Delay of endothelial cell senescence protects cerebral barrier against age-related dysfunction: role of senolytics and senomorphics

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Abstract

Accumulation of senescent cells in cerebrovasculature is thought to play an important role in age-related disruption of blood-brain barrier (BBB). Using an *in vitro* model of human BBB, composed of brain microvascular endothelial cells (BMECs), astrocytes and pericytes, this study explored the so-called correlative link between BMEC senescence and the BBB dysfunction in the absence or presence of functionally distinct senotherapeutics. Replicative senescence was deemed present at passage ≥19 where BMECs displayed shortened telomere length, reduced proliferative and tubulogenic potentials and increased NADPH oxidase activity, superoxide anion production (markers of oxidative stress), S-β-galactosidase activity and γ-H2AX staining. Significant impairments observed in integrity and function of a model of BBB established with senescent BMECs, ascertained successively by decreases in transendothelial electrical resistance and increases in paracellular flux, revealed a close correlation between endothelial cell senescence and BBB dysfunction. Disruptions in the localisation or expression of tight junction proteins, zonula occludens-1, occludin and claudin-5 in senescent BMECs somewhat explained this dysfunction. Indeed, treatment of relatively old BMEC (passage 16) with a cocktail of senolytics (dasatinib and quercetin) or senomorphics targeting transcription factor NF-κB (QNZ), p38MAPK signalling pathway (BIRB-796) or pro-oxidant enzyme NADPH oxidase (VAS2870) until passage 20 rendered these cells more resistant to senescence and totally preserved BBB characteristics by restoring subcellular localisation and expression of tight junction proteins. In conclusion, attempts that effectively mitigate accumulation of senescent endothelial cells in cerebrovasculature may prevent age-related BBB dysfunction and may be of prophylactic or therapeutic value to extend lifelong health and wellbeing.

Keywords: Endothelial cells, blood-brain barrier, senescence, senolytics, senomorphics, p38MAPK

Introduction

Ageing is a physiological process that begins during young adulthood and affects the structure and function of vascular system over time.[1,2] Endothelial dysfunction (ED) is widely regarded as the main phenotype that promotes age-dependent changes in vasculature.[3,4] Endothelium covers the entire inner surface of all blood vessels and is formed by a monolayer of endothelial cells (ECs). In addition to regulating vascular tone, vascular permeability, angiogenesis, coagulation and inflammation through synthesis and release of several vasoactive compounds, ECs also help establish specific barriers, conducting specific tasks, in different organs.[5,6] Blood-brain barrier (BBB) represents one such barrier in the central nervous system and controls the selective passage of compounds between brain parenchyma and the systemic circulation.[7] Although astrocytes, pericytes and microglial cells also contribute to the formation of BBB, brain microvascular ECs (BMECs), capable of forming tight junctions with the adjacent BMECs to prevent paracellular leakage, are regarded as the main cellular component of this unique barrier.[6] Tight junctions are multiprotein junctional complexes that consist of several transmembrane proteins and tight junction-associated scaffolding proteins, notably occludin, claudin-5 and zonula occludens-1 (ZO-1).[8] So, any biological scenario capable of affecting viability or function of BMECs during ageing process may trigger ED as an early event and eventually compromise BBB. Hence, identification of mechanisms involved in the initiation or progression of ED is of crucial importance to develop novel strategies that can delay or prevent BBB dysfunction and improve lifelong health and wellbeing.

Accumulation of senescent ECs in vasculature is increasingly recognised as an important stimulus for the development of ED during chronological ageing.[9,10] Indeed, EC senescence is implicated in both the structural and functional deterioration of the endothelium.[11,12] Consistent detection of senescent ECs, defined by permanent cell cycle arrest, shortened telomere length and positive staining for senescence-associated-β-galactosidase (SA-β-gal), in atherosclerotic

plaques attest the intrinsic role played by EC senescence in the formation and progression of vascular pathologies.[13,14] Oxidative stress, defined by the excessive availability of superoxide anion (O_2^-) and other reactive oxygen species (ROS), is thought to be a key factor for EC senescence during chronological ageing and its cell culture model, termed replicative senescence.[15,16] NADPH oxidase constitutes the main enzymatic source of oxidative stress in vasculature.[17] Detection of senescent cells in human coronary arteries but not in internal mammary arteries, known to possess very high antioxidant capacity, confirms oxidative stress as an important inducer of EC senescence and ensuing ED.[18] Restoration of tubulogenic and BBB-forming capacities of endothelial progenitor cells (EPCs), coupled with a considerable delay of senescence, by an NADPH oxidase inhibitor (VAS2870) further verify the role of this oxidase in cellular senescence and cerebral barrier dysfunction.[19]

Increasing evidence suggest that acquisition of an irreversible senescence-associated secretory phenotype (SASP), accompanied by secretion of several cytokines, chemokines and matrix metalloproteases may help spread senescence to the neighbouring cells.[20] Here, activation of p38MAPK/NF-κB pathway appears to be the main stimulus.[21,22] Indeed, selective inhibition of p38MAPK activity has been shown to collapse the senescence-associated cytokine network in human fibroblasts and to delay replicative senescence induced by dysfunctional telomeres.[22,23] Due to its ability to activate both the p53 and pRb/p16 growth arrest pathways, p38MAPK has also been shown to play an important role in the senescence growth arrest.[23] Similarly, specific targeting of NF-κB, a transcription factor that regulates the release of inflammatory cytokines and ROS, has been shown to improve endothelial function in healthy older adults to the levels observed in young controls.[24] Suppression of cellular proliferation rate, SA-β-gal activity and the expression of senescence-associated proteins, namely p16, p21 and p53, in mouse aortic ECs subjected to inflammatory cytokine interleukin-17A by an NF-κB inhibitor further confirmed the role of NF-κB in EC senescence.[25]

In addition to targeting of so-called specific mechanisms involved in cellular senescence, elimination of senescent cells by senolytics may also attenuate or even eradicate the impact of senescent cells on both the neighbouring cells and the remote tissue. The combination of dasatinib and quercetin (D+Q) has been the most commonly used senolytics in recent experimental and clinical studies.[26-28] While displaying no significant effects on non-senescent cells, D+Q selectively eliminated senescent cells in skin and adipose tissues.[28] Studies specifically focusing on ECs have shown that D+Q alleviates LPS-induced senescence in human umbilical vein ECs through inactivation of a pathway involving MAPK-NF-κB axis.[29] However, the mechanism of D+Q in replicative EC senescence-related BBB dysfunction remains unexplored.

In light of the above, using a cell culture model of chronological ageing, the current study has explored the impact of senescence on the morphological and functional characteristics of BMEC in the absence or presence of D+Q or senomorphics targeting p38MAPK, NF-κB and NADPH oxidase. Using a well-established *in vitro* model of human BBB,[11,30] it then investigated the specific mechanisms through which D+Q and p38MAPK inhibition exert their so-called cerebral barrier-protective effects. Taken together the data generated provide important novel insights into the prophylactic and therapeutic role of senotherapeutics in controlling the EC senescence-mediated BBB dysfunction and associated vascular complications.

Materials and methods

Cell culture

HBMECs were purchased from Neuromics (Minneapolis, MN, USA). Human pericytes and astrocytes were purchased from TCS CellWorks Ltd. (Buckingham, UK). All cells were cultured in a humidified atmosphere (75% N_2, 20% O_2, 5% CO_2) at 37°C in their respective media (Sciencell Research Laboratories, San Diego, USA).

Throughout the study, cells between passages 3 and 5 were used as young or non-senescent cells. Senescence was induced through repetitive culture of young cells and deemed to exist when about 70% of the cultured cells were stained positive for SA-β-Gal and γH2AX. In most experiments, this characteristic appeared between passages 19 and 21. The modulatory impact of senolytics (D+Q, 20 nM and 5 μM, respectively) and senomorphics targeting p38MAPK (BIRB-796, 5 μM), NADPH oxidase (VAS2870, 5 μM) or NF-κB (QNZ, 1 μM) on markers of senescence, BBB function, *etc* were assessed by treating passage 16 cells till passage 20 with the specific agents indicated in brackets. To ensure about the fresh supply of inhibitors, culture media were changed every other day in these experiments.

