



DIANNEXIN DOWN-MODULATES TNF-INDUCED ENDOTHELIAL MICROPARTICLE RELEASE BY BLOCKING MEMBRANE BUDDING PROCESS

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ABSTRACT

Background: Microparticles are now recognised as true biological effectors with a role in immunopathology through their ability to disseminate functional properties. Diannexin, a homodimer of annexin V, binds to PS with a higher affinity and longer blood half-life than the monomer, inhibits prothrombinase complex activity thereby diminishing coagulation and reperfusion injury mediators and prevent microvesicle-mediated material transfer. Our aim was to determine if Diannexin could modulate microparticle production by endothelial cells by interacting with the phosphatidylserine exposure occurring during the release of these vesicles.

Results: In this study we showed that fluorescently labelled Diannexin binds to calcimycin-activated endothelial cells but not to resting cells. After overnight incubation, Diannexin enters cells and their released MP carry Diannexin. Some Diannexin seems to be processed via early endosomes and later is found in lysosomes. Both unlabelled Diannexin and fluorescent Diannexin inhibit MP release from TNF-activated endothelial cells. However, Diannexin treatment does not prevent endothelial activation by TNF. In addition, the inhibitory effect of Diannexin on MP release could be observed when cells were pre-, concomitantly or post-treated with cytokines. Scanning electron microscopy showed differences in the numbers and types of protuberances at the cell surface when cells were treated or not with Diannexin. Finally, there is no apparent congruency between fluorescent Diannexin labelling and surface protuberances as shown by correlative microscopy. **Conclusions:** Altogether these data suggest that Diannexin can inhibit endothelial vesiculation by binding PS present either at the cell surface or at the level of the inner leaflet of the plasma membrane.

Key words : Microparticles, Diannexin, TNF, endothelium, inflammation, cytokines, vesiculation.

BACKGROUND

Vesiculation is the process through which cells release plasma membrane sub-micron vesicles, named microparticles (MP). It occurs after an increase of intracellular calcium, subsequent loss of phospholipid asymmetry and exposure of negatively charged phospholipids such phosphatidylserine (PS) at the outer leaflet of the plasma membrane. Vesiculation is found in all eukaryotic cells but has been particularly studied in vascular cells. A hallmark of MP is the presence of PS at their surface, which allows their identification using its ligand, annexin V(1). MP are

produced and can be detected in the blood of healthy subjects where they are thought to participate in homeostasis. In pathological conditions, the increased production of MP detected in the plasma of patients is often associated with disease severity. This is true for cardiovascular diseases, as well as inflammatory and infectious conditions(2-4). In the circulation, MP are produced mainly by platelets. Increased release of MP of endothelial origin has been associated with various inflammatory diseases such as anti-phospholipid syndrome, cerebral malaria or multiple sclerosis(5-8). Inhibition of MP production has been demonstrated using various agents which interfere with molecules involved in the production process, such as calpeptin which inhibits calpain, a calcium-dependent protease (9, 10), pantethine a key regulator of lipid metabolism (11) or in the signalling pathways controlling vesiculation such as Rho inhibitors (10, 12, 13).

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MP cargo both surface and cytosolic molecules from their cell of origin and therefore appear as real biological effectors able to disseminate various functional properties at a distance from their site of formation. Once they have interacted with their target cells, MP trigger responses from these cells which include the activation of coagulation pathways, production of inflammatory molecules or induction of apoptosis (14, 15). MP can also transfer active molecules to their target cells, providing them with a new phenotype (16, 17). MP-mediated material transfer can be inhibited by incubation of the MP with high concentrations of annexin V, although this does not modulate the binding of MP to the target cells (18).

Diannexin is a recombinant homodimer of human annexin V that binds PS with a higher affinity than that of annexin V and displays a longer half-life than its monomer. By shielding PS exposed at the surface of activated cells, Diannexin reduced the interaction with procoagulant proteins such as fVa (2 x 36 kDa) (19). In a model of liver ischemia-reperfusion, treatment with Diannexin protected the liver, whether the treatment was administered pre- or post-injury. This effect seemed to occur through protection of the sinusoidal endothelial cells (20) and a decrease in pro-inflammatory cytokines (21). In other models of ischemia-reperfusion, Diannexin reduced tubule damage and leukocyte influx, and improved renal function (22). It was cardioprotective in rabbits with severe ischemic insult (23). In addition, as annexin V could block material transfer between MP and target cells, Diannexin was able to block tumour microvesicle-mediated transfer of EGFR to endothelial cells (24).

So far, Diannexin appears to be of great importance *in vivo*, appearing to shade PS at the surface of activated cells or PS positive MP. Recently, Teoh and coll. showed in a murine model of ischemia reperfusion that MP could be taken up by hepatocytes and trigger injury, and that Diannexin could prevent MP formation and associated inflammation (25). However, how Diannexin acts on the vesiculation process, i.e., production of MP itself, is not known. In order to understand its mechanism of action, we hypothesised that Diannexin modulates MP shedding by binding to PS before or after it is translocated at the cell surface during vesiculation.

