Title

Selective PDE1 inhibition ameliorates vascular function, reduces inflammatory response, and lowers blood pressure in ageing animals


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List of abbreviations:

cGMP: cyclic guanosine monophosphate
ERCC: Excision repair cross-complementing
PDE: Phosphodiesterase
NO: Nitric oxide
L-NAME: L-NG-Nitro arginine methyl ester
EDH: Endothelium-dependent hyperpolarization
ACh: Acetylcholine
SNP: Sodium nitroprusside
KCl: Potassium chloride
VASP: Vasodilator-stimulated phosphoprotein
VSMC: Vascular smooth muscle cell
ADP: Adenosine diphosphate
IL: Interleukin
IFN-γ: Interferon-gamma
TNF-α: tumor necrosis factor-alpha
eNOS: Endothelial nitric oxide synthase

ODQ: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
Abstract

Diminished nitric oxide – cGMP-mediated relaxation plays a crucial role in cardiovascular ageing, leading to decreased vasodilation, vascular hypertrophy and stiffening, and ultimately cardiovascular dysfunction. Ageing is the time-related worsening of physiological function due to complex cellular and molecular interactions, and is at least partly driven by DNA damage. Genetic deletion of the DNA repair enzyme ERCC1 endonuclease in Ercc1Δ/Δ mice provides us an efficient tool to accelerate vascular ageing, explore mechanisms, and test potential treatments. Previously we identified the cGMP-degrading enzyme phosphodiesterase 1 as a potential treatment target in vascular ageing. In the present study, we studied the effect of acute and chronic treatment with ITI-214, a selective phosphodiesterase 1 inhibitor on vascular ageing features in Ercc1Δ/Δ mice. Compared to wild-type mice, Ercc1Δ/Δ mice at the age of 14 weeks showed decreased reactive hyperemia, diminished endothelium-dependent and –independent responses of arteries in organ baths, carotid wall hypertrophy, and elevated circulating levels of inflammatory cytokines. Acute ITI-214 treatment in organ baths restored the arterial endothelium-independent vasodilation in Ercc1Δ/Δ mice. An 8-week treatment with 100 mg/kg/d ITI-214 improved endothelium-independent relaxation in both aorta and coronary arteries, at least partly restored the diminished reactive hyperemia, lowered the systolic and diastolic blood pressure, normalized the carotid hypertrophy, and ameliorated inflammatory responses exclusively in Ercc1Δ/Δ mice. These findings suggest PDE1 inhibition would provide a powerful tool for nitric oxide – cGMP augmentation and have significant therapeutic potential to battle arteriopathy related to ageing.

Key Words: Phosphodiesterase 1; DNA damage; nucleotide excision repair, Ercc1; ageing; NO – cGMP pathway; vascular dysfunction; hypertension; cardiovascular disease; mice.

Significance Statement:

The findings implicate the key role of PDE1 in vascular function and might be of clinical importance for the prevention of mortalities and morbidities related to vascular complications during ageing, as well as for progeria patients that show a high risk of cardiovascular disease.
Introduction

Ageing is the time-related worsening of physiological function due to complex cellular and molecular interactions. The substantial increase in the average human life span brought by improved nutritional, sanitary, and healthcare conditions over the last decades is accompanied by an elevation in the significant age-related cardiac, arterial and microvascular disease burden in a growing number of countries worldwide. Focusing on the vasculature, such pathological conditions include worsened vasodilation, increased arterial stiffness, reduced blood flow in small resistance arteries, and dysfunctional endothelium (Donato et al., 2018; Fajemiroye et al., 2018; Oue et al., 2018).

The onset of cardiovascular disease (CVD) is triggered by vascular alterations characterized by impaired vasodilation and the overproduction of inflammatory markers. A strong body of evidence from epidemiological studies shows that ageing itself is the major independent risk factor for cardiovascular diseases (Versari et al., 2009; Widmer and Lerman, 2014; Bautista et al., 2015; Almeida et al., 2017). Unlike risk factors such as dyslipidemia and hypertension, for which suitable models exist to study their primary risk contributions to vascular dysfunction and response to prevention or intervention maneuvers, ageing lacks such models and has thus been harder to study. This limitation was recently overcome by the discovery that accumulating DNA damage is a central causative mechanism for ageing, along with the development of accelerated ageing mouse models based on genetically reduced DNA repair (Wu et al., 2017; Golshiri et al., 2020). In several of these models, risk factor-free accelerated vascular ageing was demonstrated. One such model, the Ercc1Δ/Δ mouse, lacks the proper function of ERCC1 (an endonuclease that plays a role in the repair of helix-distorting DNA adducts, cross-links, and homologous recombination), and develops all major non-atherosclerotic vascular ageing features found in elderly humans (Wood, 2010; Faridounnia et al., 2018). This includes increased blood pressure and vascular stiffness, and reduced vasodilation (Durik et al., 2012). The model has been used as a convenient tool to test the effects of dietary and pharmacological interventions. We have used this model to explore mechanisms of aberrant nitric oxide (NO) - cGMP signaling in smooth muscle cells, which plays a central role in vascular ageing (Wu et al., 2017; Golshiri et al., 2020).

