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Phenotypic switching prevention and proliferation/migration inhibition of vascular smooth muscle cells by the ruthenium nitrosyl complex *trans*-[Ru(NO)Cl(cyclam)](PF$_6$)$_2$

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Keywords
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Abstract

Objectives Vascular smooth muscle cell (VSMC) migration and proliferation at sites of vascular injury are both critical steps in the development of intimal hyperplasia (IH). Local delivery of nitric oxide (NO) largely prevents these events. Among the NO donors, tetraazamacrocyclic nitrosyl complexes, such as *trans*-[Ru(NO)Cl(cyclam)](PF$_6$)$_2$ (cyclamNO), gained attention for their features, which include the possibility of being embedded in solid matrices, and ability to participate in a nitrite/NO catalytic conversion cycle.

Methods Methods used to evaluate cyclamNO activity: safety margin by NR and MTT; cell proliferation by 3H-thymidine incorporation and proliferating cell nuclear antigen (PCNA) expression; antimigratory properties by transwell and wound healing; prevention of cell phenotypic switching under platelet-derived growth factor type BB (PDGF-BB) stimuli by analysis of alpha smooth muscle actin (α-SMA) expression.

Key findings Cell proliferation and migration induced by PDGF-BB were significantly inhibited by cyclamNO. The ~60% reduction on expression of contractile protein α-SMA induced by PDGF-BB revealed VSMC phenotypic switching which is significantly prevented by cyclamNO. Compared to the NO donor sodium nitroprusside, cyclamNO showed to be significantly less cytotoxic.

Conclusions With great potential to maintain VSMC functionality and prevent IH-associated events, cyclamNO might be a promissory drug for several applications in cardiovascular medicine, as in stents.

Introduction

Intimal hyperplasia (IH) and lumen stenosis are the key characteristics of a number of different vascular disorders.[1] Although the underlying mechanisms are multifactorial, excessive growth and migration of vascular smooth muscle cells (VSMC) are major contributors to pathological vascular remodelling in response to vascular damage or disease, leading to subsequent intimal thickening and neointimal formation.[2] Over the decades, several alternatives have been evaluated to prevent IH. The use of systemic drug therapy with different compounds demonstrated low tolerance and efficacy, besides narrow therapeutic ranges.[3] These outcomes have led to the concept of local drug delivery and the introduction of drug-eluting stents (DES), aiming to improve efficacy and minimize adverse systemic effects.[3] Currently, the main therapy to directly prevent VSMC activation is the local delivery of antiproliferative and immunosuppressive drugs (*e.g.* paclitaxel, sirolimus) by percutaneous DES implantation.[3] However, according to several studies in humans and animal models, the efficacy and safety of these devices are limited, in part due to the local toxicity of the drug, antiproliferative effect on endothelial cells, delayed healing and persistent inflammatory stimuli, contributing to late intimal growth and thrombosis, besides the need for

prolonged antiplatelet therapy or revascularization procedures.[4–8] Subsequent stent types with newer structures, polymers and different drugs with improved release kinetics have emerged to improve both efficacy and safety.[9,10] Therefore, despite great advances in the field of vascular biology and pharmacology, currently it remains significant problems with insufficient effective therapeutic strategies and associated with patient morbidity.[9–11]

Under physiological conditions, VSMC residents in internal elastic lamina, the outermost part of tunica intima, express several differentiation markers, such as high levels of myofilaments, reflecting their contractile phenotype, and with proliferation index and secretory activity extremely low.[2] However, conditions like vascular damage or endothelial dysfunction promote an extensive release of inflammatory cytokines and growth factors that triggers the VSMC phenotype transition and promote pathological vascular wall remodelling, which includes VSMC growth/migration, extracellular matrix over production, IH and luminal narrowing, resulting in stenosis, occlusions and frequent episodes of thrombosis.[2,12–14] Given the role of nitric oxide (NO) in maintaining a normal vascular environment, many researchers have hypothesized that replacement of NO at the site of injury would prevent development of IH by inhibition of VSMC proliferation/migration.[11,12,15,16]

Nitric oxide is a multifunctional biological agent and one of the most important cellular processes mediators.[17] Produced by healthy endothelium, through constitutive nitric oxide synthase, NO diffuses across the VSMC membranes and stimulate the production of cyclic guanosine monophosphate (cGMP) by the activation of the enzyme soluble guanylyl cyclase (sGC). Beyond being a powerful vasodilator, NO has a key role in mediating vascular remodelling; however, following injury, damaged endothelium reduces the production and responsiveness to NO and consequently NO/sGC/cGMP signalling-mediated effects.[12] Previous studies show that maintenance of NO signalling immediately after arterial damage can prevent pathological remodelling and related events.[11,12,18]