Senescence-associated β-galactosidase activity

HBMECs were seeded in 12-well plates and subjected to the outlined experimental conditions. The SA-β-gal activity were evaluated using a beta-galactosidase staining kit (APExBIO, USA) as per the manufacturer's instructions. In brief, HBMECs were washed with warm PBS and fixed with the fixation solution at room temperature for 10 min. The cells were then washed and incubated in staining solution mix overnight at 37℃. The cells were viewed under a light microscope and the cells with blue colour were regarded as senescent. The number of cells stained positive or otherwise for SA-β-gal was counted manually in five randomly chosen areas on each slide and the percentage of SA-β-gal positive cells were calculated considering all the readings from three independent experiments

Establishment of triple cell culture model of human BBB

Triple cell culture model of human BBB, composed of HBMECs, astrocytes and pericytes, was used throughout the study due to well-established crosstalk amongst different cell layers and thus its close functional resemblance to BBB *in vivo*.[17,30] To this end, approximately 1×10^5 HAs were seeded on the basolateral side of the transwell inserts (polyester membrane, 12 mm diameter, 0.4 μm pore size, Corning, UK). Once the cells adhered to the inserts, the inserts

were reverted to the original orientation and cultured in a fresh 12-well plate to 90% confluence. $5x10^4$ young or $10x10^4$ senescent HBMECs were then seeded onto the apical side of the inserts containing astrocytes. Since senescent cells lose their capacity to proliferate, it was essential to seed a significantly higher number of senescent HBMECs in these studies to promote appropriate monolayer formation. Once, full confluence is attained in both layers, the inserts were transferred to fresh 12-well plates containing confluent pericytes, to set up the triple cell culture model of human BBB.

Assessment of the BBB integrity and function

The integrity and function of the BBB were evaluated successively by measurements of the transendothelial electrical resistance (TEER) and the paracellular flux of sodium fluorescein (NaF, 376 Da) or Evan's blue-labelled albumin (EBA, 67 kDa). TEER was measured using a EVOM resistance meter and STX electrodes (World Precision Instruments, Hertfordshire, UK). After reading the TEER values, the inserts were rinsed with Hank's Balanced Salt Solution (HBSS, Sigma) and transferred into fresh 12-well plates containing 2 ml of HBSS. 500 µl of 50 µg/ml NaF or 165 µg/ml EBA was added in the luminal chamber of each insert and the plates with the inserts were incubated for 1 hour. After this, 400 µl of solutions from the luminal and abluminal chambers were collected and 100 µl of each solution was added into a 96-well plate in triplicate. Both NaF and EBA levels were measured using a FLUOstar Omega microplate reader (BMG Labtech Ltd, UK) and the flux across the barrier was calculated as before.[31]

Measurement of NADPH oxidase capacity and superoxide anion level

NADPH oxidase activity and superoxide anion production were assessed through lucigenin chemiluminescence and cytochrome C reduction assays, respectively as previously described.[30] Briefly, for the chemiluminescence assay, approximately 50 µg of BMEC homogenates were incubated at 37°C in assay buffer containing potassium phosphate buffer

(300 mM, pH 7.0, Sigma), ethylene glycol tetraacetic acid (50 mM, Sigma), sucrose (1 M, Sigma) and lucigenin (200 μM, Sigma). The contributions of nitric oxide synthase (NG-nitro-L-arginine methyl ester, 10 mM, Sigma), mitochondrial respiratory chain complex 1 (rotenone, 10 mM, Sigma), xanthine oxidase (allopurinol, 10 mM, Sigma) and cyclooxygenase (indomethacin, 10 mM, Sigma) to overall O_2^- production was eliminated by adding the specific inhibitors in brackets to assay buffer. Following ~15 min of incubation with these inhibitors, NADPH (100 μmol/L, Calbiochem, UK) was added to start the reaction which was monitored every minute for 2 hours and the rate of reaction was calculated using a luminometer (FLUOstar Omega, BMG Labtech, Aylesbury, UK).

For the cytochrome-*C* reduction assay, approximately 100 μg of homogenates were incubated for 1 h at 37°C with assay buffer containing HEPES (1 M, Calbiochem, UK), ethylene glycol tetraacetic acid (50 mM, Sigma), sucrose (1 M, Sigma), mannitol (1 M, Sigma) and cytochrome *C* (800 μM, Sigma). O_2^- production was then studied as the reduction of cytochrome *C* and monitored with the change in absorbance at 550 nm using a plate reader (FLUOstar Omega, BMG Labtech, Aylesbury, UK).

Proliferation assay

The proliferative capacity of HBMECs was evaluated by using the WST-1 (4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.3-benzene disulfonate) kit (Abcam, UK). In principle, the kit is used to quantify the cleavage of tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. So, the higher the absorbance readings are, the larger the numbers of viable cells are. In brief, $5x10^3$ HBMECs were seeded in 96-well plates and cultured for 24 h in a humidified atmosphere, as described above. The cells were then subjected to experimental conditions, and the media were subsequently replaced with 100 μL fresh medium containing 10 μL of WST-1. The plates were incubated for 2 h at 37°C prior to reading the absorbance (480 nm) using a FLUOstar Omega microplate reader (BMG Labtech Ltd, UK).

Tube formation assay

Tube formation assay was performed to evaluate the angiogenic capacity of the cells. For this, HBMECs were seeded in 96-well plates pre-coated with Matrigel (Corning, US) and incubated at 37℃ for 4 hours. The total number and length of tubule network, calculated as the sum of number or length of segments and branches detected in the analysed area, were assessed using Angiogenesis Analyzer plugin ImageJ software (version 1.52k, NIH, Maryland, USA). Images, acquired from 5 randomly selected fields of each well, were analysed under a light microscope.

Immunocytochemistry

To detect the localisation of tight junction proteins, occludin, claudin-5 and ZO-1, to examine the level of DNA damage marker, γ-H2AX and to evaluate the level of stress fibre formation, HBMECs (9×10^4) were seeded on glass coverslips and cultured to about 90% confluence in 12-well plates. The cells were then fixed and permeabilised with 4% paraformaldehyde for 20 minutes and 0.1% Triton X-100 for 5 minutes before incubating overnight at 4℃ with primary antibodies specific for tight junction proteins (1:200, all from ThermoFisher, UK) or γ-H2AX (1:250, Abcam, UK). On the following day, the coverslips were washed and incubated with relevant secondary antibodies for 1 h at room temperature. Nuclei were then detected in these cells by DAPI (4,6-diamidino-2-phenylindole) staining. To detect stress fibres, HBMECs were stained with rhodamine phalloidin (Abcam, UK) for 60 minutes at room temperature. The coverslips were then mounted on glass slides using mounting medium (Vector Laboratories, Peterborough, UK) and visualised by fluorescence microscopy (Zeiss Axio Observer, Carl Zeiss Ltd, Cambridge, UK). ImageJ software (version 1.52k, NIH, Maryland, USA) was used to quantify the florescence signal. The data were calculated as before and presented as corrected total cell fluorescence.[17] In brief, the junctional areas between cells were selected as the regions of interest (ROI) to measure the intensity of the fluorescent signal and the total fluorescent signals in each image were divided by the area of ROI to acquire the average fluorescent density

of the tight junction protein for each image. The number of nuclei stained positive or otherwise for γ-H2AX was counted manually in five randomly chosen areas on each slide and the percentage of γ-H2AX-positive cells were calculated considering all the readings from three independent experiments. The number of stress fibres traversing the cells were manually counted on five randomly selected areas on each slide and the number of stress fibres were comparatively analysed considering data gathered from three independent experiments.

Quantitative PCR

Genomic DNA from HBMECs were extracted with the Nucleon BACC1 Genomic DNA Extraction Kit (GE Healthcare, UK). Telomere length of the genomic DNA samples was measured by real-time PCR using the Absolute Human Telomere Length Quantification qPCR Assay Kit (ScienCell Research Laboratories, USA). The average telomere length on each chromosome end was calculated as per the manufacturer's instructions using the formulae based on the Cq value acquired by Agilent Mx3000P QPCR System (Santa Clara, USA).