In ageing-related CVD, the second messenger signaling molecules 3′, 5′-cyclic adenosine and guanosine monophosphate (cAMP, cGMP) play a vital role, being involved in dysfunctional vasomotor activity, vessel tone, permeability, proliferation, and fibrosis. Both cAMP and cGMP are also implicated as negative mediators of the transformation of quiescent cardiac fibroblasts to active myofibroblasts (Yoshifumi et al., 1996; Fukuhara
et al., 2005; Insel et al., 2012; Golshiri et al., 2019; Ehsan Ataei et al., 2020). The levels of these second messengers are determined by the balance between their formation and the phosphodiesterase (PDE) enzymes that catalyze their hydrolysis and inactivation. PDE’s consist of 11 main members expressed as more than 100 isoforms that are encoded by at least 21 distinct genes and classified based on substrate specificities and regulatory factors. The cell and tissue-specific expression of PDE’s provide a great opportunity for better organ targeting and more specific therapeutic interventions (Lugnier, 2006; Ehsan Ataei et al., 2020). In Ercc1Δ−/− mice, in combination with human studies, we identified PDE1 as a potential contributor to vascular ageing (Bautista et al., 2015). The PDE1 family members are dual-substrate enzymes that hydrolyze and inactivate both cGMP and cAMP and are activated by a Ca\(^{2+}\)/calmodulin-binding domain (Ehsan Ataei et al., 2020). PDE1 is expressed as three identified isoforms (1A, 1B, and 1C). It is highly expressed in the smooth muscle cell, and appears to contribute to the lack of vasodilation capacity in Ercc1Δ−/− arteries, as demonstrated with the non-selective PDE inhibitor vinpocetine (Bautista et al., 2015). The potentially important role of PDE1 in vascular function and its correlation with several risk factors in cardiovascular diseases, including heart pressure-overload, hypertrophy, pulmonary arterial hypertension (PAH), and post-myocardial infarction, has been highlighted in several key studies (Fiona et al., 2007; Miller et al., 2009; Miller et al., 2011; Hashimoto et al., 2018; Dey et al., 2020). Systematic research employing potent and selective PDE1 inhibitors in animal models of the accelerated phenotype of ageing is currently lacking. However, the recent development of the highly selective PDE1 inhibitor, ITI-214, which possesses a similar affinity for all three isoforms of PDE1, and is well-tolerated in human safety studies, and under clinical development for the treatment of heart failure and neurodegenerative disease, has opened new opportunities (Li et al., 2016; Snyder et al., 2016; Wennogle et al., 2017; Hashimoto et al., 2018; Pekcec et al., 2018).

Considering our recent findings on the role of PDE1 in reduced vasodilation in Ercc1Δ−/− accelerated ageing mice and findings of others in healthy mice (Bautista et al., 2015), we hypothesized that selective PDE1 inhibition could reduce blood pressure, improve vascular function and ameliorate the resistance in small arteries through elevation of cAMP and cGMP second messengers. In this study, we demonstrate that acute and chronic treatment with the PDE1 selective inhibitor ITI-214 can improve age-dependent vascular function and reduces the elevated level of inflammatory cytokines in plasma, suggesting a potential general mechanism of improved signaling through inhibition of PDE1.
Material and methods

Drugs and reagents: All chemicals and reagents were purchased from Sigma-Aldrich and ITI-214 was provided by Intra-Cellular Therapies.

Animals: Ercc1Δ−/− and Ercc1+/+ F1 mice with a hybrid C57BL6J::FVB background were generated by cross-breeding of parents with a pure C57BL6J and FVB background. The genotype and phenotype features of the model have been described before (Weeda et al., 1997; Vermeij et al., 2016). In short, in Ercc1Δ−/− mice the ERCC1 gene is not completely deleted, it is partially inactivated by introducing, into the encoded protein, a carboxy-terminal UvrC-homologous region. The encoded protein contains a seven amino-acid carboxy-terminal truncation (equivalent to deleting six residues of the human protein). The ERCC1 knockout mice all die before day 38, whereas the maximum life span of Ercc1Δ−/− mice is considerably longer (6 months). The hybrid background of the experiment mice prevents strain-specific phenotypes. Breeding was performed at the Erasmus MC animal facility. Mice were housed in individually ventilated cages in a controlled environment (20–22°C, 12h light:12h dark cycle) with access to normal chow and water ad libitum. The animals were weighed and visually inspected every day to warrant their well-being. All animal studies were performed in accordance with the Principles of Laboratory Animal Care and with the guidelines approved by the independent Dutch Animal Ethical Committee.