The biological importance of NO had launched searches for NO carriers capable to deliver NO efficiently and in a controlled manner in physiological medium and aiming biological applications.[19–29] NO donors are pharmacologically active substances that release NO, spontaneously or induced by chemical or photochemical reactions, differing by varied chemical and photochemical reactivities in physiological milieu and NO-release kinetics.[19–31] Among these NO donors, ruthenium tetraammine and tetraazamacrocycle nitrosyl complexes have favourable features, such as water solubility, stability even in aqueous solutions, low toxicity and for the possibility to control the reactivity of the coordinated NO by the careful choice of ligands around the RuNO moiety.[22,24,29,32] Such features make them attractive candidates as NO delivery agents, not only for therapeutic applications, as in cardiovascular devices, but also as pharmacological tools to investigate the role of NO in physiology and pathophysiology.

The activation of ruthenium tetraammine and tetraazamacrocycle nitrosyls, such as *trans*-[Ru(NO)Cl(cyclam)] $(PF_6)_2$ (cyclamNO, Figure 1), towards release of NO can be initiated after their irradiation by light or through reduction by various reducing agents present in the physiological environment, resulting in a dissociation of the complex in NO and its corresponding aqua or hydroxo Ru(III) complex, depending on *p*H (photochemically) or the aqua Ru (II) complex (by reduction), $[RuOH_2]$.[22,24,29,32] Several biological effects of such compounds, including potential applications, have been reported,[22,24,29,32] and their biological effects have been in most cases attributed to NO release from the complex cation after being reduced or photolysed in the biological medium.[22,24,29]

Here, we used the ruthenium NO carrier, cyclamNO, mostly for two further reasons. One is that cyclamNO can be embedded into solid matrices,[22,24,29,32–34] which has great impact for biological applications. In this case, immobilization systems may increase the residence time of the complex *in vivo* as well as avoiding premature release of NO before reaching the target and undesirable reactions such as nucleophilic attack on nitrosyl, which would restrain the action of the complex.[22,24,29,32–34] Indeed, preliminary results from our group showed that cyclamNO can be imbedded in thin solid films deposited over surgical stainless steel surface, used in stents.[35] The other reason is that it is capable of being involved in a catalytic nitrite/NO conversion cycle.[29,36] Reduction of the cyclamNO cation, *trans*-$[Ru(NO)Cl(cyclam)]^{2+}$, *in vitro,* is followed by a fast chloride release ($k_{-Cl} \sim 1.5\ s^{-1}$ at 25 °C), followed by a slower NO release ($k_{-NO} = 6.1 \times 10^{-4}\ s^{-1}$ at 25 °C), forming the diaqua complex *trans*-$[Ru(cyclam)(H_2O)_2]^{2+}$.[36] The diaqua complex formed can, moreover, act as scavenger of nitrite (NO_2^-), forming the nitro complex *trans*-$[Ru(NO_2)(cyclam)(H_2O)]^+$, which in turn, is converted back to the ruthenium nitrosyl complex *trans*-$[Ru(NO)(OH)(cyclam)]^{2+}$ in physiological *p*H (Equations 1–6); as

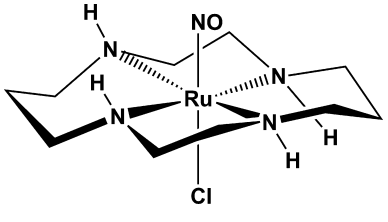

Figure 1 Structural representation of cyclamNO cation *trans*-$[Ru(NO)Cl(cyclam)]^{2+}$.

nitrite is one of the major NOx pools in blood,[37] these reactions constitute a catalytic nitrite to NO conversion, raising the efficiency of these complexes as NO donors,[22,24,29] a feature that differentiates the ruthenium tetraammine and tetraazamacrocycle nitrosyl complexes from most other NO donors, as organic donors, several metal complexes or sodium nitroprusside, a clinically used NO donor with side effects that must be overcome.[22,24,29] Therefore, conceivably, that conversion would make the stent, with the embedded cyclamNO, have a long-lasting effect.