Statistical analysis

Data are displayed as mean ± standard error of the mean (SEM) from a minimum of three independent experiments. Statistical analyses were performed by unpaired *t*-test. GraphPad Prism 9.0 statistical software package (GraphPad Software Inc., La Jolla, Ca, USA) and SPSS Statistics 27.0 (IBM, New York, USA) were used to perform these analyses. $P<0.05$ was considered as significant.

Results

Late-passage endothelial cells exhibit typical characteristics of cellular senescence

Given the non-specificity of markers used to detect senescence in *in vitro* settings, in the present study, a series of markers targeting cellular morphology, cell cycle progression and metabolic activity were simultaneously studied. Initial studies looking at the changes in endothelial phenotype showed that BMECs subjected to repetitive cell culture acquired larger and flattened

morphology and developed thick actin stress fibres traversing the cells at passage ≥19. In this context, relevant studies exploring the effects of BIRB-796, QNZ and VAS2870 or D+Q on phenotypic changes showed that treatments with these agents, except for QNZ, even at a reasonably late stage (from passage 16 (p16) to p20) lead to deceleration of morphological changes and stress fibre formation (**Figure 1a-c**).

Subsequent studies aiming to provide further evidence for BMEC senescence demonstrated significantly higher SA-β-gal activity in late-passage (p20) versus low-passage (p5) and mid-passage (p16) cells (**Figure 2a-b**). However, as other conditions e.g. serum starvation may also induce an increase in SA-β-gal activity,[32] the level of γ-H2AX, a sensitive marker for double-stranded DNA breaks, was also investigated in this study. As with the SA-β-gal activity, the γ-H2AX staining was also significantly higher in p20 BMECs compared to younger counterparts (**Figure 3a-b**). Similar to morphological studies, treatments with all agents, other than QNZ, drastically reduced the number of SA-β-gal-positive cells compared to senescent group. Even so, SA-β-gal activity remained well above the levels observed in p5 and p16 cells. Interestingly, while all agents significantly decreased γ-H2AX staining, the maximal effect was obtained with VAS2870 and QNZ (**Figures 2a-b and 3a-b**).

Scrutiny of cell proliferation as an index of cell cycle progression revealed a significant decline in BMEC proliferative capacity with increasing passage numbers. Despite halting these decreases, treatments with senolytics and senomorphics failed to return the proliferation rates to the levels seen in low-passage cells (**Figure 4a**). These studies also showed that treatments with QNZ significantly diminished the number of viable BMECs compared to controls i.e. p16 cells (**Figure 4b**). Because of this potential apoptotic effect, we did not pursue QNZ in the subsequent experiments. Moreover, due to a recent study illustrating that suppression of oxidative stress with vitamin C or VAS2870 delays EPC senescence and augments their

functional capacity, NADPH oxidase was also not targeted in the following functional studies.[19]

From this point on, BMECs with high proliferative capacity and less than 3% SA-β-gal positive staining were defined as young cells. These included cells up to and including p6. HBMECs that showed no sign of proliferation for a week in culture with complete medium and had over 70% SA-β-gal- and γ-H2AX-positive staining were defined as senescent. These included cells at and above p19.

Considering that telomeres play an essential role in preserving the integrity of chromosomes during the cell division cycle and undergo programmed shortening in culture conditions,[33] the telomere length was investigated by quantitative PCR and shown to be substantially shorter in senescent versus young cells. Inability of D+Q or BIRB-796 to influence telomere length suggested that telomeres were already irretrievably lost in p16 BMECs (**Figure 5a**).

In addition to above, senescent cells also possessed significantly greater levels of NADPH oxidase activity and $O_2^{\cdot -}$ production, supporting the notion that oxidative stress plays a pivotal role in the process of cell senescence.[15] Marked inhibitions observed in both enzyme activity and $O_2^{\cdot -}$ release, the foundation molecule of all ROS,[34] by VAS2870 confirmed that senescent cells were metabolically active and responsive to external stimuli (**Figure 5b-c**).

Senescent endothelial cells lose their angiogenic potential and fail to form BBB

Angiogenesis is a vital process by which new blood vessels form and thus contribute to growth, development and regeneration of the damaged tissue.[35] As evidenced by significant decreases in number and length of new tubules appeared on matrigel, senescence negatively affected the capacity of ECs for angiogenesis. Here, suppression of p38MAPK by BIRB-796, rather than

elimination of senescent cells via D+Q, appeared to be more effective in diminishing the impact of senescence on tubule formation in *in vitro* settings (**Figure 6a-c**).

Through lateral migration and consequent replacement of dead or dying ECs, resident ECs play an important role in preventing structural and functional vascular damage.[6] The data generated up to this point suggested that senescent BMECs were unlikely to be as protective or functional as their younger counterparts. The overt inadequacy of senescent BMECs to form a functional model of human BBB, evidenced by decreased transendothelial electrical resistance (TEER) and increased paracellular flux of sodium fluorescein (NaF) across the barrier, supported this hypothesis. Interestingly, treatments with BIRB-796 and D+Q completely neutralised the disruptive effects of senescence on cerebral barrier. Contrary to increased permeability of NaF, the flux of Evans blue-labelled albumin (EBA), a high molecular weight permeability marker, remained unchanged across all experimental conditions thereby implying the formation of relatively small inter-endothelial cell openings between senescent BMECs (**Figure 7a-c**).

Tight junctions are disrupted in replicative senescent endothelial cells

To determine whether possible changes in tight junctional complex formation might explain the abovementioned BBB-related findings, subcellular localisation and abundance of ZO-1, occludin and claudin-5 were investigated. Since senescent cells lost their capacity to proliferate, a significantly higher number of senescent cells were seeded in these studies to promote appropriate monolayer formation. Intermittent interruptions in plasma membrane staining of ZO-1 offered some explanation for the impaired integrity and function of the BBB established with senescent BMECs. Unlike ZO-1, occludin and claudin-5 were found to locate mostly to cytoplasm and nuclei in young and senescent cells, respectively. Although treatments with BIRB-796 and D+Q could somewhat prevent the loss of ZO-1 in cells undergoing senescence, they could not prevent the emergence of abovementioned phenotypic changes i.e. larger and flattened morphology. Neither BIRB-796 nor D+Q altered the subcellular localisation of

occludin or claudin-5 observed in senescent cells despite returning the level of the former protein to the levels seen in young cells without affecting that of the latter (**Figure 8a-b**).

Discussion

BBB regulates the passage of an array of selective components between brain parenchyma and the systemic circulation.[5-7] Due to its pivotal roles in nutrient supply and waste disposal, it is essential to monitor the function of BBB at all times to conserve neurovascular homeostasis. Increased permeability of the BBB is implicated in chronological ageing and can be severely neurotoxic.[36,37] Indeed, continual detection of senescent cells at specific sites of age-related pathologies, like atherosclerosis, confirms the idea that cellular senescence contributes to organismal ageing and age-related pathologies.[12,14] Although formed through complex interactions between neurovascular cells and the underlying basement membrane, dysfunction of BMECs alone may be sufficient to compromise BBB integrity during the ageing process.[38] Bearing these in mind and using a cell culture model of chronological ageing, the current study specifically assessed the impact of BMEC senescence on BBB integrity and function. Relevant studies reporting a strong inverse correlation between donor age and propagative lifespan of skin fibroblasts confirm that replicative cellular senescence occurs during chronological ageing and can be reliably used as an experimental model to explore the molecular mechanisms of ageing.[6,39]