Study design: In total, 34 Ercc1Δ−/− mice at the age of 6 weeks, and 28 of their WT littermates (Ercc1+/+) of the same age were randomized into four groups: one group of Ercc1Δ−/− mice and one of the WT were given normal drinking water; another group of Ercc1Δ−/− and WT mice received the PDE1 inhibitor ITI-214 (100 mg/kg/day) via drinking water for 8 weeks. Both male and female animals were used. Blood pressure and superficial blood flow were measured one week prior to sacrifice. At the age of 14 weeks, the mice were sacrificed, the tissues were collected, snap-frozen, and wire myography and pressure myography experiments were done.

Sacrifice and tissue harvest: At the age of 14 weeks, the animals were sacrificed by cardiac puncture and lethal blood withdrawal from the vena cava under anesthesia. Blood was centrifuged at 2500 rpm at 4° C for 10 minutes and plasma was collected and stored at -80 ° C. The thoracic aorta, coronary and carotid artery were carefully isolated and stored in cold Krebs-Henseleit buffer solution for organ bath experiments.
Wire myography: The thoracic aorta was isolated and cleaned in cold oxygenated (with 95% O₂ and 5% CO₂) Krebs-Henseleit buffer solution (in mmol/L: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 8.3; pH 7.4) for ex vivo wire myography experiments. Two-mm segments of the thoracic aorta were mounted in 6-mL chambers of wire myography device (Danish Myograph Technology, Aarhus, Denmark). After the normalizing procedure, the maximum contractile responses were determined using 100 mmol/L KCl. After 4 times washing steps with a 5-minute interval for each step, 30 nmol/L U46619 or 30 mmol/L KCl were applied to preconstrict the vessel segments and evaluate the relaxation concentration-response curves (CRCs) to acetylcholine (ACh) and sodium nitroprusside (SNP) (respectively). The two-mm coronary segments were also mounted following the same procedure, the maximum contractile responses were determined using 60 mmol/L KCl, and CRCs to ACh and SNP were performed after preconstriction with 30 nmol/L U46619. L-NAME 100 μmol/L, TRAM34 10 μmol/L, and apamin 100 nmol/L were given 10 minutes before U46619, to investigate the involvement of nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) pathway in the relaxation responses (Golshiri et al., 2020). To assess the acute effects of PDE1 inhibition, 100 nmol/L ITI-214 was added to the organ bath 15 minutes before preconstriction.

Analysis of ITI-214 Levels in Plasma: ITI-214 levels were determined in the mouse plasma using liquid-liquid extraction with subsequent high-performance liquid chromatography and tandem mass spectrometry detection. A nominal concentration range of 1-1000 ng/mL for ITI-214 was chosen to quantitate the study samples. Samples were kept frozen at -70 ± 10 °C prior to analysis. A 20 μL matrix aliquot was diluted with 100 μL water, followed by extraction using acetonitrile fortified with 1 ng/mL of internal standard and 1% of formic acid. Analytes were isolated through an Ostro sample preparation plate. The eluate was injected into a Shimadzu HPLC system and detected via MS/MS using positive ion electrospray through a Sciex API 6500 mass spectrometer. A linear, 1/concentration weighted, least-squares regression algorithm was used to quantitate unknown samples.

cGMP measurement in Plasma: The plasma level of cGMP was measured using an Enzyme-Linked Immunosorbent Assay (ELISA) kit from Enzo Life Sciences (Farmingdale, NY, USA) and versa max microplate reader. 20 μL of the plasma was used to run the acetylated version of the kit according to the manufacturer protocol. All standards and samples have run in duplicate.

Blood pressure and vasodilator function (in vivo): A Laser Doppler perfusion imaging system (Perimed, PeriScan PIM 3 System) was used to assess in vivo hindleg vasodilator function, as described before. In short,
mice were anesthetized by 2.8% isoflurane/O₂ ventilation (Penlon, Sigma Delta vaporizer), while keeping the body temperature at 37.0 °C. The hindleg, while kept in a fixed position, was occluded for 2 minutes with a tourniquet. Upon release of the tourniquet, reactive hyperemia was measured for 10 minutes. Results were expressed as the area under the response curve (Durik et al., 2012; Bautista et al., 2015).

The in vivo blood pressure was measured in conscious animals by an experienced technician, using the tail-cuff technique (CODA High-Throughput device from Kent Scientific) after 5 daily sessions: 4 training sessions and a subsequent measurement session to record 40 measurement cycles. The average of the valid cycles was used for comparison (Golshiri et al., 2020).

**Pressure myography:** After the sacrifice, the carotid arteries were isolated from 14-week-old Ercc1Δ/Δ and WT animals and cleaned from all the fat tissues. The carotid arteries were mounted in pressure myograph (Danish Myograph Technology, Aarhus, Denmark) in calcium-free buffer (in mmol/L: NaCl 120, KCl 5.9, EGTA 2, MgCl2 3.6, NaH2PO4 1.2, glucose 11.4, NaHCO3 26.3; pH 7.4), to assess the passive properties of the vessels. The intraluminal pressure of the vessel was increased stepwise by 3-minute, 10 mm Hg steps, starting from 10 mm Hg and ending at 120 mm Hg. At the end of each step, the lumen diameter and wall thickness of the vessel were measured (Durik et al., 2012).

