth-inhibiting effect on activated endothelial cells responsible for the generation of a new vessel by the angiogenesis process. However, some problems with the solubility of HT C8 and HT C12 compounds under the conditions required for using them in the rest of the assays led us to discontinue the study of these two compounds.

In order to investigate the mechanisms of inhibition of endothelial growth by the action of hydroxytyrosyl alkyl ethers HT C1 to C6, studies of their effect on the cell cycle of BAEC were carried out. The results obtained show that only in the case of cells treated with HT C6 there is an increase in the subpopulation in subG1 phase, suggesting an induction of apoptosis by the action of the compound.

In the process of new vessel formation, endothelial cells must migrate to the angiogenic stimulus and finally differentiate to give rise to the new vessel. With the wound-healing assay, we have been able to verify that all tested hydroxytyrosyl alkyl ethers are able to inhibit endothelial cell migration. Once again, the most potent effect is observed with HT C6, which inhibits the *in vitro* mobility of BAEC at concentrations much lower than those needed by HT itself and other derivatives to achieve the same effects (Fortes, García-Vilas, Quesada & Medina, 2012; López-Jiménez, Gallardo, Espartero, Madrona, Quesada & Medina, 2018).

The formation of tubular-like structures on Matrigel by endothelial cells is considered a model for the study of the final phase of angiogenesis. Our data show that the four tested hydroxytyrosyl alkyl ethers (HT C1, C2, C4 and C6) at a half of their respective IC_{50} values were able to inhibit completely the formation of endothelial tubular-like structures on Matrigel. Since in all the previous assays, the effects produced by HT C6 were observed at lower concentrations than those required by HT C1, C2 or C4, we decided to proceed further with the characterization of the targets and mechanisms of the antiangiogenic effects of HT C6. Our results show that this compound is not capable of disrupting the tubular structures previously formed by BAEC at concentrations that

were twice as high as those necessary to completely inhibit the tube formation process. This suggests a preferential effect on activated endothelial cells, and not on those that are quiescent, in the already formed vessel, ruling out another possible activity for HT C6 as a vascular disruptor.

The potential pro-apoptotic effect of HT C6 on BAEC suggested by the cell cycle experiments was confirmed by the detection, in the cells treated with the compound, of hyperpigmented nuclei, characteristic of apoptotic cells, in which condensation has occurred in nuclear chromatin. These effects match with those described for HT and some of its derivatives on these same cells, although in the case of HT C6 they are exerted at concentrations that are up to two orders of magnitude lower than those required by them, revealing a greater potency of this compound to induce apoptosis of endothelial cells (Fortes, García-Vilas, Quesada & Medina, 2012; López-Jiménez, Gallardo, Espartero, Madrona, Quesada & Medina, 2018).

Apoptosis can be activated or inhibited by physiological or pathological stimuli, being involved in the development of some diseases such as Parkinson's disease or cancer. Regarding pathological angiogenesis, the induction of apoptosis in endothelial cells has been related to the mechanism of action of some antiangiogenic compounds, such as dimethyl fumarate, aeroplysinin-1, toluquinol, AD0157, or damnacanthal, among others (García Caballero, Marí-Beffa, Medina & Quesada, 2011, Martínez Poveda, Rodríguez-Nieto, García-Caballero, Medina & Quesada, 2012, García Caballero, Marí-Beffa, Cañedo, Medina & Quesada, 2013; García Caballero, Cañedo, Fernández-Medarde, Medina & Quesada, 2014; García-Vilas, Pino-Ángeles, Martínez-Poveda, Quesada & Medina, 2017), so we explored this way of action for HT C6. The results obtained show that HT C6 produces an activation of effector caspases 3 and 7 in endothelial cells, which reinforces the hypothesis that the inhibition of endothelial growth by HT C6 may be due, at least in part, to the activation of apoptosis in these cells. It has also been proven that the incubation of endothelial cells with HT C6 causes an increase in the cleaved forms of two substrates characteristic of apoptosis such as lamin-A, essential for the integrity of the nuclear envelope, and PARP-1, a nuclear enzyme involved in DNA repair, which reinforces the reasoning described above.