$$trans-[Ru^{II}(NO^{+})CI(cyclam)]^{2+}+e^{-}\xrightarrow{E_1} trans-[Ru^{II}(NO^{o})CI(cyclam)]^{+} \quad (1)$$

$$trans-[Ru^{II}(NO^{o})CI(cyclam)]^{+}\xrightarrow[+H_2O]{k_{-Cl}} trans-[Ru^{II}(NO^{o})(H_2O)(cyclam)]^{2+}+Cl^{-} \quad (2)$$

$$trans-[Ru^{II}(NO^{o})(H_2O)(cyclam)]^{2+}\xrightarrow[+H_2O]{k_{-NO}} trans-[Ru^{II}(H_2O)_2(cyclam)]^{2+}+NO \quad (3)$$

$$trans-[Ru^{II}(H_2O)_2(cyclam)]^{2+}+NO_2^{-}\xrightarrow{k_{+NO_2^-}} trans-[Ru^{II}(NO_2)(H_2O)(cyclam)]^{+} \quad (4)$$

$$trans-[Ru^{II}(NO_2)(H_2O)(cyclam)]^{+}+2H^{+}\rightleftharpoons trans-[Ru^{II}(NO)(H_2O)(cyclam)]^{3+}+H_2O \quad (5)$$

$$trans-[Ru^{II}(NO)(H_2O)(cyclam)]^{3+}\rightleftharpoons trans-[Ru^{II}(NO)(OH)(cyclam)]^{2+}+H^{+} \quad (6)$$

Hence, here we assess the cytotoxicity and efficacy on inhibition of VSMC proliferation, migration and phenotypic modulation, under growth-stimulated conditions by platelet-derived growth factor type BB (PDGF-BB), of *trans*-[Ru(NO)Cl(cyclam)]$(PF_6)_2$, focusing on its efficacy on prevention of these events and also aiming to provide support for the development of new RuNO-based therapeutics, such as ruthenium nitrosyls embedded in surface coatings of surgical stainless steel, to be used as NO-eluting stents.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), SNP, MTT, NR, DMSO, L-NAME, PDGF-BB, H_2O_2, RIPA buffer, *p*-coumaric acid, luminol and tris were all purchased from Sigma Chemicals, St Louis, MO, USA. Trypsin 0.25% solution, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Vitrocell, Campinas, SP, BR. Tritiated thymidine and liquid scintillation cocktail were from Amersham Biosciences Inc, Little Chalfont, BKM, UK. Mouse monoclonal anti-PCNA primary antibody (#ab29) and rabbit polyclonal anti-α-SMA antibody (#ab5694) from Abcam, Cambridge, MA, USA. Rabbit polyclonal anti-GAPDH (#sc25778) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Cell culture

In conduction of this research, the authors adhered to the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation (protocol number 2916-1 approved in October/2012). Adult New Zealand rabbits (weight: 2.5–3.0 kg) were supplied by Central Animal House Services of University of Campinas. Animals were euthanized by sodium pentobarbital overdosing (100 mg/kg), and the thoracic aorta was removed and dissected free of connective tissue under sterile conditions. Subsequently, the tissue was extensively segmented and incubated for 4 h at 37 °C in serum free DMEM containing 1 mg/ml of collagenase type IA and 0.1 mg/ml of elastase. After the enzymatic digestion period, the cells were centrifuged, trypisinized and cultured in DMEM medium with the addition of 10% of FBS and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). Immunostaining for the smooth muscle cell markers α-SMA and smooth muscle myosin heavy chain was performed for the characterization of VSMC. The cells were kept in a water-jacketed incubator and humidified with 5% CO_2, maintained at 37 °C. Cells were used between 2nd and 4th passages and, prior to experiments, the cells were made quiescent by omitting FBS for 24 h.

Synthesis of *trans*-[Ru(NO)Cl(cyclam)]$(PF_6)_2$ and cell treatments

The *trans*-[Ru(NO)Cl(cyclam)]$(PF_6)_2$ complex was synthesized as previously described,[36] using $K_2[Ru(NO)Cl_5]$ and cyclam as precursors. The concentrations of cyclamNO used in experimental protocols were similar to other studies with nitrosyl complexes.[38,39] For cytotoxicity assays, the 0.1 mM, 0.5 mM, 1 mM and 1.5 mM concentrations of cyclamNO and SNP were adopted, and for the other experiments, only the 0.1 mM concentration was used for both complexes. The cell medium was used as vehicle and all solutions were prepared just before the treatments, filtered in a Millipore membrane (0.22 μm), and, then, kept away from light because cyclamNO and SNP are light sensitive.[29,40] PDGF-BB concentration in all experiments was 20 ng/ml diluted in cell medium.