Given the non-specificity of commonly used markers for senescence, this study, as indicated before, employed a panel of interrelated markers aiming to detect changes cell phenotype, cell cycle progression and metabolic activity.[40] Since accurate identification of specific mechanisms involved in BMEC senescence and dysfunction is critically important for strict regulation of ageing-related cerebrovascular alterations and clinical conditions, such as vascular dementia, this comprehensive approach was of paramount importance.[41] Acquisition of a flatter and enlarged morphology in cells that ceased to proliferate, in culture media

supplemented with all necessary growth factors, confirmed that senescence occurred in BMEC after about 19-20 population doublings. Marked reductions in protein degradation and RNA turnover, leading to increases in intracellular RNA and protein content may, to a degree, account for the cellular enlargements observed in this study. Persistent DNA damage response arising from the exhaustion of DNA replication cycles and telomere shortening due to repetitive cell culture may also contribute to cellular enlargements by activating transcription factor p53 and cyclin-dependent kinase inhibitor p21 and thus blocking the cell cycle at G1-S interphase.[42] Further scrutiny of cellular morphology by analyses of cytoskeletal organisation through actin microfilaments staining verified that senescent cells took up a larger and flattened phenotype and developed thick actin stress fibres traversing the cells. The cytoskeleton primarily helps cells maintain their shape and internal organisation which enables cells to perform essential functions like proliferation and motility. Under normal circumstances, the cortical actin bands participate in formation of tight junctions and prevent paracellular flux. Once formed, thick contractile actin stress bundles pull junctional proteins inward whereby break-up junctional complexes and create intercellular gaps in that activations of small GTP-binding protein RhoA and transcriptional factor NF-κB play a role.[43,44] Detection of remarkably higher SA-β-gal staining, NADPH oxidase activity, $O_2^{\cdot -}$ production and γ-H2AX deposition coupled with permanent DNA damage in cells manifested morphological changes substantiates the notion that senescence is a complex multifactorial process which affects and is affected by various biological phenomena, e.g. oxidative stress.[15,33]

Despite availability of nutrients, growth factors and space to grow into, quiescence of senescent BMEC indicates that senescent cells also develop functional abnormalities. These are often associated with survival and cell division. Given that proliferation and migration of BMECs are important prerequisites for replacement of the dead or dying ECs, age-dependent progressive loss of BMECs and their functionality are linked to perturbation of vascular

integrity and homeostasis.[6] Accumulation of metabolically active senescent BMECs in cerebrovasculature can further worsen the vascular impairment by adversely affecting the function of circulating EPCs through agents, including ROS, cytokines and chemokines that they synthesise and release.[15] By differentiating into mature ECs and through their paracrine effect, EPCs are known to assist vascular re-endothelialisation and regeneration.[6,11,19]

Attenuation of angiogenesis by senescent BMECs may also exacerbate cerebrovascular damage. Observation of fewer and shorter tubule formation on matrigel with senescent BMECs proved that senescence adversely affects angiogenesis possibly through a mechanism involving the inhibition of certain pro-angiogenic factors.[45,46] Failure of senescent BMECs to establish a functional model of BBB, when co-cultured with astrocytes and pericytes, provided further evidence for the dysfunctionality of senescent cells. In support of a recent study documenting that mixing replicative senescent EPCs with young BMECs elicits BBB damage in *in vitro* settings[19] this study also showed a substantial breakdown in integrity and function of BBB established with senescent BMECs and attributed this to disintegration of ZO-1 staining in plasma membrane of senescent cells. Considering that ZO-1 mediates the connections between actin cytoskeleton and other tight junction proteins, these relatively small sporadic disruptions may conceivably have a huge impact on overall BBB function.[47] Decreased availability and somewhat unexpected cytoplasmic and nuclear localisation of claudin-5 and occludin in senescent BMECs may also contribute to BBB dysfunction. Cytoplasmic and nuclear localisation of occludin has previously been noted in other cerebral cells, such as astrocytes and neuroepithelial cells. In these cells, occludin appeared to play crucial roles relating to RNA metabolism and nuclear functions.[48] In another study, non-plasma membrane localisation of tight junction proteins has been correlated with the decreased telomerase activity in senescent cells.[49] While substantially reduced telomere length in senescent BMECs may shed some light on the non-plasma membrane localisation of occludin and claudin-5 observed in this study,

these findings cannot satisfactorily explain the complete disappearance of these proteins in BMEC plasma membrane.

To assess whether manipulation of senescence may extend the life span and proliferative capacity of ECs, we treated relatively old but non-senescent BMECs (p16) with a cocktail of senolytics, D+Q or the inhibitors of p38MAPK, NF-κB or NADPH oxidase. Due to its role in senescence growth arrest via activation of p53 and pRb/p16 pathways, p38MAPK has previously attracted some attention.[23] In the present study, attenuation of senescence-related changes in cellular morphology, proliferative capacity, tubulogenesis, SA-β-gal activity, γ-H2AX staining and complete restoration of BBB function with BIRB-796 proposes p38MAPK signaling pathway as a potential therapeutic target for the management of chronological senescence. Inhibition of p38MAPK may also halt the spread of senescence to the neighbouring cells through suppression of SASP via a mechanism involving NF-κB transcriptional activity.[20] Hence, treatments with an NF-κB inhibitor i.e. QNZ rather unsurprisingly diminished the impact of senescence on cell morphology, stress fibre formation and γ-H2AX staining. However, significant drops in number of viable cells in QNZ-treated experimental groups casts serious doubt on the applicability of NF-κB inhibitors as efficacious therapeutics.

Oxidative stress, emerging from an imbalance between the pro- and anti-oxidants, represents the most commonly encountered pathology in senescent cells.[15] Although lower (physiological) levels of O_2^- regulate cell proliferation, migration and gene expression, prolonged exposure to high levels of O_2^- and other ROS promotes senescent phenotype through damaging all major macromolecules i.e. DNA, protein and lipid.[15,18,35] Naturally, suppression of NADPH oxidase, the main enzymatic source of endothelial O_2^-, by VAS2870 effectively delayed the appearance of senescent phenotype, improved proliferative potential of p16 BMECs and corroborated the results of a recent study exhibiting delay of EC senescence and BBB dysfunction with VAS2870.[19] Restoration of normal endothelial function in dysfunctional arterial segments with

NADPH oxidase inhibitors and ROS scavengers further affirm the role of NADPH oxidase, O_2^- and oxidative stress in endothelial dysfunction.[50-52]

Selective removal of senescent cells by senolytics is thought to subside the burden of these cells on their microenvironment (neighbouring cells) and remote tissue and improve cerebrovascular function as a consequence. The combination of D+Q is regarded as an effective cocktail of senolytics with significant anti-ageing effects.[53] While dasatinib, a small-molecule tyrosine kinase inhibitor, induces apoptosis and attenuates the viability of senescent adipocytes,[28] quercetin displays potent anti-inflammatory, anti-oxidant and immunoprotective effects and therefore alleviates platelet aggregation, oxidative stress and vascular permeability.[54] Once combined, D+Q exerts further beneficial effects, reduces secretion of pro-inflammatory cytokines, prevents the accumulation of macrophages, attenuates the production of major SASP factors and potentiates the proliferative capacity of progenitor cells to reduce the burden of senescent cells in skin and adipose tissues.[26,28,55] In accordance with these observations, treatments with D+Q, in the current study, markedly delayed BMEC senescence and improved their proliferative, angiogenic and barrier-forming capacity.

There are some limitations to this study. As alluded above, despite using a variety of antibodies raised against different epitopes of occludin and claudin-5, we could not visualise these proteins on BMEC plasma membrane. Albeit in full agreement with the manufacturing companies' immunocytochemistry reports for the respective proteins, the lack of plasma membrane staining was unexpected and deserve further attention in future studies. It is of note here that 23 proteins have thus far been recognised as the members of claudin family in human tissues. Although claudin-1, claudin-12, claudin-25, claudin-34c1 also appear to be involved in the formation of cerebral tight junctions, claudin-5 constitutes the main family member in brain capillary tissues and is closely associated with the formation and functionality of the BBB.[56-59] Hence, this particular protein has been targeted in the present study. However, it is

possible that investigation of other aforementioned family members may have yielded plasma membrane staining. Given that p38MAPK/NF-κB pathway acts in part through regulation of SASP, it would have been useful to study the impact of BIRB-796 and D+Q on the composition of SASP. In future, it is important to confirm the correlative link between EC senescence and age-related BBB dysfunction *in vivo*. Similarly, the efficacy of senolytics and senomorphics need to be explored in the future translational studies to substantiate the link between EC senescence and chronological ageing. However, given the lack of cell-specificity of senolytics and senomorphics at the present time, relevant future studies must either employ an *in vivo* model of BMEC dysfunction or utilise agents that are specific for this particular cell line to distinguish the effects of BMEC senescence from those of peripheral senescence.