Altogether, the *in vitro* results presented in this work confirm that hydroxytyrosyl alkyl ethers (HT C1 to C6) produce antiangiogenic effects that are stronger as the length of the alkyl chain increases. These antiangiogenic properties affected endothelial cell

viability, migration and formation of tubular-like structures. Furthermore, our results demonstrate that HT C6 is a new angiogenesis inhibitor, which acts on several key steps of the process at concentrations much lower than those of the natural HT and other derivatives previously studied, leading to the apoptosis of endothelial cells.

In addition to the in vitro evidences of the inhibitory activity of HT C6 in key steps of angiogenesis, the compound exhibits a potent antiangiogenic effect *in vivo* in the CAM assay, confirming that the structural modification of HT, to give rise to the compound HT C6, results into a notable increase in its antiangiogenic activity.

In conclusion, in this work we explored a series of HT derivatives (hydroxytyrosyl alkyl ethers) as potential antiangiogenic compounds, showing that all of the tested derivatives exhibited inhibitory effect in endothelial cell survival, with an interesting dependence of the length of the alkyl chain. Although some of the studied derivatives showed inhibitory effects in angiogenesis-related processes *in vitro*, our data points to HT C6 as the most potent compound in the series, inducing apoptosis in endothelial cells, and inhibiting physiological angiogenesis *in vivo*. Our results open the door for a more in-depth characterization of the mechanism of action of HT C6 in angiogenesis inhibition, mainly focused in the study of cell survival and proliferation pathways underlying the observed induction of apoptosis in endothelial cells. The presence of the aliphatic chain in the HT derivatives increases the hydrophobicity of these molecules compared to HT, and this chemical characteristic could be essential in the interaction with intracellular targets that finally leads to apoptosis induction in endothelial cells by HT C6.

Another question that deserves to be further investigated in the future is the antioxidant potential of these compounds, since all the hydroxytyrosyl alkyl ethers used in this work maintain the ortho-diphenolic group intact and thus, a higher antioxidant activity of these compounds than the HT itself could be expected. Interestingly, these HT derivatives have been described as potent antioxidant molecules in the context of platelet activation in vivo (Muñoz-Marín, De la Cruz, Reyes, et al., 2013), and the contribution of this antioxidant activity to the mechanism of action of HT C6 in angiogenesis inhibition cannot be discarded.

Finally, the antiangiogenic potential of the HT derivatives with longer alkyl chains of the series (HT C8 and C12) is an interesting issue that remains to be evaluated, in order to determine if the working hypothesis is accepted as well for these compounds. This work reinforces the idea that the molecular structure of HT is an interesting starting

point for the rational design of new synthetic molecules exhibiting more potent antiangiogenic activity than HT, focussing the attention in the hydroxytyrosyl alkyl ethers derivatives as candidate drugs for the treatment of angiogenesis-dependent diseases.

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Figure legends

Figure 1. *Hydroxytyrosyl alkyl ethers reduce endothelial cell survival in vitro showing differential effects on cell cycle in these cells*. (A) Molecular structure of hydroxytyrosol and the six derivative compounds used in this work. (B) Representative survival curves of endothelial cells (BAEC) after 72 hours in presence of increasing doses of the compounds, obtained by using the MTT method. Tested compounds: HT C1 (-●-), HT C2 (-■-), HT C4 (-▲-), HT C6 (-▼-), HT C8 (-◆-), HT C12 (-○-). Data in plots are mean values for four replicates. Error bars were omitted from plots for a better visualization of the results in the graph. (C) IC_{50} values calculated in BAEC for each HT derivative. Data are given as means±S.D. for three independent MTT experiments, each with 4 replicates for each tested concentration. (D) Representative histograms of cell cycle in BAEC in presence of DMSO (negative control), 2-methoxyestradiol (positive control) and the compounds HT C1, C2, C4 and C6 at their corresponding IC50 values, measured by flow cytometry after 24 hours of treatment and propidium iodide staining. (E) Quantitative analysis of cell populations in the different phases of cell cycle and in SubG1 for each experimental condition. Data are expressed as means ± SD for three independent experiments (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Figure 2. *Hydroxytyrosyl alkyl ethers decrease endothelial cell migration.* (A) Representative pictures of wound-healing assay performed in BAEC in presence of DMSO (control condition), HT C1, HT C2, HT C4 or HT C6 at their respective IC50 concentrations, 7 hours after the treatments. Discontinued lines represent the cell-free area at time 0 in each experimental condition and migration fronts are pointed by the continuous lines. (B) Quantification of the recovered area at 7 hours in wound healing-assay performed in presence of DMSO (control condition) or three concentrations of HT C6. Data are shown as percentages of recovered area at time 7 h, expressed as the mean ± SD for three independent experiments (**$p < 0.01$; ***$p < 0.001$).