METHODS

Human brain endothelial cells culture

Human brain microvascular endothelial cells,

hCMEC/D3, were cultured as previously described (13) on collagen-coated multi-well plates for flow cytometry analysis, Labtek II chamber slides for fluorescence microscopy studies and glass coverslips for electron microscopy work. Cells were grown to confluence in EBM-2 medium complete with 5% FCS, ascorbic acid (5 µg/ml, Sigma), hydrocortisone (1.4 µM, Sigma), CDLC (1:100 dilution, Invitrogen), HEPES (100 mM, Invitrogen) and β-FGF (1 ng/ml, Sigma). hCMEC/D3 were used from passage 28 to 40.

Endothelial TNF stimulation and Diannexin treatment

TNF (50 ng/ml, Peprotech) was added overnight to induce MP production and at 10 ng/ml to induce upregulation of adhesion molecules as previously described (13).

A stock solution of Diannexin was prepared from lyophilised Diannexin provided by A.C. Allison, then diluted extemporaneously in complete culture medium to final concentrations of 5, 15 and 45 nM.

Effects of Diannexin on MP release were analysed according to the following treatments (i) pre-treatment: cells were incubated with Diannexin (15 nM) for 5 and 2 h prior to addition of TNF (50 ng/ml), (ii) concomitant treatment: cells were incubated with Diannexin (15 nM) at the same time as TNF (50 ng/ml), (iii) post-treatment: cells were incubated with TNF (50 ng/ml) for 0.5 and 2 h prior to addition Diannexin (15 nM). In each case, control wells received either medium, TNF or Diannexin.

Detection of MP by flow cytometry

MP were quantified in the culture supernatant of hCMEC/D3 cells treated or not with Diannexin +/- TNF after labelling with specific endothelial mAbs against endoglin (PE-CD105, Beckman Coulter) or ICAM-1 (PE-CD54, Beckman Coulter).

Analysis of hCMEC/D3 treated with Alexa Fluor 488- Diannexin

Conjugation of Diannexin to Alexa Fluor 488 (AF) was performed according to manufacturer instructions (Invitrogen).

hCMEC/D3 were incubated overnight with AF-Diannexin (15 nM), washed with medium to remove excess Diannexin and observed by microscopy. Imaging and analysis were performed with the Olympus IX71 microscope, 40 x magnification. In parallel, cells were cultured on collagen-coated 24 well plates and treated similarly. After the overnight incubation,

supernatants were collected and cells were detached for flow cytometry analysis.

Labelling of phosphatidylserine exposure after calcimycin activation

hCMEC/D3 were stimulated for 45 min with calcimycin (A23187, 30 μ M), then quickly washed with complete medium and labelled with AF-Diannexin for 15 min and observed on the Olympus IX71 microscope, 40 x magnification.

hCMEC/D3 surface phenotyping by flow cytometry

After treatment with Diannexin +/- TNF, cells were detached via trypsin/EDTA treatment then incubated with PE-conjugated anti-CD54 mAb for 20 min and then analysed by flow cytometry. Mean fluorescence intensity and percentage of positive cells was recorded.

Intracellular localisation of Diannexin

hCMEC/D3 were incubated with AF-Diannexin (15 nM) at different incubation times (30 min, to overnight). LysoTracker Red (50 nM, Invitrogen) was used for the labelling of lysosomes and was added 30 minutes before washing and analysis in PBS. For early endosome labelling, hCMEC/D3 were fixed with 1% (v/v) PFA for 20 min, washed once in PBS, permeabilized with ice cold methanol for 3 min and then rinsed gently twice in PBS. Early endosome antigen 1 (EEA-1) was detected using a mouse monoclonal IgG1 to EEA-1 or Rab 5 revealed with Alexa Fluor 546 conjugated goat anti-mouse secondary antibody (1:400, Invitrogen). Both primary and secondary antibodies were diluted in 1% (w/v) BSA in PBS and incubated for 30 min at room temperature. Finally, cells were washed in PBS, quickly rinsed in water and mounted in Prolong hardening mounting medium (Invitrogen). Samples were analyzed by fluorescence microscopy on the IX71 inverted Olympus microscope.

Scanning electron microscopy of hCMEC/D3 treated with Diannexin

hCMEC/D3 were pre-treated or not for 2h with Diannexin prior to TNF activation or left resting. Cultures were washed in 0.1M sodium cacodylate/0.1M sucrose/pH 7.4 buffer solution and fixed in sodium cacodylate buffered 2% (v/v) glutaraldehyde for 30 min. Buffer washes were performed prior to post-fixation in 1% osmium tetroxide/0.1M sodium-Cacodylate for 1 h. Subsequent buffer and water washes were required prior to dehydration in 30%, 50%, 70%, 80%, 90% and 99% ethanol, with each step

performed twice for 5 min. Cells were dehydrated twice in ultrapure ethanol for 10 min each, treated with hexamethyldisilazane (HMDS) for 3 min, air-dried briefly and then placed in a desiccator. Coverslips were mounted onto SEM specimen stubs with double sided carbon tape, lined with silver dag and platinum coated. Samples were imaged with the Zeiss Ultra Plus by secondary electron detection at 10 kV. Coded images were analysed using Image J software. Numbers of cells presenting MP at their surface and numbers of MP per cells were counted. Statistical analyses were performed between the different groups and then images were decoded.