Determination of cyclamNO cytotoxicity

The cytotoxic effects of cyclamNO were evaluated by the NR and MTT assays as previously described.[41,42] Cells were cultured in 96-well plates at confluent density, starved and then treated with different doses of cyclamNO. The NO donor SNP, with recognized cytotoxicity, was used as control. After 24 and 48 h of treatment, the cell medium was replaced by MTT (0.8 mg/ml) or NR (100 μg/ml) dye dissolved in serum free medium and incubated for 4 and 2 h, respectively. After this period, for the MTT assay, the cells were washed in PBS followed by the addition of DMSO and stirred for 1 h for complete dissolution of the formazan crystals. The absorbance was recorded at 560 nm using the microplate spectrometer system (Biotek Instruments, Winooski, VT, USA). For the NR assay, washing with PBS was followed by the addition of an elution medium (ethanol : acetic acid, 1 : 1, v/v) and agitation for 20 min for complete dye dissolution and the absorbance was recorded at 540 nm. Results were analysed as the percentage of viable cells considering 100% of viable cells in control (not treated).

Assessment of cyclamNO antiproliferative properties

Radiolabelled thymidine incorporation is a recognized method to assay the ability of a new agent to stimulate or inhibit cell proliferation.[43] Cells were cultured in 24 wells until confluence, starved and treated for 48 h with cyclamNO (0.1 mM) in the absence or presence of PDGF-BB (20 ng/ml) to induce cell proliferation. In the last 4 h of treatment, 1 μCi/ml of 3H-thymidine was added to the cell medium. After washing three times with 5% trichloroacetic acid, cells were lysed in 0.5 M NaOH solution, transferred to glass vials, the liquid scintillation cocktail was added and the radioactivity measured in a liquid scintillation counter (Beckman, Fullerton, CA, USA). Radioactivity was expressed as disintegrations per minute and converted to percentage of viable cells in comparison with control (100%).

The expression of PCNA, a protein expressed in the early G_0/G_1 and S phase of cell cycle, was analysed by Western blotting technique.[44] Cells were cultured in a six-well plate and at confluence treated with 0.1 mM of cyclamNO or 20 ng/ml of PDGF-BB. After 48 h, cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.2, 0.15 M NaCl, 1.0 mM EDTA, 0.1% SDS, 1.0% Triton X-100, 1.0% sodium deoxycholate). Equal amounts of cell lysates (30 μg of protein) were resolved in a SDS–polyacrylamide gel electrophoresis and blotted in nitrocellulose membranes, incubated with the antibodies : anti-PCNA (diluted 1 : 1000) or GAPDH (diluted 1 : 5000) and later exposed to chemiluminescence reagents (201 μM *p*-coumaric acid, 1.24 mM luminol, 1.5 M tris, pH 8.8, 0.018% H_2O_2) and photographed in a photodocumentation system (GboxChemi XR, Syngene International, Karnataka, BLR, India). Bands were quantified by densitometry using NIH's software, Image J (Bethesda, MD, USA).

Assessment of cyclamNO antimigratory properties

Transwell migration assays were performed using transwell insert, with filter membranes of 8-μm pore size (Corning Inc., Corning, NY, USA), to induce a directional migration.[45] Cells were starved and seeded (5×10^4 cells per well) in DMEM with 0.5% of FBS into the upper chamber of the insert and treated with cyclamNO (0.1 mM). In the bottom chamber, DMEM with 0.5% of FBS or DMEM supplemented with PDGF-BB (20 ng/ml) was added. After 4 h, non-migrating cells on the upper side of the membrane were removed with a swab. Migrated cells were stained with haematoxylin and eosin, photographed in an electronic microscopic (Leica Microsystems, Wetzlar, Hessen, DE, USA) at a 400× magnification with a digital camera and counted in the full membrane.

Non-directional migration was quantitatively evaluated by wound-healing assay.[46] Cells were cultured in a 24-well plate until confluence. A wound was made by scraping off the cell monolayer with a sterile pipette tip across the centre of the well. Another wound was made to create a cross without cells to facilitate capture of images in different times. Floating cells were removed by extensive washing with PBS. CyclamNO (0.1 mM) was added to cell medium in the presence or absence of PDGF-BB (20 ng/ml). The wounded regions were photographed in a microscope at 200× magnification with a digital camera in times zero and 16 h after stimuli, and the number of cells migrated into the wound area was quantified using Image J Software.