Figure 3. *Hydroxytyrosyl alkyl ethers inhibit the formation of endothelial tubular-like structures on Matrigel.* (A) Representative pictures of endothelial tubular-like structures on Matrigel in presence of DMSO (negative control of inhibition), 2 μM staurosporine (positive control of inhibition) or HT C1, HT C2, HT C4, HT C6 at doses corresponding

to their MIC. (B) Representative pictures of vascular disruption assay of structures formed by endothelial cells on Matrigel exposed to DMSO (negative control of vascular disruption), 0.2 μM CA4P (positive control of vascular disruption) and HT C6 at its IC50 concentration.

Figure 4. *HT C6 induces chromatin condensation in endothelial cells.* (A) Representative pictures of BAEC in presence of DMSO (negative control of apoptosis), 10 μM 2-ME (positive control of apoptosis) and three doses of HT C6, after 16 hours of treatment. Cell nuclei were stained with Hoechst 33342 dye. (B) Quantification of cell nuclei with condensed chromatin in the different conditions. Data are shown as mean ± SD for three independent experiments (*$p < 0.05$; ***$p < 0.001$).

Figure 5. *HT C6 promotes the activation of caspases 3 and 7, and cleavage of PARP-1 and lamin-A, in endothelial cells.* (A) Caspases 3 and 7 activity assay, measured by specific luminescent substrate in BAEC incubated 16 hours in presence of DMSO (negative control of apoptosis), 10 μM 2-ME (positive control of apoptosis) and three doses of HT C6. Data are shown as mean ± SD for three replicates (***$p < 0.001$; ****$p < 0.0001$); similar results were obtained in three independent experiments. (B) Representative Western-blots of cleaved PARP-1 and cleaved lamin-A in cell extracts of BAEC incubated for 16 hours in presence of DMSO (negative control), 10 μM 2-ME (positive control) and three doses of HT C6. GAPDH was used as internal loading control. Similar results were obtained in three independent experiments (Lamin-A) and two independent experiments (PARP-1). (C) Densitometric quantification of the blots of lamin-A (data from three independent experiments, left, expressed as mean ± SD for three replicates; **$p < 0.01$) and PARP-1 (data from one representative experiment, right).

Figure 6. *HT C6 shows antiangiogenic activity in vivo.* (A) Representative pictures of chorioallantoic membrane (CAM) assay testing HT C6 at different doses. DMSO was used as negative control and 5 nmol/CAM aeroplysinin-1 was used as positive control of angiogenesis inhibition. Dotted circles show the location of methylcellulose discs in the CAM. (B) Number of positive CAM (with impaired vasculature development) over

total CAM number, and percentage of angiogenesis inhibition corresponding to each dose of HT C6.

HIGHLIGHTS

- A series of hydroxytyrosyl alkyl ethers show antiangiogenic potential in vitro.
- Studied compounds inhibit endothelial cell growth, migration and tube formation.
- Hydroxytyrosyl hexyl ether is a potent antiangiogenic compound in vitro and in vivo.
- Hydroxytyrosyl hexyl ether induces apoptosis in endothelial cells.