**Analysis of Plasma Cytokine Levels:** Plasma protein levels of IL-1β, IL-2, IL-6, IL-10, TNF-α, and IFN-γ were measured using a V-Plex Meso Scale Discovery (MSD) Multiplex spot assay Mouse Neuroinflammation 1 panel. All samples were diluted at a ratio of 1:4 with diluent 41 - provided in the MSD kit. Samples and Standards were run in duplicates or triplicates according to manufacturer instructions and analyzed with MSD Discovery Workbench software (Meso Scale Discovery, Gaithersburg, MD).

**Data Analysis:** Relaxation to ACh and SNP are expressed relative to the contraction produced by 30 nmol/L U46619 or 30 mmol/L KCl, which were set at 100% in each individual aortic or coronary rings. Data are shown as the percentage of relaxation, expressed as the mean ± S.E.M. The number of each individual experiment is shown for each of the rings. Statistical analysis was conducted using IBM SPSS statistics (IBM Corporation, version 25) and (GraphPad Prism, version 8.0.1; GraphPad Software Inc., San Diego, CA). Data were analyzed using Student’s paired t-test and general linear model repeated measurements. P values less than 0.05 were considered significant.
Results

ITI-214 acutely improves the diminished aortic NO responsiveness in Ercc1Δ/− mice: In agreement with previous studies in this model (Bautista et al., 2015), SNP responsiveness is diminished in Ercc1Δ/− mice vs. WT. The acute addition of ITI-214 to the organ bath enhanced the SNP response in Ercc1Δ/− mice (Figure 1).

IT-214 levels in the blood are in the normal range during chronic treatment: To evaluate the amount of drug consumption by drinking water and confirming sufficient drug intake and drug concentration in the blood, the plasma level of ITI-214 was measured after 8 weeks of treatment (100 mg/kg/day). The mean plasma concentration of ITI-214 is about 140 ng/mL, which is comparable to the 60 ng/mL concentration we have used in our acute study in the organ baths. It also corresponds to free plasma concentrations of 1-2 nmol/L ITI-214 (due to >99% plasma protein–bound), which is above the Ki for recombinant PDE1, but far below the Ki of other PDEs (Hashimoto et al., 2018).

ITI-214 treatment restores the endothelium-independent but not the endothelium-dependent response of the aorta in Ercc1Δ/− mice: The well-known diminished aortic ACh response in Ercc1Δ/− mice vs. WT was not affected by ITI-214 treatment (Figure 3A). Yet, it did restore the disturbed response of the aorta to the endothelium-independent vasodilator SNP in Ercc1Δ/− mice (Figure 3B).

ITI-214 does not alter the contribution of NO or EDH during exposure of the aorta to acetylcholine: To explore the signaling pathways underlying the decreased endothelium-dependent vasodilation, we studied the contribution of NO and EDH to the ACh relaxation in aortic rings, using the NO inhibitor L-NAME and the EDH inhibitor cocktail TRAM34 and apamin. In both vehicle-treated Ercc1Δ/− and WT mice, L-NAME significantly decreased the ACh response (Figure 4A, B). The effect of L-NAME was less pronounced in Ercc1Δ/− than in WT. TRAM34/apamin had no effect in Ercc1Δ/− mice and non-significantly reduced the ACh response in WT mice when added on top of L-NAME. The residual response in the presence of both L-NAME + TRAM34/apamin was not different between Ercc1Δ/− and WT. Altogether, we demonstrate that the NO-mediated
relaxations were hampered in Ercc1Δ/− vs. WT. ITI-214 did not change the relative contribution of endothelium-derived NO and EDH in both WT or Ercc1Δ/− (Figure 4 C, D).

**ITI-214 treatment restores the endothelium-independent but not the endothelium-dependent response of the coronary artery in Ercc1Δ/− mice:** To explore the role of PDE1 in the disturbed vasodilatory responses of small arteries, we also studied coronary arteries of vehicle- and ITI-214-treated mice. The difference in vasodilator response in Ercc1Δ/− mice versus WT littermates (Figure 5A, B) of coronary arteries was far more pronounced compared to aorta segments (Figures 1A and 2A). Similar to the aorta, chronic ITI-214 treatment did not improve the ACh response (Figure 5C, D), while it fully restored the response to SNP in Ercc1Δ/− mice (Figure 5E, F).

**ITI-214 treatment does not change the cGMP level in plasma:** The chronic treatment with ITI-214 did not change the plasma level of cGMP in both Ercc1Δ/− mice and WT littermates. The SNP responses in Ercc1Δ/− mice and WT littermates can be fully blocked by addition of the sGC inhibitor ODQ (Figure 6A, B).