Correlative microscopy

Cells were treated with TNF +/- AF-Diannexin

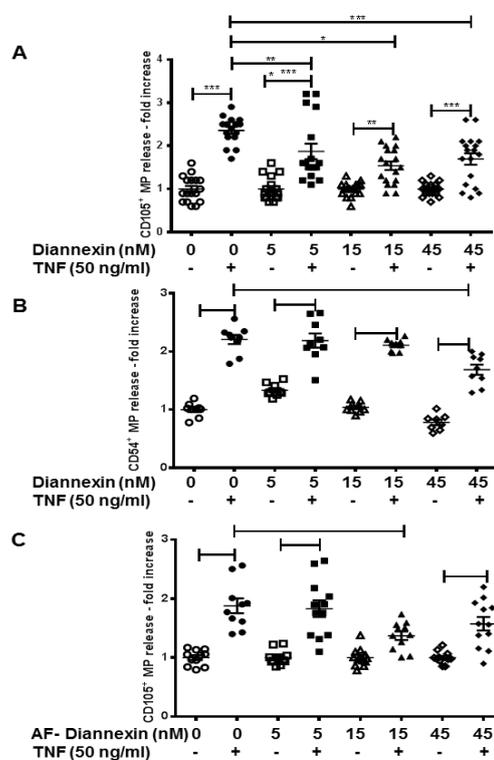


Figure 1: Inhibition of TNF-induced vesiculation by Diannexin. Resting (open symbols) or TNF-stimulated (plain symbols) brain endothelial cells were treated with increasing doses of Diannexin (0, 5, 15, 45 ng/ml) 2h prior to activation. After an overnight, cell culture supernatants were collected and an MP quantified by flow cytometry using anti-CD105 staining (A, C) or anti-CD54 staining (B). Data are expressed as fold increase compared to resting condition from 3 independent experiments (***) = $P<0.0001$, ** = $P<0.001$, * = $P<0.05$; Kruskal-Wallis test and Dunns post-test for multiple comparisons). A and B show the effects of unlabelled Diannexin on MP release. C shows effects of Alexa fluor-labelled Diannexin on MP release.

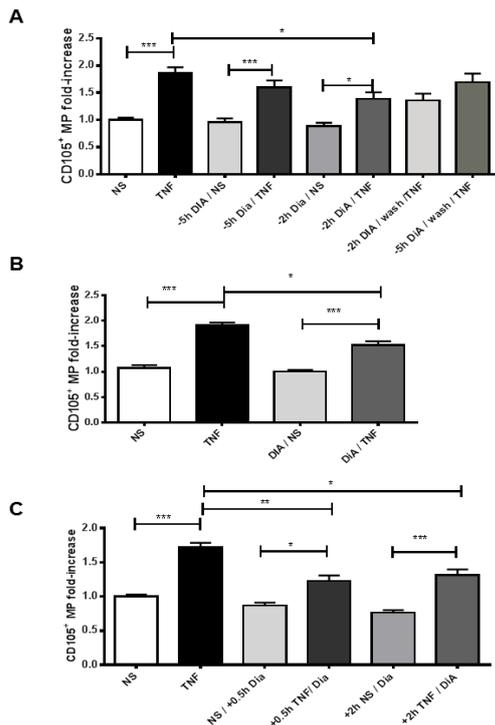


Figure 2: Kinetics of Diannexin treatment on MP release. Resting or TNF-stimulated brain endothelial cells were treated with a single dose of Diannexin (15 ng/ml) at various time points prior (A), concomitantly (B) or post-TNF activation (C). After an overnight, cell culture supernatants were collected an MP quantified by flow cytometry using anti-CD105 staining. Data are expressed as fold increase compared to resting condition from 3 independent experiments (***) = $P < 0.0001$, ** = $P < 0.001$, * = $P < 0.05$; Kruskal-Wallis test and Dunns post-test for multiple comparisons).

as described above in immunofluorescence analysis. Monolayers were scored with a pipette tip and samples were mounted in PBS for immediately viewing with the Zeiss LSM 510 Meta confocal microscope. Images were collected at 63X oil and the positions of areas of interest were annotated for later use. Following viewing, mounting slides were removed and samples were washed with PBS and subsequent sodium cacodylate buffer dilutions. Samples were processed for electron microscopy as described above. Image processing was performed in Adobe Photoshop with the aid of low magnification reference images.