In conclusion, this experimental study supports the concept that ECs go into senescence during ageing process which ultimately damages the integrity of the BBB. The study reports that inhibition of p38MAPK and removal of senescent cells by senolytics successfully delay the senescence of BMEC and fully restore BBB integrity and function. Taken together, these findings pave the way for the development of effective novel strategies that may delay or abate age-related BBB dysfunction and improve lifelong health and wellbeing as a consequence.

Declarations

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Data Availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors Contributions

JY performed most of the experiments and carried out the data analysis. RRA performed oxidative stress-related studies. UB designed and supervised the study, interpreted the data and wrote the manuscript. All authors approved the final version for publication.

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Figure legends

Figure 1. Exposure of human BMECs to an *in vitro* model of chronological ageing, mimicked by replicative senescence, induces profound alterations in cellular morphology (a) and cytoskeletal organisation compared to young cells (b). Compared to young cells (p5), relatively old (p16) and senescent cells (p20) possess larger and flattened morphology and thick actin stress fibres. Treatment of p16 cells with inhibitors for p38MAPK (BIRB-796), NF-κB (QNZ), NADPH oxidase (VAS2870) or a cocktail of senolytics (dasatinib and quercetin; D+Q) until p20 attenuated the effect of replicative senescence on both morphology (a) and cytoskeletal organisation as ascertained by reductions in number of stress fibres indicated by white arrows (b, c). Scale bars: 100 μm. The images of cellular morphology and stress fibres were captured using x20 and x40 magnification. Data are expressed as mean±SEM from three independent experiments. **P*<0.05 compared to p5 BMECs. $^{\#}$ *P*<0.05 compared to p20 BMECs.

Figure 2. The number SA-β-gal positive cells increases by replicative senescence. Exposure of human BMECs to repetitive cell culture markedly increased the number of SA-β-gal-positive cells in late passage (p20) cells compared to young (p5) and mid-passage (p16) cells. Treatment of p16 cells with senomorphics targeting p38MAPK (BIRB-796) or NADPH oxidase (VAS2870) or a cocktail of senolytics (dasatinib and quercetin; D+Q) until p20 diminished the number of cells that underwent senescence. Treatment with QNZ, an NF-κB inhibitor significantly elevated the number of SA-β-gal-positive cells (a, b). Data are expressed as mean±SEM from three independent experiments. The SA-β-gal stainings were visualised using 20x magnification. **P*<0.05 compared to p5 BMECs. $^{\#}$*P*<0.05 compared to p20 BMECs.

Figure 3. Treatments with senotherapeutics diminish senescence-induced DNA damage. Replicative senescence, realised by repetitive cell culture, markedly increased the percentage of γ-H2AX-positive cells in late passage (p20) human BMECs compared to young (p5) and

mid-passage (p16) cells. Treatments of p16 cells with senomorphics targeting p38MAPK (BIRB 796), NF-κB (QNZ) or NADPH oxidase (VAS2870) or senolytics (dasatinib and quercetin; D+Q) until p20 diminished the percentage of γ-H2AX-positive cells (a, b). Scale bars: 50 μm. The γ-H2AX stainings were visualised using 40x magnification. Data are expressed as mean±SEM from three independent experiments. **P*<0.05 compared to p5 BMECs. $^{\#}$ *P*<0.05 compared to p20 BMECs.

Figure 4. Senotherapeutics reduce the impact of senescence on BMEC proliferative capacity. The proliferation rate of BMECs, assessed by WST-1 assay, decreased with increasing passage numbers. Treatments of p16 cells with senomorphics targeting p38MAPK (BIRB 796), NF-κB (QNZ) or NADPH oxidase (VAS2870) or senolytics (dasatinib and quercetin; D+Q) until p20 significantly decreased the suppressive effect of senescence on proliferation (a). However, treatments with QNZ significantly reduced the number of viable cells compared to p16 cells, control group (b). Data are expressed as mean±SEM from three independent experiments. **P*<0.05 compared to p5 BMECs. $^{\#}$ *P*<0.05 compared to p20 BMECs. †*P*<0.05 compared to p16 BMECs.

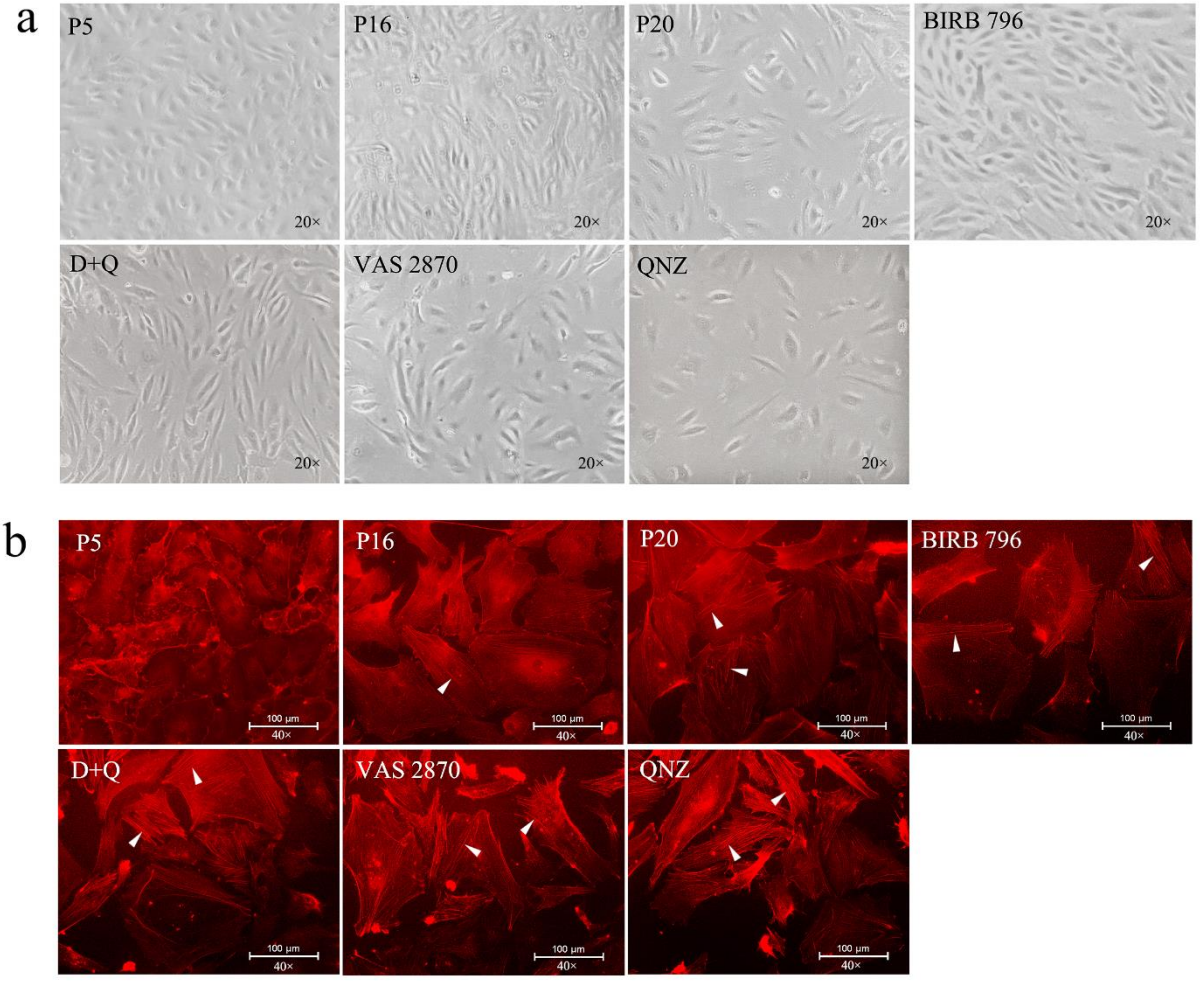
Figure 5. Senescence is associated with reduced telomere length and increased oxidative stress. Compared to young cells (p5), the average telomere length is markedly reduced in senescent (p20) cells. Treatments of p16 cells with BIRB-796, a p38MAPK inhibitor and a senomorphic or a cocktail of senolytics (dasatinib and quercetin; D+Q) until p20 failed to negate the effect of senescence on telomere length (a). Increases in NADPH oxidase activity and superoxide anion generation confirm that senescent cells have oxidative stress. Treatment with VAS2870, an inhibitor of NADPH oxidase, neutralise the effect of senescence on both parameters (b, c). Data are expressed as mean±SEM from three independent experiments. **P*<0.05 compared to p5 BMECs. $^{\#}$ *P*<0.05 compared to p20 BMECs.