**ITI-214 treatment lowers blood pressure in Ercc1Δ/− mice:** Blood pressure measured by tail-cuff in conscious mice was identical in untreated Ercc1Δ/− mice (SBP:117.85±19.98; DBP:83.35±18.40) and their WT littermates (SBP:107.21±15.37; DBP:73.48±17.83). Treatment with ITI-214 reduced both systolic and diastolic blood pressure in Ercc1Δ/− mice (SBP:93.38±10.21; DBP:64.46±7.99) but not WT (SBP:108.97±18.19; DBP:73.30±21.17) (Figure 7A, B).

**ITI-214 treatment normalizes the increased wall/lumen ratio in carotid arteries of Ercc1Δ/− mice:** Ercc1Δ/− showed an increased wall-to-lumen ratio at 100 mm Hg compared to WT (Figure 7C), which was restored by ITI-214.

**ITI-214 treatment improves microvascular blood flow in Ercc1Δ/− mice:** Since ITI-214 improved NO-mediated dilation in the coronary artery, a mid-sized artery, we explored the effect of chronic ITI-214 on microvascular blood flow. Reactive hyperemia to a 2-minute hind leg occlusion, measured by laser Doppler, was significantly lower in Ercc1Δ/− compared to WT (Figure 7D) while it has been partly restored and the difference was no longer present after ITI-214 chronic treatment.
**ITI-214 treatment lowers the elevated levels of inflammatory cytokines in Ercc1Δ/− mice:** The plasma levels of IL-1β, IL-6, TNF-α, IL-2, and IL-10 were elevated in Ercc1Δ/− mice vs. WT, while IFN-γ was reduced. ITI-214 significantly reduced the levels of the former 3 (Figure 8).

**Discussion**

Age-related CVD is the leading worldwide cause of mortality and morbidity among all acquired diseases. Functional and structural alterations in vascular tissue are at the early basis of the etiology of CVD, and characterization and intervention in the pathogenic mechanisms that govern these alterations are key in the development of pharmacotherapy (Ghebre et al., 2016; Donato et al., 2018; Fajemiroye et al., 2018). PDE1 was previously identified by us as a potential drug target in vascular ageing to rescue decreased NO – cGMP signaling, a pivotal pathogenic mechanism in the ageing cardiovascular system (Bautista et al., 2015). In the present study we explored this hypothesis by investigating the acute and chronic effects of PDE1 inhibition with ITI-214 on main ageing features of the macro- and microcirculation and inflammatory status, in a mouse model of accelerated ageing, Ercc1Δ/−, that enables pharmacotherapy during the entire course of the ageing process. We show that ITI-214 treatment improves the decreased vasodilation of aorta, coronary artery, and the microcirculation in Ercc1Δ/−. In addition, ITI-214 selectively lowered blood pressure and medial hypertrophy in these mice when compared to WT. Interestingly, the treatment reduced the elevated level of pro-inflammatory cytokines whereas anti-inflammatory cytokine levels are preserved. No sex-related differences were observed in any of the measured parameters.

Previously, we tested the possible involvement of PDE1 in disturbed ageing-related vasodilation employing the non-selective PDE1/PDE5 inhibitor vinpocetine in aortic rings of Ercc1Δ/− (Bautista et al., 2015). Vinpocetine led to an acute increase in vasodilation, which might have been due to PDE1 inhibition, but might have involved also PDE5. Our present study with ITI-214 demonstrates that acute inhibition of PDE1 alone is sufficient to improve endothelium-independent responses to SNP in the aorta of Ercc1Δ/− mice. The result is in agreement...
with an earlier study by Khammy et al. in rat mesenteric arteries (Khammy et al., 2017), showing that the role of PDE1 is not limited to mice. Notably, the effect of ITI-214 is selective for Ercc1Δ/ and more pronounced in coronary arteries, revealing a potential vessel type-specific effect. The greater effect might be simply explained by the higher degree of dysfunction observed in the coronary artery in Ercc1Δ/ as compared to the aorta. The selectivity of the effect for Ercc1Δ/ might be explained by the fact that SNP responses are already maximal in WT mice. ITI-214 did not improve the responses to Ach and the correction of response to SNP shows the same pattern (data not shown). This might be explained by the fact that Ach responses in both WT and Ercc1Δ/ are for a large part independent of NO, as was demonstrated by L-NAME. Especially in Ercc1Δ/, in which endothelium-dependent Ach-mediated NO is virtually absent, an acute effect of ITI-214 is not likely to occur. In summary, NO – cGMP signaling is strongly reduced in aged mice, and greatly improved by ITI-214. The acute effect of ITI-214 in all likelihood strongly depends on the relative and absolute contribution of NO that can be released by the endothelium during chronological ageing or disease conditions.