Statistical Analyses

Statistical analyses were performed using Graph Pad Prism 5 software. All data are represented as mean \pm SD unless otherwise stated.

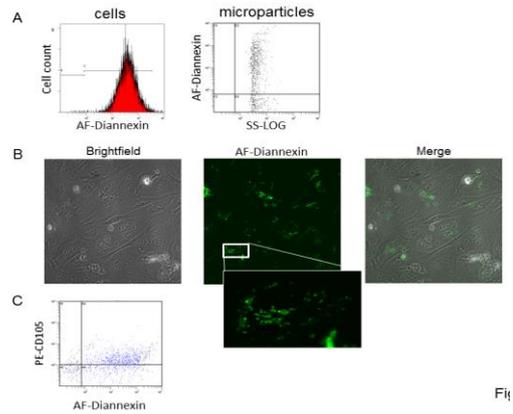


Figure 3: Kinetics of interaction of fluorescently labelled Diannexin with endothelial cells and subsequent MP release. Resting cells were incubated overnight with AF-Diannexin, culture supernatant was collected and cells detached and analysed by flow cytometry. (A) left histogram shows 100% of the cells labelled with Diannexin; right cytogram shows that a large majority of the events falling in the MP size gate are labelled with AF-Diannexin. (B) shows that after incubation with AF-Diannexin all cells are fluorescent showing a perinuclear pattern. (C) confirms that the majority of AF-Diannexin positive events in the MP gate are effectively MP as they can be double labelled with endothelial marker CD105-PE.

RESULTS

Diannexin and AF-Diannexin inhibit MP release from TNF-activated hCMEC/D3

Effect of Diannexin on release of CD105-positive MP. To analyse the effect of Diannexin treatment on MP release, supernatants of hCMEC/D3 pre-treated for 2 h \pm Diannexin at 5, 15 and 45 nM were collected after overnight incubation with or without TNF (50 ng/ml). MP present in the supernatant were labelled with an anti-CD105 mAb and then counted by flow cytometry. A 2.5-fold increase was observed after stimulation with TNF (Fig. 1A, $p < 0.0001$). While no difference was observed between the numbers of MP collected from resting cells treated or not with Diannexin, a significant decrease in the numbers of TNF-stimulated MP was observed in the presence of Diannexin. Interestingly, for all doses of Diannexin, a significant fold-increase in MP release could still be observed in the supernatants of cells that had received Diannexin + TNF stimulation compared to Diannexin alone.

Effect of Diannexin on release of ICAM-1 positive MP. To substantiate this inhibitory effect, we detected MP in the various supernatants after labelling with another surface probe (Fig. 1B). MP