Figure 6. Senescence adversely affect the angiogenic capacity of BMECs. Senescent (p20) BMECs displayed significantly fewer and shorter tubules on matrigel compared to young (p5) cells. Treatment with senomorphic BIRB-796, a p38MAPK inhibitor, increased the number and length of tubules. In contrast, treatment with a combination of senolytics (dasatinib+quercetin, D+Q) only improved the number of tubules without affecting their length (a-c). The tubules were visualised using 10x magnification *P<0.05 compared to p5 BMECs. #P<0.05 compared to p20 BMECs.

Figure 7. The presence of senescent BMECs adversely affect blood-brain barrier integrity and function. Co-culture of senescent (p20), but not young (p5), BMECs with astrocytes and pericytes in an *in vitro* model of human BBB decreased barrier integrity (a) and increased paracellular flux of a low molecular weight (NaF) but not high molecular weight (EBA) permeability marker across the barrier (b, c). Treatments with senomorphic BIRB-796, a p38MAPK inhibitor and a cocktail of senolytics (dasatininb and quercetin, D+Q) neutrailised the deleterious effects of senescence on both BBB integrity and function (a-c). Data are expressed as mean±SEM from six independent experiments *P<0.05 compared to p5 BMECs. #P<0.05 compared to p20 BMECs.

Figure 8. Senescence is associated with disruptions in tight junction protein localisation and expression. Compared to young (p5) cells, the plasma membrane localisation of ZO-1 is interrupted in senescent (p20) human BMECs which was somewhat prevented by treatments with senomorphic BIRB-796, a p38MAPK inhibitor or a cocktail of senolytics (dasatinib+quercetin, D+Q) (a). Unlike ZO-1, both occludin and claudin-5 appear to localise to cytosol and nuclei and treatments with either agent did not greatly influence their subcellular localisation (a). Senescence reduced the abundance of all three proteins in BMEC. Treatments with BIRB-796 and D+Q returned the level of occludin to those seen in p5 cells. They

somewhat improved the level of ZO-1 but failed to alter that of claudin-5. Data are expressed as mean±SEM from three independent experiments. Scale bars: 50 μm. All images were captured using 40x magnification. *P<0.05 compared to p5 BMECs. $^{\#}P$<0.05 compared to p20 BMECs.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VAS 2870
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Number of stress fibre per cell
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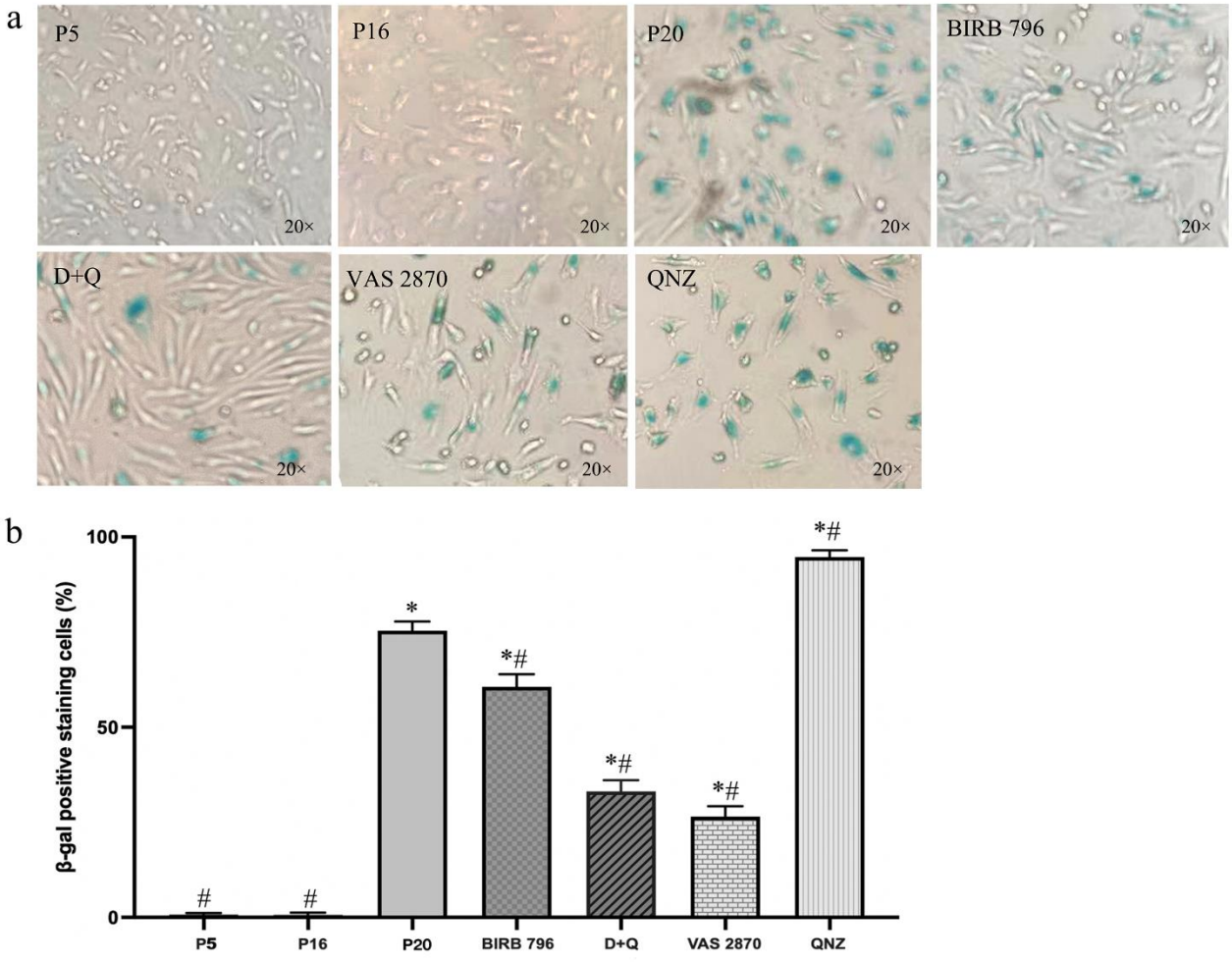
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β-gal positive staining cells (%)
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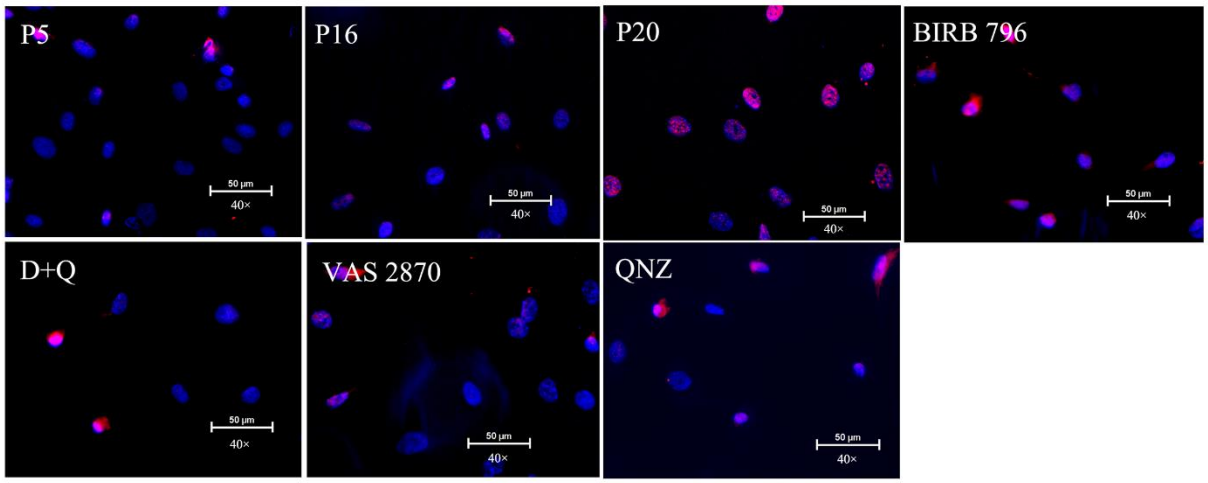
a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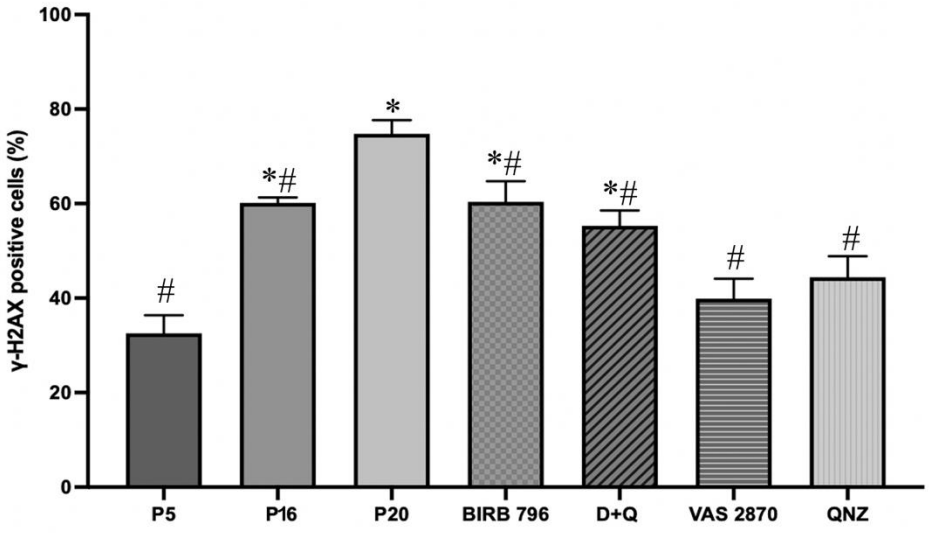
b