To our knowledge, this is the first study that explores the consequences of chronic selective PDE1 inhibition on vasomotor function in ageing mice, or, for that matter, in any model of vascular disease. The endothelium-dependent and -independent vascular responses were diminished in Ercc1Δ/ mice. The results in the aorta are in line with our previous studies in the same model of accelerated ageing. For the first time, we show the vascular dysfunction in the coronary artery of Ercc1Δ/ mice. Chronic ITI-214 improved endothelium-independent vasodilation in the aorta, coronary artery, and the cutaneous microcirculation of Ercc1Δ/ mice, suggesting that PDE1 is relevant for a large array of arteries. In the aorta, we have used a single high dose of SNP after Ach CRC, since we know from previous studies that the maximum response to this single high dose of SNP after the Ach CRC duplicates the maximum reached after a full SNP CRC (Golshiri et al., 2020). Acute administration of ITI-214 to the organ bath yielded no further SNP response in rings of ITI-214-treated mice (data not shown), supporting that ITI-214 was still present in the aortic rings after isolation. The blood pressure was identical in Ercc1Δ/ and WT mice while we have previously shown a modest increase in blood pressure in 16 weeks old Ercc1Δ/ mice. This suggests that vasomotor dysfunction might play a role as the early etiological events related to the blood pressure increase. The effect of chronic inhibition of PDE1 in the microcirculation is likely to be related to the blood pressure effect that was observed, as blood pressure regulation predominantly takes place through small resistance arteries. In agreement, both the effect on blood pressure and reactive hyperemia were selective for Ercc1Δ/ as compared to WT. The plasma cGMP levels did not change in treated animals and considering the tissue-specific distribution of PDE1, we did not expect an effect on plasma cGMP levels.
However, since ITI214 is able to improve SNP responses, and the SNP responses can be fully blocked by the sGC inhibitor ODQ, it is evident that ITI214 is acting through NO/cGMP signaling and improving cGMP responses. Moreover, we found that an alternative hypothesis for improvement of vascular function, blood pressure lowering, can be excluded. The observed improvement in vascular function is independent of blood pressure-lowering effects, as we have previously shown that systolic and diastolic blood pressure lowering by the Ang II type 1 receptor antagonist losartan, did not improve vascular aging features in the Ercc1Δ/Δ model (Wu et al., 2017). Similar to our present study it was recently shown in hypertensive Dahl salt-sensitive rats on 4% NaCl chow that another selective PDE1 inhibiter (BTTQ) has an antihypertensive effect, both acutely as well as chronically (Dey et al., 2020). In these rats, a vasodilator response of isolated, preconstricted mesenteric arteries to BTTQ was observed in Dahl SS rats on 0.4 % NaCl. Similar to our study, ACh responses were not increased by chronic PDE1 inhibition, which was tested after 21 days in rats fed 4 NaCl%. However, the Dahl SS rats in this study did not appear to display endothelial dysfunction since responses reached 90% of the preconstriction with 10 µmol/L phenylephrine. Unfortunately, no low salt control was included to establish this. Therefore, it remains to be determined what will be the effect of chronic PDE1 inhibition in models with moderately decreased endothelium-dependent relaxations. Nevertheless, PDE1 inhibition may be an option when seeking novel anti-hypertensive treatments, especially in the elderly.

Apart from vasomotor improvements, we also showed the effects of PDE1 chronic inhibition on carotid artery wall hypertrophy. ITI-214 treatment restored the increased wall-to-lumen ratio of Ercc1Δ/Δ mice to the same ratio as WT. Intimal and medial thickening is attributable to SMC proliferation and migration. Previous studies in cultured VSMC have shown that NO – cGMP decreases proliferation and migration of VSMC, and it is a well-known observation that endothelial denudation of the vessel wall or chronic NO inhibition in vivo leads to increased wall thickness (Fingerle et al., 1990; Rajabrata et al., 1996; Fischer et al., 2004). In early studies on cardiomyocyte hypertrophy and atherosclerotic plaque formation in the vasculature, PDE1 was identified as a potential drug target to attenuate hypertrophic remodeling (Miller et al., 2009; Miller et al., 2011; Cai et al., 2013). More recent studies in PDE1C-knockout mice confirmed this possibility (Knight et al., 2016). The anti-hypertrophic effect of PDE1 inhibition can relate to both cAMP and cGMP increases, which appear to act independently (Sergei et al., 2003). Both cyclic nucleotides control the diameter and wall thickness of arteries because of their effects on vascular relaxation and proliferation. PDE1A and C appear to act separately and specifically on either of these functions depending on the subcellular location of these enzymes. Moreover, species-specific differences have been found, as illustrated elsewhere (Sergei et al., 2003). It has also been
shown that endogenous cGMP-dependent signaling is able to negatively regulate cardiac hypertrophy by suppressing $G_{q/11}$ activation and normalizing $Ca^{2+}$ signaling (Marie-Aude et al., 2004; Miller et al., 2009). Thus, PDE1 is a very versatile member of the PDE family for the regulation of cardiovascular function.