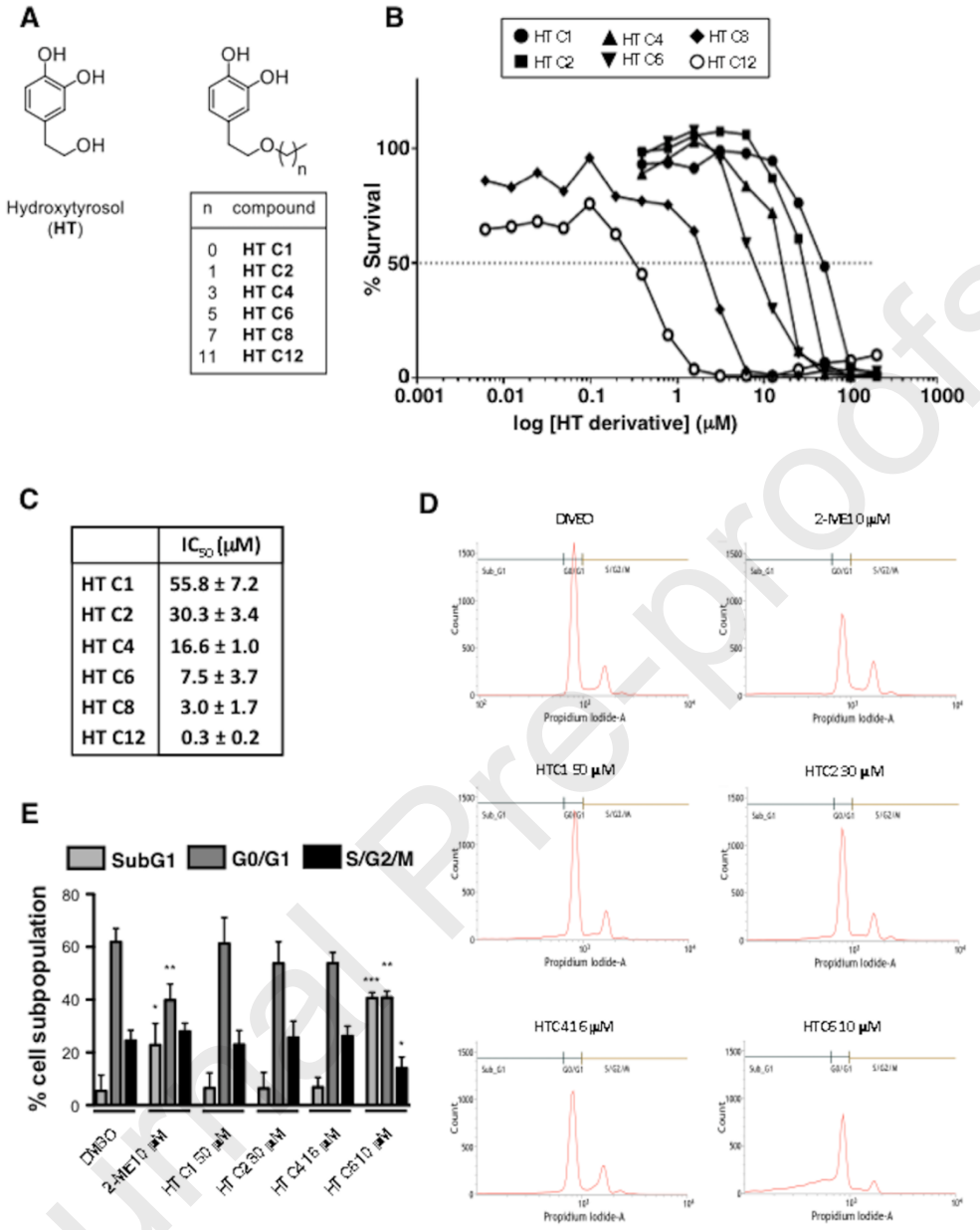
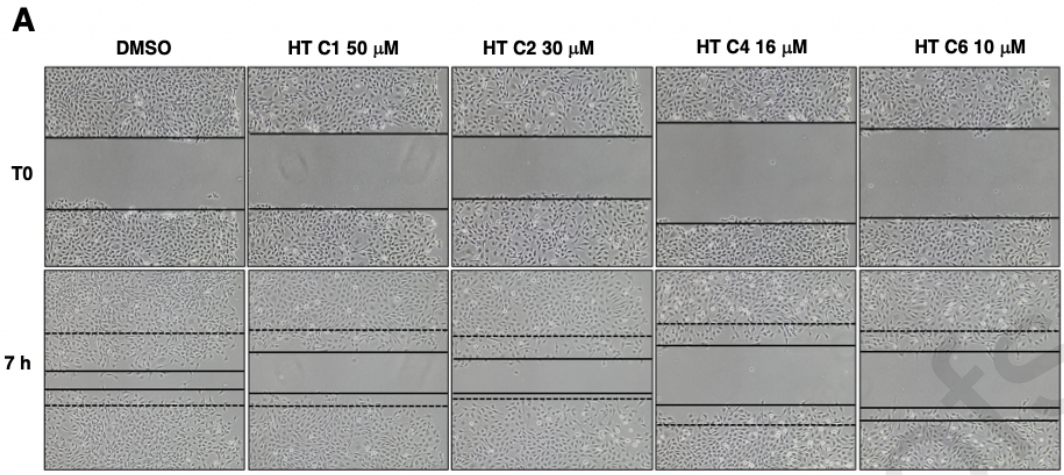