γ-H2AX positive cells (%)
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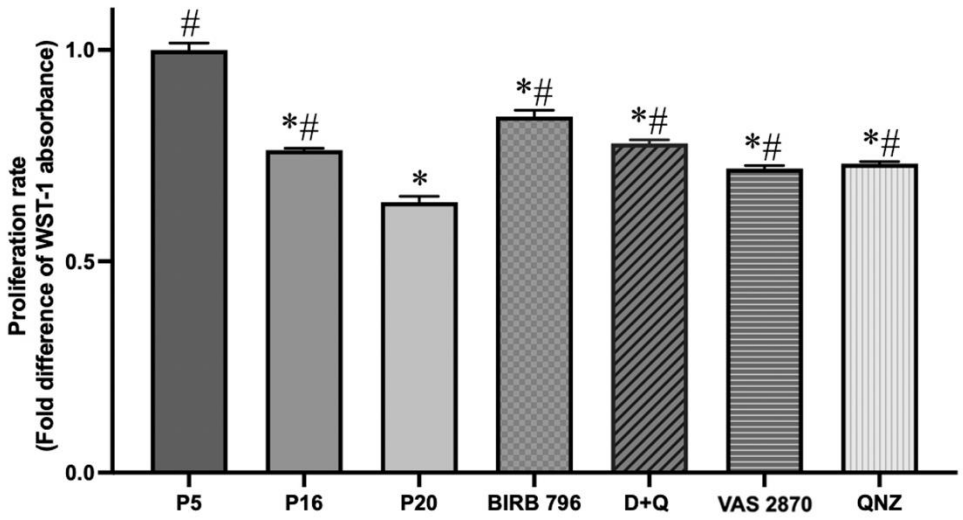
Proliferation rate
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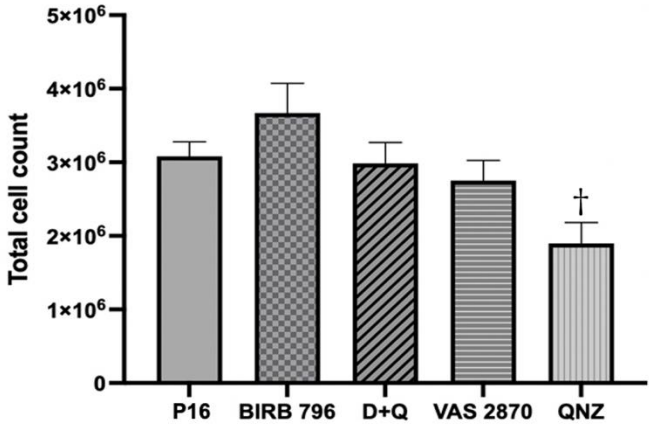
Total cell count
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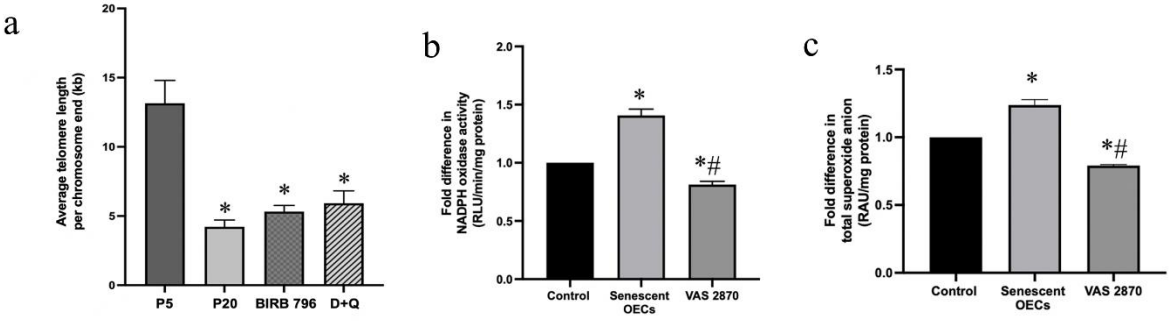
a
Average telomere length per chromosome end (kb)
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BIRB 796
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Fold difference in NADPH oxidase activity (RLU/min/mg protein)
2.0
1.5
1.0
0.5
0.0
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Control
Senescent OECs
VAS 2870
c
Fold difference in total superoxide anion (RAU/mg protein)
1.5
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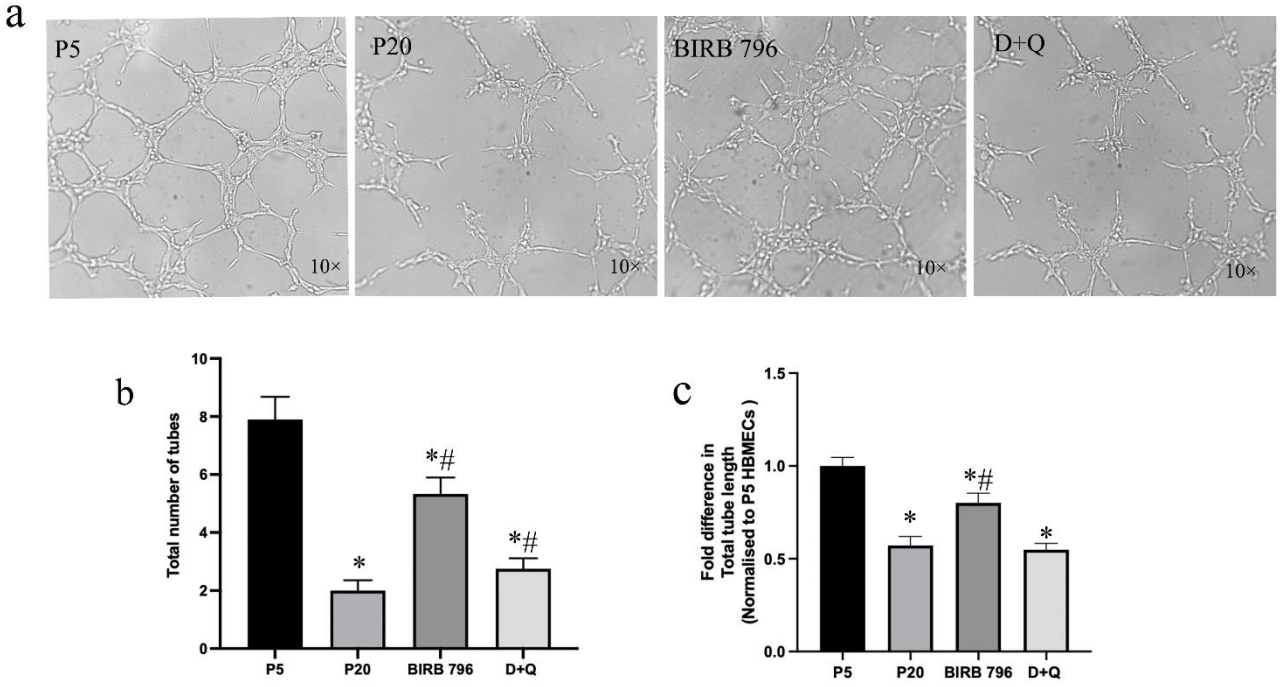