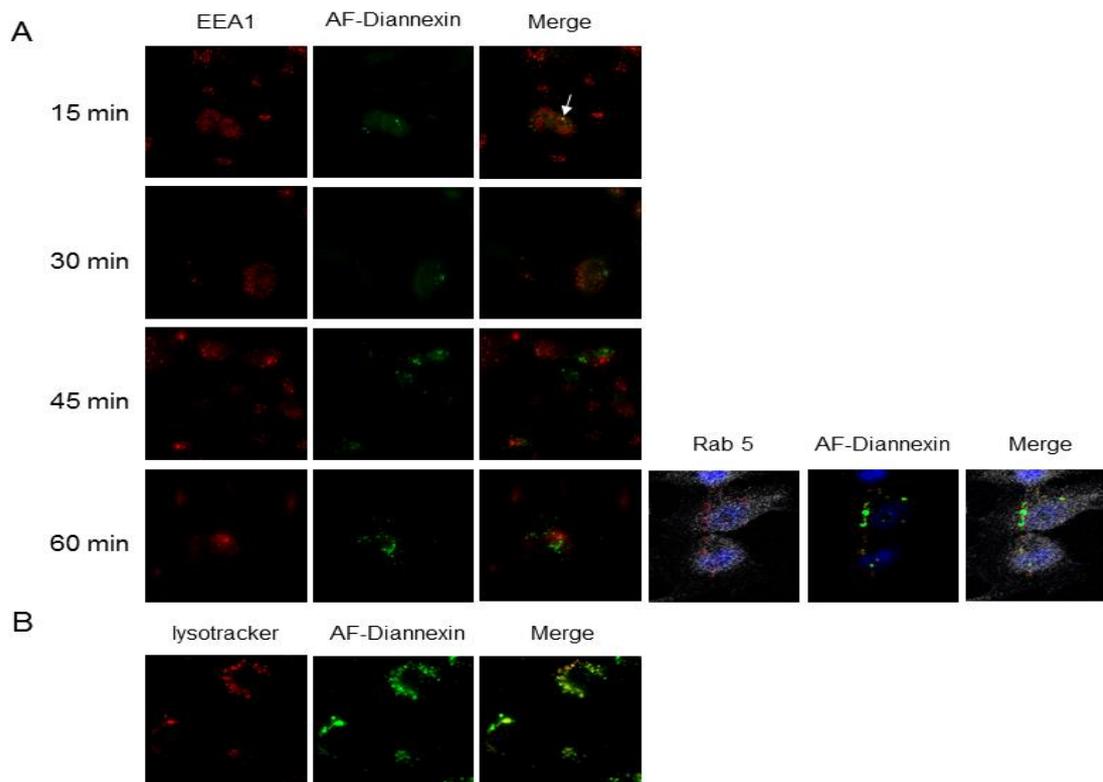


Figure 4: Intracellular localisation of Diannexin. Resting cells were labelled with AF-Diannexin (green) for 15, 30, 45 and 60 min, fixed and labelled with early endosome markers EEA1 (red) or Rab5 (white). When indicated nucleus was counterstained with DAPI. Cells were also incubated overnight with AF-Diannexin and stained with live lysosome dye lysotracker (red) and imaged within 20 min. (A) shows staining for EEA1 at 15, 30, 45 and 60 min and Rab 5/DAPI at 60 min only. Arrow indicates co-localisation between EEA1 and AF-Diannexin at 15 min. Red circles on Rab5 and merge images show the location of AF-Diannexin with regards to Rab5. (B) shows partial co-localisation of the lysosome marker and AF-Diannexin after overnight incubation.

collected in the conditions described above were labelled for ICAM-1 using a PE-labelled anti-CD54 mAb. TNF stimulation induced a dramatic increase in MP release, due to both an increase in numbers of MP and upregulation of the antigen at the surface of the mother cells by the cytokine. Treatment with Diannexin at 45 nM did significantly reduce the numbers of TNF-induced MP.

Effect of AF-Diannexin on release of CD105-positive MP. As fluorescently labelled Diannexin (AF-Diannexin) will be used in further experiments, we assessed the effect of Diannexin after conjugation. In an experimental setup similar to that described above, AF-Diannexin readily inhibited the TNF-induced vesiculation when cells were treated with 15 nM of AF-Diannexin. No effect was observed at 5 nM and a trend to decrease was seen at 45 nM (Fig. 1C).

Effect of pre-, concomitant or post-treatment with Diannexin on MP release

Cells were treated with Diannexin either before (pre-), at the same time (concomitant) or after

(post-) TNF addition. As controls, cells were left either untreated, treated with TNF or with Diannexin alone.

Diannexin pre-TNF treatment: a significant increase in MP numbers was seen upon TNF only stimulation, as expected (Fig. 2A, $p < 0.005$). When cells were treated with Diannexin 5 h or 2 h prior to TNF, a significant increase in MP release could still be observed compared to cells treated with Diannexin alone vs Diannexin/TNF ($p < 0.005$ vs $p < 0.05$, respectively). However, the TNF-induced MP release was significantly reduced when cells treated with Diannexin 2h prior TNF activation compared to cells treated with TNF only (Fig. 2A, $p < 0.05$). When cells were pre-treated with Diannexin for 2 and 5 h, then washed prior to TNF activation, no effect on MP release could be observed.

Diannexin/TNF concomitant treatment: When cells were treated with Diannexin and TNF simultaneously, they produced significantly fewer MP than cells stimulated by TNF only, but still more than resting conditions (Fig. 2B, $p < 0.005$).

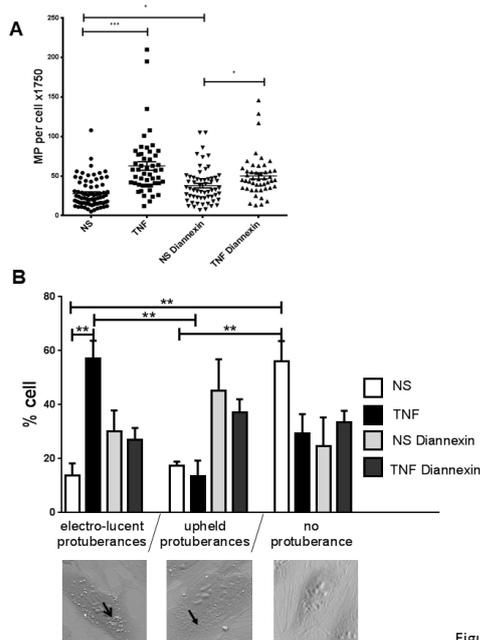


Figure 5: Diannexin treatment affects the cell surface morphology. Resting and TNF-stimulated cells were treated with Diannexin, fixed and analysed by scanning electron microscopy. (A) Numbers of MP were counted at the surface of the cells (10 cells at x1750 magnification were counted). (B) Protuberances at the surface of the cells were categorise as lector-lucent (left), upheld (middle) or absent (right) and counted. Graph shows percentages of MP counted in each category for all experimental conditions.

Diannexin post-TNF treatment: When cell were treated with Diannexin 0.5 h and 2 h post TNF activation (50ng/ml), a significant increase in MP release was still observed when compared with Diannexin +/- TNF (Fig. 2C, $p < 0.05$ and $p < 0.005$, respectively). However, the increment in MP release in TNF/Diannexin treated cells was significantly lower than that seen with TNF alone ($p < 0.005$, $p < 0.05$, respectively).