A major player both in arteriopathy as well as high blood pressure is inflammation (Baudry et al., 1996; Alexander et al., 2002; Orshal and Khalil, 2004; Dexter et al., 2006; Joey, 2006). Pro-inflammatory cytokines can interact with important blood pressure-regulatory systems, such as the renin-angiotensin system, and also increase the formation of a number of endothelial cell substances, such as endothelin; reduce acetylcholine-induced vasodilatation; and destabilize the mRNA of endothelial nitric oxide synthase, which ultimately affect vascular function and blood pressure regulation (Alexander et al., 2002; Jena et al., 2002; LaMarca et al., 2005). Hypertrophy can also be stimulated by cytokines such as IL-1β, IL-6, and TNF-α. These cytokines trigger remodeling of the cytoskeleton and change the adhesiveness of the cell to the matrix, additionally, these pro-inflammatory cytokines are involved in restraining the expression and activity of eNOS, production of oxygen-derived free radicals by neutrophils, VSMCs and ECs, and reduction of NO bioavailability (Sprague and Khalil, 2009; Tian et al., 2016; Chen et al., 2018). We suggest that the reduced level of pro-inflammatory cytokines by PDE1 inhibition might be an additional anti-hypertrophic mechanism. During ageing, a sustained increase in cytokines is associated with vascular dysfunction and vascular disease such as atherosclerosis and hypertension (Peeters et al., 2001; Mirhafez et al., 2014; Moss and Ramji, 2016; Steven et al., 2019; Tyrrell and Goldstein, 2020). We here show that Ercc1Δ/Δ have increased circulating levels of certain cytokines, which is attenuated by PDE1 inhibition. PDE1 inhibition significantly reduced IL-1β, IL-6, and TNF-α pro-inflammatory cytokines while it did not reduce the elevated level of anti-inflammatory cytokine IL-10. The anti-inflammatory effects of ITI-214 were recently shown by O’Brien et al. in an in-vitro model of an immortalized murine microglial cell line, BV2 cells, possibly by inhibition of ADP dependent migration via amplified signaling through cAMP and subsequently increased phosphorylation of VASP (O’Brien et al., 2020). We now demonstrate the anti-inflammatory properties of PDE1 inhibition in vivo in a mouse model of progressive ageing. Thus, apart from vasodilatory and anti-hypertrophic effects through cGMP, PDE1 inhibition also might involve anti-inflammatory effects, which may be cAMP-dependent. It remains to be determined if ITI-214 mediated reduction in inflammation contributes to augmentation of smooth muscle SNP responsiveness or is an independent event. Moreover, It is currently not known what levels inflammatory factors should reach to have an impact on non-atherosclerotic vascular aging. A complicating observation in this respect is that multiple cytokines are involved, and their temporal-quantitative pattern might be very complex, making it difficult to
mimic the conditions in vivo. Thus, it remains unclear whether this statistically significant reduction in proinflammatory cytokines would increase vasodilator capacity. Nevertheless, inflammation, with increased IL-6 and TNF-α as a major mediator, has been implicated in the pathophysiology of hypertension, in particular of end-organ damage (Xiao et al., 2015), and in this light, the present observation that ITI-214 decreases these cytokines is valuable as a future direction, when uncovering the mechanisms of treatment effects.

In summary, we demonstrated the role of PDE1 in vasodilation, particularly involving NO-cGMP signaling and vascular hypertrophy in an accelerated ageing mouse model. We showed selective PDE1 inhibition by ITI-214 can attenuate diminished vasodilation and reduced microcirculatory blood flow caused by ageing, and reduce hypertrophy and the level of pro-inflammatory cytokines. These findings suggest PDE1 inhibition would have significant therapeutic potential to battle arteriopathy related to ageing. It is also worth mentioning that the development of isomer-specific PDE1 inhibitors can shed light on mechanisms involved in PDE1 inhibition and provide more opportunities for future investigations. Application in coronary artery disease might be of specific interest since ITI-214 is currently under development in patients with heart failure to improve cardiac function by enhancing cardiac contractility and dilating systemic arteries without inducing abnormal heart rhythms (Identifier: NCT03387215).
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Authorship Contributions:

Participated in research design: Golshiri, Ataei Ataabadi, Davis, Danser, Snyder, Roks.

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Contributed new reagents or analytic tools: Snyder, van der Pluijm, Brandt, MaassenVanDenBrink.

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Figure Legends:

**Figure 1:** Endothelium-independent responses in isolated aortic rings from Ercc1<sup>Δ/−</sup> mice (open circles) versus rings from WT littermate (filled circles) measured ex vivo in small wire organ baths, the acute effect of ITI-214 administration to organ bath in WT and Ercc1<sup>Δ/−</sup> mice is in green lines. Relaxations are calculated relative to the contraction produced by KCl 30 mmol/L in each ring, which are set at 100%. Values are expressed as means ± SEM; n, number of mice; #, p<0.05, two-way ANOVA followed by Bonferroni's post hoc test on Emax, Ercc1<sup>Δ/−</sup> (vehicle) vs. WT (vehicle) and Ercc1<sup>Δ/−</sup> (vehicle) vs. Ercc1<sup>Δ/−</sup> (vehicle + acute ITI-214).