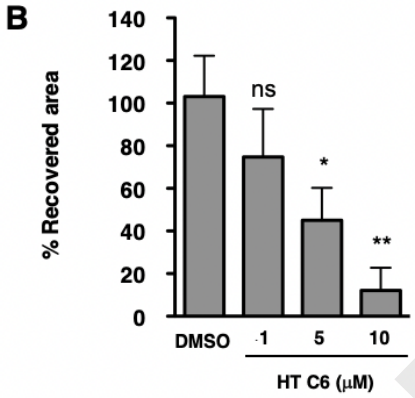
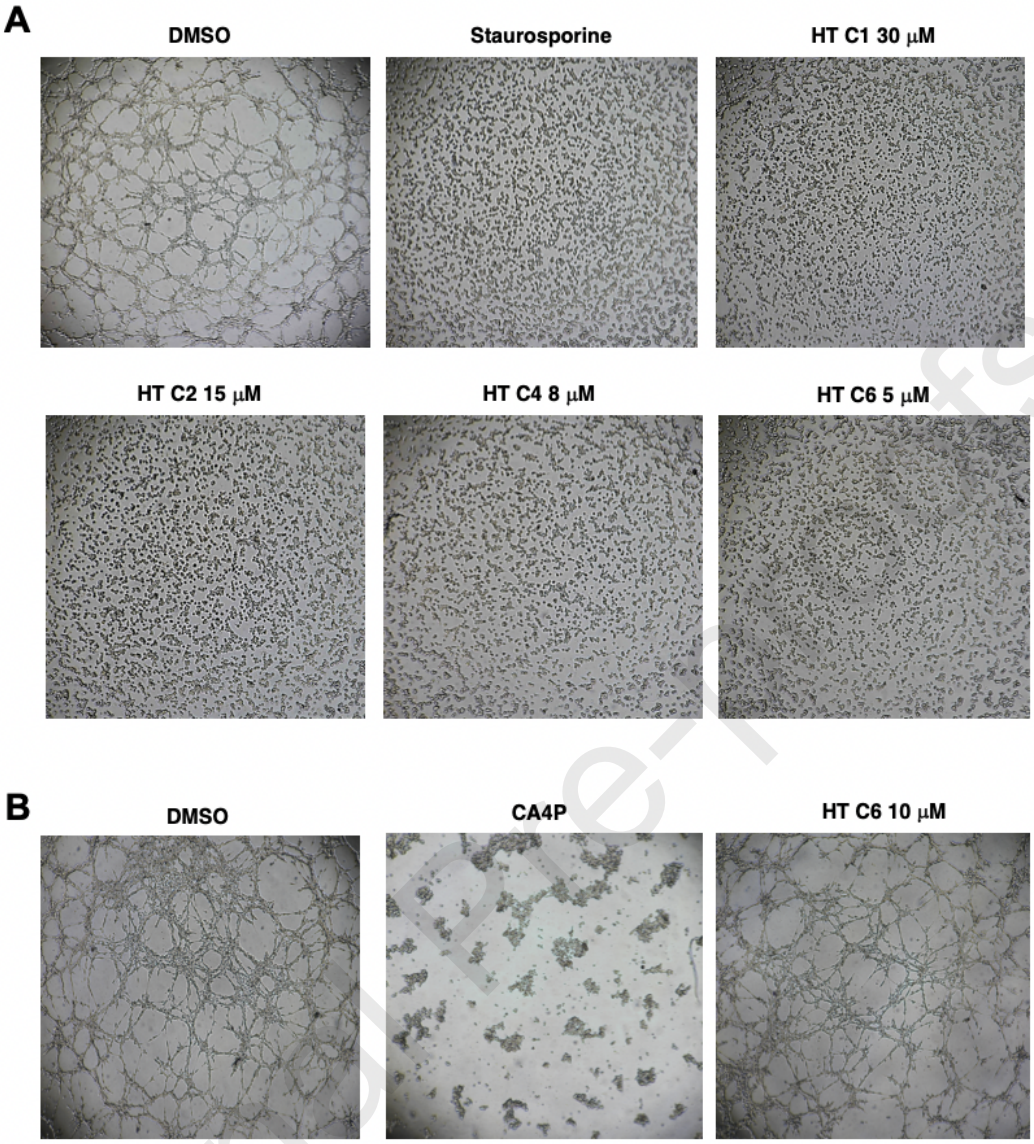
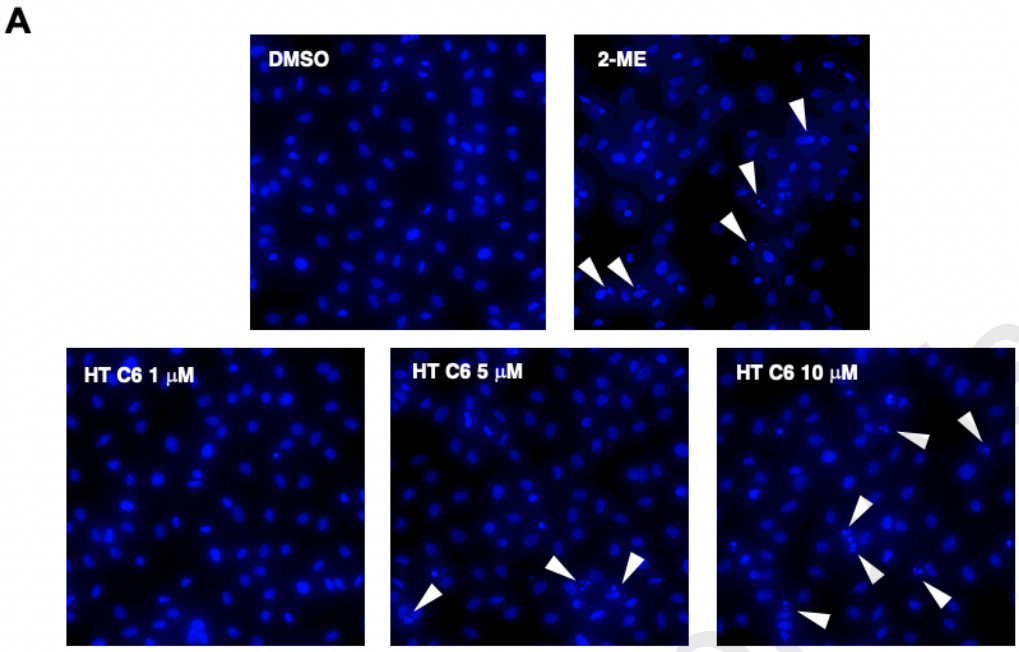