AF-Diannexin enters cells after overnight incubation and released MP are labelled

Diannexin has been described to label PS at the surface of red blood cells. To visualise this binding to the endothelial cell membrane, Diannexin was labelled with Alexafluor 488 (AF-Diannexin). As shown in Fig.1Asuppl., after 20 min incubation with AF-Diannexin, the resting endothelial monolayer showed no AF-Diannexin staining while cells activated with calcimycin (A23187) showed a membrane staining pattern, especially on cells that have started to round up, confirming that AF-Diannexin specifically binds to exposed PS. As a control, cells were also labelled with FITC-annexin V and a similar pattern was observed, albeit with a

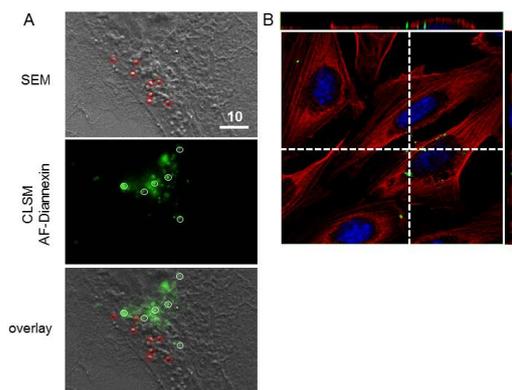
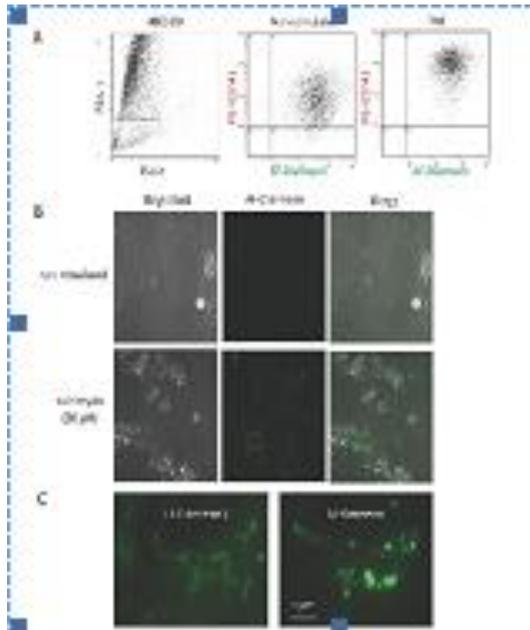


Figure 6: Confocal-scanning electron microscopy correlative analysis of Diannexin and surface protuberances. Cells were incubated with AF-Diannexin fixed, imaged by confocal microscopy. (A) Cells were then prepared for scanning electron microscopy. The same cells were then imaged and both micrographs were overlaid. Surface electro-lucent protuberances were outlined in red circles while AF-Diannexin staining was outlined in white circles. Overlay shows no congruency between the two structures. (B) Cells were then labelled for γ -actin that has an apical distribution in these cells. Projections on x/y axes show distribution of AF-Diannexin directly below the plasma membrane.

lower fluorescence intensity (Fig. 1Bsuppl.). This is to be expected, as annexin V is FITC-but not AF-labelled and asDiannexin is known to have a higher affinity for PS than annexin V monomers.

To evaluate whether the effect of Diannexin on hCMCE/D3 vesiculation is due to an inhibition of the activation status of the cells, surface expression of ICAM-1 was assessed by flow cytometry (Fig. 1C suppl.). Cells were pre-treated with AF-Diannexin 2 h prior to TNF stimulation (10ng/ml). After an overnight incubation, a similar degree of ICAM-1 up-regulation was observed at the surface of the cells.

Although AF-Diannexin does not bind to resting cells at the time the surface staining can be observed on activated cells (Fig. 1. suppl.), when AF-Diannexin was left in the medium overnight, the whole cell population appeared labelled, as determined by both flow cytometry and fluorescence microscopy (Fig. 3A left and 3B, respectively). Microscopy showed fluorescent staining localised in the peri-nuclear region of the cells (Fig. 3B, insert). When the cell culture supernatant was collected, flow cytometry detected AF-labelled events that are compatible with MP, in both forward and side scatter (Fig. 3A, right panels). In addition, when the supernatant was labelled with an antibody against the endothelial MP surface marker CD105, we



Supplementary figure 1: AF-Diannexin does not prevent up-regulation of ICAM-1 and has a similar pattern of staining as annexin V. (A) Cells were stimulated with TNF (10 ng/ml) and expression of ICAM-1 was assessed by flow cytometry. After overnight all cells were double stained for ICAM-1 and AF-Diannexin and ICAM-1 showed up-regulation of its surface expression. **(B)** Cells were stimulated with calcimycin (A23187), labelled with AF-Diannexin and imaged in widefield (left), green fluorescence (middle) and overlaid (right). Top images show normal cell morphology and absence of AF-Diannexin staining on resting cells while bottom images show cells rounding as expected following calcimycin treatment and AF-Diannexin labelling on the curved portions of the cells. **(C)** FITC-Annexin V and AF-Diannexin show similar staining pattern after calcimycin treatment.

observed that most of AF positive events were positive for CD105, confirming that these are MP from endothelial origin (Fig. 3C).