Senescent OECs
VAS 2870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D+Q
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b
Total number of tubes
10
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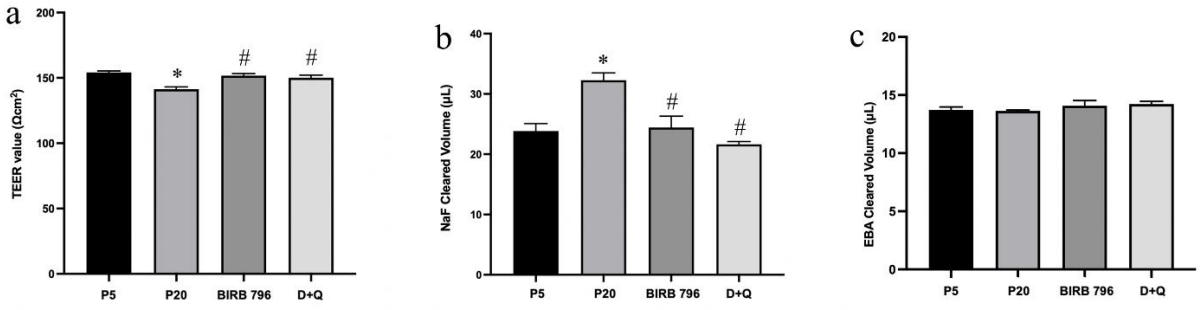
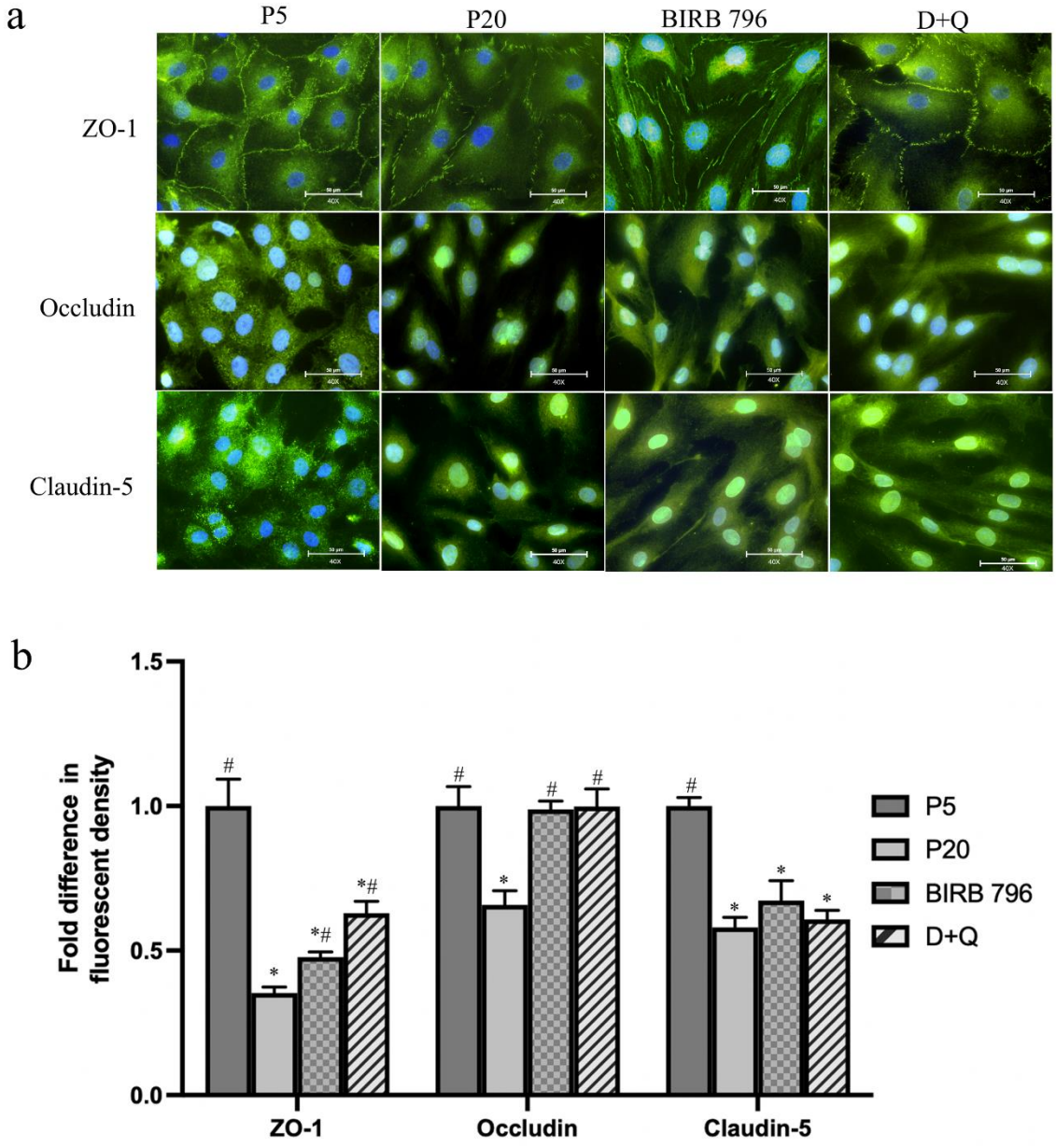
2
0
*
*#
*#
P5
P20
BIRB 796
D+Q
c
Fold difference in Total tube length (Normalised to P5 HBMECs)
1.5
1.0
0.5
0.0
*
*#
*
P5
P20
BIRB 796
D+Q


a
TEER value (Ωcm²)
200
150
100
50
0
*
#
#
P5
P20
BIRB 796
D+Q
b
NaF Cleared Volume (µL)
40
30
20
10
0
*
#
#
P5
P20
BIRB 796
D+Q
c
EBA Cleared Volume (µL)
20
15
10
5
0
P5
P20
BIRB 796
D+Q


a
P5
P20
BIRB 796
D+Q
ZO-1
Occludin
Claudin-5
b
Fold difference in fluorescent density
1.5
1.0
0.5
0.0
ZO-1
Occludin
Claudin-5
P5
P20
BIRB 796
D+Q