**Figure 2:** The plasma concentration of the ITI-214 in both WT and Ercc1<sup>Δ/−</sup> mice after 8 weeks of treatment.

**Figure 3:** Endothelium-dependent and -independent responses in isolated aortic rings from Ercc1<sup>Δ/−</sup> mice (open circles, bars) versus rings from WT littermate (filled circles, bars) (A, B). The endothelium-dependent and -independent effects of 8 weeks of treatment with ITI-214 in drinking water on WT and Ercc1<sup>Δ/−</sup> mice in green lines/bars, measured in aortic rings in ex vivo organ bath experiments. Data are calculated relative to the precontraction. Values are expressed as means ± SEM; n, number of mice; *, p < 0.05, GLM for repeated measures; #, p<0.05, two-way ANOVA followed by Bonferroni’s post hoc test.

**Figure 4:** The impact of the NO synthesis inhibitor L-NAME (100 μmol/L) and EDH inhibitors TRAM34 (10 μmol/L) and apamin (100 nmol/L) on the relaxations induced by ACh in aortic rings precontracted by U46619. Relaxations are calculated relative to the contraction produced by U46619 in each ring, which is set at 100%. Values are expressed as means ± SEM; n, number of mice; *, p< 0.05; GLM for repeated measures.
Figure 5: Endothelium-dependent and -independent responses in isolated coronary rings from Ercc1Δ/− mice (open circles) versus rings from WT littermate (filled circles), measured ex vivo in small wire organ baths (A, B). The endothelium-dependent and -independent effects of 8 weeks of treatment with ITI-214 in drinking water on WT (C, E) and Ercc1Δ/− mice (D, F) (green lines), measured in coronary rings in ex vivo organ bath experiments. Relaxations are calculated relative to the contraction produced by U46619 in each ring, which is set at 100%. Values are expressed as means ± SEM; n, number of mice; *, p< 0.05; GLM for repeated measures.

Figure 6: cGMP level in the plasma (A) and the effect of ODQ on endothelium-independent response (B) in Ercc1Δ/− mice (open circles) versus WT littermate (filled circles). The effects of 8 weeks of treatment with ITI-214 in drinking water are shown in green. Values are expressed as means ± SEM; the number of mice per group is 5-10. Statistical differences were analyzed by two-way ANOVA followed by Bonferroni’s post hoc test (#, p < 0.05).

Figure 7: Systolic and diastolic blood pressure measured by tail-cuff in conscious Ercc1Δ/− mice versus their WT littermates (A, B). The ratio of wall thickness to lumen diameter at 100 mm Hg, as measured by pressure myography technique (C). Cutaneous reactive hyperemia in Ercc1Δ/− mice versus their WT littermates measured with laser Doppler, expressed as area under the curve (AUC) (D). The number of mice per group is 6-10. Statistical differences were analyzed by two-way ANOVA followed by Bonferroni’s post hoc test (#, p < 0.05).

Figure 8: The concentration of inflammatory cytokines in plasma of Ercc1Δ/− mice and WT littermate. The number of mice per group is 5-10. Statistical differences were analyzed by two-way ANOVA followed by Bonferroni’s post hoc test (#, p < 0.05).
Figure 1

- WT (Vehicle) (n=17)
- Ercc1Δc (Vehicle) (n=16)
- WT (Vehicle + acute ITI-214) (n=18)
- Ercc1Δc (Vehicle + acute ITI-214) (n=14)

Relaxation (%) vs. log SNP (mol/L)

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Figure 2
Figure 3
Figure 4

A

WT (Vehicle) (n=20)
WT (L-NAME) (n=18)
WT (L-NAME + Tram/Apamin) (n=16)

B

Ercc1Δ/- (Vehicle) (n=18)
Ercc1Δ/- (L-NAME) (n=13)
Ercc1Δ/- (L-NAME + Tram/Apamin) (n=12)

C

WT (ITI-214) (n=8)
WT (L-NAME) (n=7)
WT (L-NAME + Tram/Apamin) (n=7)

D

Ercc1Δ/- (ITI-214) (n=15)
Ercc1Δ/- (L-NAME) (n=10)
Ercc1Δ/- (L-NAME + Tram/Apamin) (n=9)
Figure 5
Figure 6

A

Plasma

cGMP (pmol/ml)

WT (Vehicle)  WT (ITI-214)  Ercc1Δ/Δ (Vehicle)  Ercc1Δ/Δ (ITI-214)

B

SNP 0.1mM

Relaxation (%)

WT (Control)  WT (ODQ)  Ercc1Δ/Δ (Control)  Ercc1Δ/Δ (ODQ)

Figure 6
Figure 7
Figure 8