Intracellular localisation of Diannexin

To determine the intracellular localisation of AF-Diannexin, the kinetics of uptake was analysed in parallel with the staining pattern of specific markers for early endosomes (EEA1, rab 5, 7 and 11) and lysosomes (lysotracker). As expected, very little staining was observed in cells incubated with AF-Diannexin for 15 and 30 min (Fig. 4A). A more marked perinuclear staining was observed from 45 min onwards with a maximum after overnight incubation. At 15 min, very rare AF events co-localised with EEA1 staining (white arrow), however, taking into account the weak AF-Diannexin staining, around 30% of AF-Diannexin co-localised with EEA-1. This was no longer observed at later time points. However, when the

endosome marker rab 5 was assessed, it appeared as very fine dots (compared to the vesicular aspect of EEA1) and could be seen sitting directly beside the AF-Diannexin, but no co-localisation could be observed after a 60 min incubation (Fig. 4A, right panels, red circles to delineate AF-Diannexin labelling in regards to rab5 staining). When AF-Diannexin was left overnight it appeared to mostly co-localise with lysotracker, suggesting a processing of the molecule via the lysosome pathway. No co-localisation was observed with rab-7 and rab-11.

Diannexin treatment affects the cell surface morphology

We have previously shown that increased numbers of cell surface protuberances were associated with an increased number of MP present in the culture supernatant, suggesting that these protuberances are indeed budding MP (13). These protuberances were enumerated at the surface of the hCMEC/D3 irrespective of their size or electro-lucent. Between 7 and 9 images at x1750 magnification were counted using Image J software. Significantly more peri-nuclear protuberances were observed at the surface of TNF-stimulated cells compared to resting cells (Fig. 5A). Treatment of resting cells with Diannexin was associated with a higher number of protuberances at the cell surface and TNF+Diannexin stimulation still induced the release of a higher number of MP compared to resting conditions.

However when the fine morphology of the cell surface was analysed in all experimental conditions, we could observe and counted the different types of protuberances were present (Fig. 5B). Electro-lucent protuberances (Fig. 5B, left, open arrow) were predominantly observed on TNF-stimulated cells (60%) while protuberances that seemed "upheld" under the surface (Fig. 5B, centre, black arrow) were observed in higher numbers on cells treated with Diannexin, either resting or TNF-stimulated (35% and 30%, respectively). Cells without protuberances were mainly found in resting conditions (60%, Fig. 5B, right).

Absence of congruency between AF-Diannexin labelling and surface protuberances

To evaluate whether the presence of these peri-nuclear protuberances (13) was related to internalised Diannexin, we performed correlative microscopy on cells treated with AF-Diannexin. When overlaying AF-Diannexin intracellular labelling and SEM of budding vesicles, no co-

localisation could be observed, suggesting that no electro-lucent protuberance is formed where AF-Diannexin is localised (Fig. 6A).

In addition, orthogonal projections of z-stacks showed that some AF-Diannexin appeared to be still at the plasma membrane while some was localised below the plasma membrane, as seen when imaging γ -cytoplasmic actin that forms an apical sub-membranous meshwork in these cells (Fig. 6B) (13).

DISCUSSION

The annexin homodimer, because of its higher affinity to PS than its monomer (26), has been originally described as an anti-thrombotic agent with cell protective effects in cases of ischaemia reperfusion injuries (22)(20, 23), reduced inflammatory cell infiltration and inhibition of apoptosis of β -cell in grafts of islet cells (27). Diannexin is also known for its ability to shade PS at the surface of cells, and therefore prevent the interactions of this phospholipid, successfully inhibiting coagulation and protecting cells after reperfusion injuries. Also, Diannexin cloaking the PS at the surface of vesicles was preventing their pro-angiogenic properties during tumour development (24). More recently, a direct relationship between Diannexin and microparticles was shown by Teoh and coll *in vivo*(25)but the mechanisms by which Diannexin is able to do so have not been assessed. This is what we attempted to do in this study where in summary we have demonstrated that (i) Diannexin treatment of TNF-stimulated endothelial cells significantly inhibits the release of MP, (ii) Diannexin does not prevent upregulation of adhesion molecules at the surface of the treated cells but by decreasing the number of MP carrying these adhesion molecules will limit their interactions, (iii) although Diannexin is rapidly internalised, its effect can still be observed after 24 h, at a time when Diannexin can be observed below the cell surface, possibly bound to the PS at the inner leaflet of the plasma membrane but is not associated with the surface protuberances (iv) the morphology of the cell is altered after treatment with Diannexin: fewer protuberances are observed after TNF activation and when protuberances are present they appear upheld.