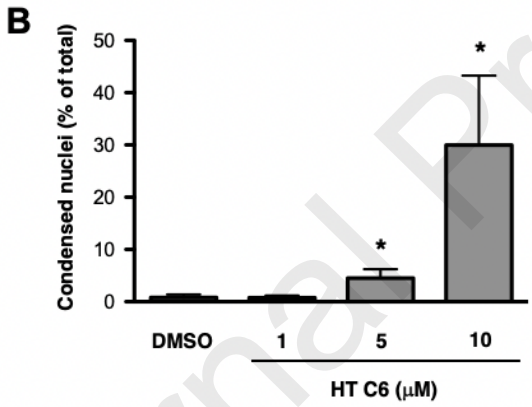
Figure 1











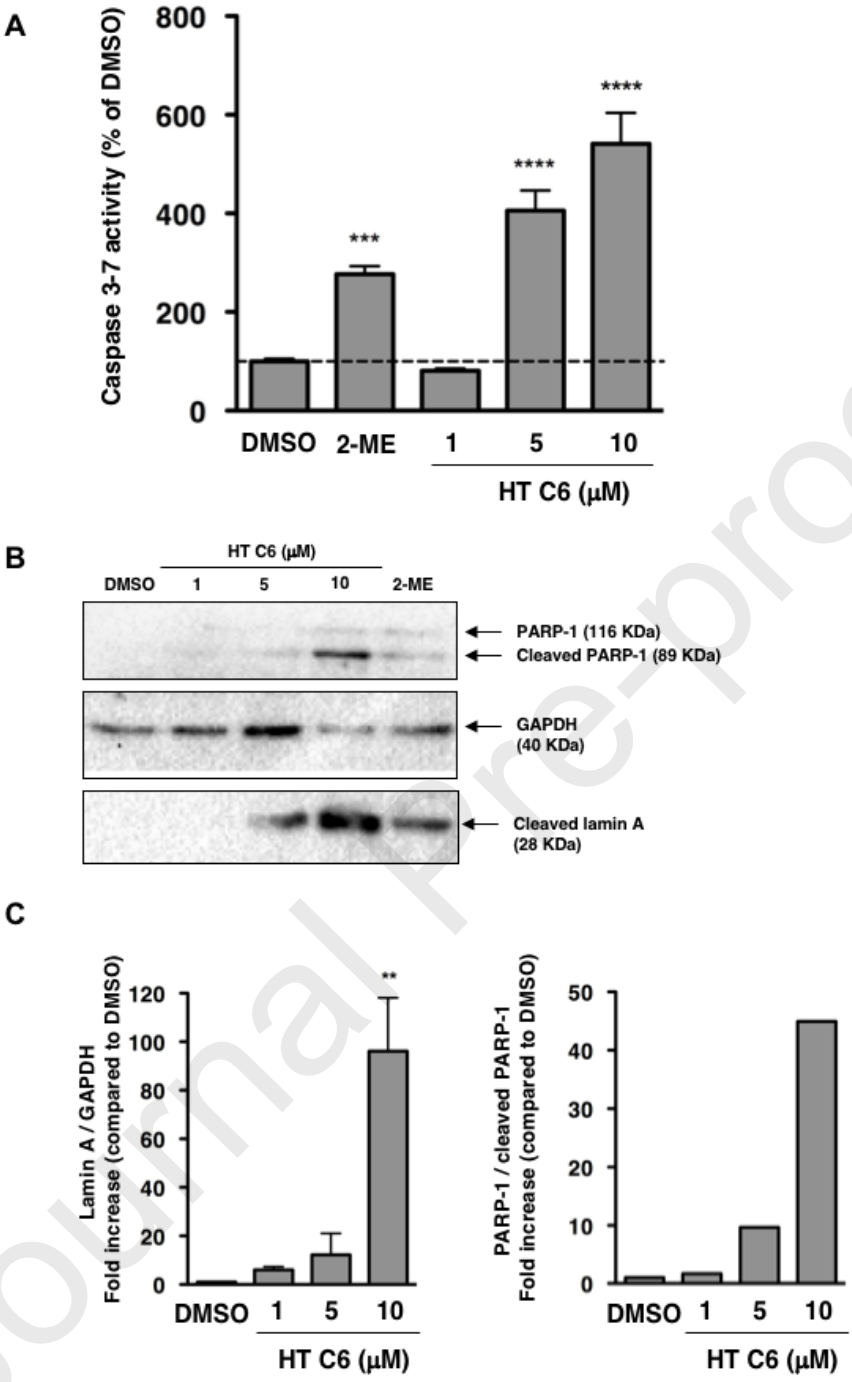
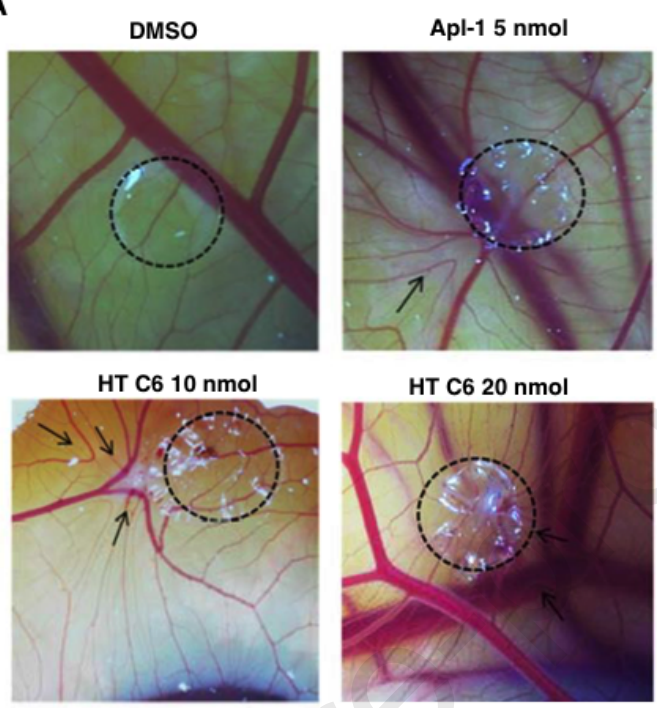


Figure 5

A



B

Dose (nmol/CAM)	Positive/total (%)
0	0/5 (0)
1	2/10 (20)
5	7/13 (54)
10	6/10 (60)
20	5/6 (83)

Figure 6