MP have been described by many, including our group, as biological effectors involved in disease pathogenesis. However the fine mechanisms associated with MP release are still not fully understood. In our study, Diannexin only showed an inhibitory effect on MP released in stimulated conditions. This suggests that the

mechanism by which Diannexin interferes is not associated with the release of MP in resting conditions. MP released under physiological conditions have been often described as "protective" or as having a positive functional effect (2, 10).Diannexin demonstrated protective effects *in vivo* and, recently, treatment with Diannexin during inflammatory conditions was associated with a reduction of MP release *in vivo*(20). Therefore, we can hypothesise that treatment with Diannexin will only demonstrate its effects in pathogenic conditions and will not alter the production of beneficial MP.

In the present study, treatment of TNF-activated endothelial cells with Diannexin did not affect the ability of these cells to respond to pro-inflammatory signals, as shown by the up-regulation of surface adhesion molecules, suggesting that although MP release was significantly decreased, cells would still be able to interact with leucocytes. Whether the consequences of these interactions would still be present remains to be determined. We have also demonstrated that MP released after TNF+Diannexin treatment carry the same detectable levels of adhesion molecules, namely ICAM-1, also suggesting that these MP could interact with a target cell. However, due to the reduced number of MP released, the net amount of ICAM-1 receptors available for interaction with its ligand would be significantly lower. Also, when using fluorescently-labelled Diannexin we showed that MP released by Diannexin-treated cells were fluorescent, suggesting that Diannexin was present either at the surface of the MP or more likely contained within the MP, as we demonstrated that Diannexin was quickly internalised by the endothelial cells. After being internalised, Diannexin was detected by confocal microscopy below the plasma membrane, as assessed by γ -actin labelling, and one can hypothesise that it was bound to the PS present at the inner leaflet of the phospholipid bilayer, therefore preventing additional externalisation of PS. This was further demonstrated by correlative microscopy that showed no congruency between internalised Diannexin and membrane protuberances that are likely to later be shed as MP. This suggests that where protuberances were formed, no Diannexin was present to prevent the phenomenon. It therefore seems that Diannexin could not only cloak the PS present at the surface of the cells but also the one expressed on the inner surface of the plasma membrane, thus preventing both further interaction with external PS and exposure of those internalised. This was also visualised by electron

microscopy, where Diannexin treatment of the cells was correlated with a significant increase of “upheld” protuberances that was itself associated with a significant decrease of shed MP found in the supernatant of cell cultures. The inhibitory effect of Diannexin was observed whether the cells were treated with the inhibitor prior, concomitantly with or after TNF stimulation, suggesting that the sole presence of the molecule was able to prevent the effect of TNF. This was confirmed by the fact that a pre-treatment by Diannexin followed by a wash of the molecule prior to TNF stimulation was enough to remove the inhibitory effect.

We have recently shown that treatment of endothelial cells with the Rho-kinase inhibitor Y27632 is associated with a decrease in MP production linked to changes in β and γ actin isoforms (13). Here, the apical γ -actin was not modified in the presence of Diannexin, suggesting that these two inhibitory effects occur via different mechanisms, with Diannexin most likely physically preventing exposure of PS and subsequent MP shedding while inhibitors such as Y27632 act on the signalling pathway controlling MP release.

As previously mentioned, Diannexin is internalised and can be found associated with early endosomes and later with lysosomes, however this association is not 100% and some Diannexin can definitively be detected below the plasma membrane. In addition, cells are still fluorescent 24h after being treated with labelled Diannexin, suggesting that not all the Diannexin has been processed in the lysosomes. We cannot exclude the possibility that Diannexin itself has been lysed and that only the Alexa fluor remains, however the confocal images suggest otherwise, with a staining pattern below the plasma membrane compatible with Diannexin bound to internal PS.

CONCLUSIONS

In conclusion, Diannexin is a promising molecule that has already proven its efficacy in ischaemia reperfusion injuries but has also a potential as an adjunct therapy in inflammatory diseases where MP have a potential deleterious effect. Importantly, by not altering the level of MP released by resting cells, Diannexin can be envisaged as a treatment that will not have the side effects that a molecule affecting physiological levels and properties would possess.

AUTHOR'S CONTRIBUTIONS

VC designed and performed all the cell cultures, flow cytometry and fluorescence labelling

experiments, tagged the Diannexin and wrote the manuscript.

SLL performed the electron microscopy preparation and imaging, confocal and correlative microscopy imaging experiments and critically edited the manuscript.

BW participated in the flow cytometry and critically edited the manuscript.

ACA provided the Diannexin and critically assessed the first versions of the manuscript

GERG conceived the study, supervised the work, helped design and critically edited the manuscript.

COMPETING INTERESTS

VC, SL, BW and GERG have no competing interests

ACA from Alavita Inc. who developed the Diannexin gifted the Diannexin for this study.

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