Phenotypic regulation of VSMC by cyclamNO

Alpha-SMA expression in VSMC was evaluated to demonstrate the prevention of cell phenotypic switching by cyclamNO under PDGF-BB stimuli. Cells were cultured at six-well plate until confluence, treated with cyclamNO (0.1 mM) or PDGF-BB (20 ng/ml), and after 48 h, the protein expression was determined by Western blotting technique, as described above, using the antibodies anti-α-SMA (diluted 1 : 1000) and anti-GAPDH (diluted 1:5000) as loading control.

Data analysis

Summarized data are presented as mean ± SEM (standard error of mean) from individual experiments. Statistical significance was calculated by one-way analysis of variance followed by Tukey's multiple comparison test (GraphPad Software Inc., San Diego, CA, USA), and *P*-values <0.05 were considered significant.

Results

CyclamNO is less cytotoxic than SNP

Experimental protocols to determine the vascular cytotoxic potential of cyclamNO and SNP, evaluating the VSMC viability through lysosomal and mitochondrial integrity, were carried out first. As seen in Tables 1 and 2, in the presence of SNP, the cells markedly showed toxic effects. The percentages of viable cells were ~33% in MTT assay and ~57% in NR assay, in 24 h at the highest concentration (1.5 mM). The SNP IC_{50} value in VSMC is nearly 0.5 mM,[47] and at 1.5 mM, elimination of 100% of cell population after 48 h exposition, in both assays, is observed. The cytotoxicity induced by cyclamNO is significantly smaller than that induced by equimolar SNP ($P < 0.05$) in both assays, more markedly after 48 h of exposition. Cells suffered a gradual loss of viability in dose-dependent manner, and the percentage of viable cells, even at the highest concentration used (1.5 mM), was about 70% after 24 or 48 h of exposition to cyclamNO in both assays. The IC_{50} value for cyclamNO in VSMC was not reached in the concentration range used (0.1–1.5 mM). In other studies, with different lines of cell cultures, the IC_{50} value is only achieved at higher concentration levels of cyclamNO, superior to 2 mM.[39] These results indicate that cyclamNO can be more safely used than SNP.

CyclamNO inhibits PDGF-BB-induced proliferation in rabbit VSMC

The capacity of cyclamNO to maintain VSMC function was evaluated by proliferation assays with 3H-thymidine and PCNA expression, using PDGF-BB as stimuli. With 3H-thymidine, as seen in Figure 2a, after 48 h of exposition, PDGF-BB induced intense proliferation in VSMC, and showed an increase of ~100% in thymidine incorporation (197.5% ± 8.3, $P < 0.05$), in comparison with control. However, the addition of cyclamNO resulted in significant reduction of this response (145.7% ± 4.7, $P < 0.05$).

Furthermore, evaluation of cell proliferation by PCNA protein expression analysis (Figure 2b) showed that PDGF-BB was able to induce an elevation of almost three times in the expression of this proliferation marker in comparison with control (1.53% ± 0.2 and 0.69% ± 0.4, respectively). In contrast, the elevated PCNA expression induced by PDGF-BB was significantly prevented by cyclamNO (0.78% ± 0.1, $P < 0.05$) (Figure 2b).

CyclamNO had no significant effect in control cells in both proliferation assays. Together, these results revealed the potential use of cyclamNO to inhibit VSMC proliferation, even in the presence of potent proliferative stimuli.

CyclamNO inhibits PDGF-BB-induced migration in rabbit VSMC

Transwell and wound-healing assays were used to directly investigate the inhibitory response of cyclamNO on rabbit VSMC cell migration induced by PDGF-BB. Directional migration after 4 h of stimuli with PDGF-BB was significantly prevented by cyclamNO (Figure 3a,b). Compared to control, the addition in the upper chamber of insert of PDGF-BB alone induced ~2.3× migrant cells, whereas cyclamNO only and the mixture of PDGF-BB and cyclamNO resulted in ~1.5× and ~1.3× migration ($P < 0.05$), respectively.

Table 1 Cytotoxic effects of cyclamNO and SNP assessed by MTT assay after 24 or 48 h of treatment

	MTT (% of viable cells)			
	After 24 h		After 48 h	
	cyclamNO	SNP	cyclamNO	SNP
0.1 mM	98 ± 1.5	93 ± 4.3	93 ± 3.0*	62 ± 2.9
0.5 mM	89 ± 2.6*	75 ± 8.1	80 ± 3.8*	52 ± 4.1
1.0 mM	78 ± 5.9*	59 ± 5.9	72 ± 5.0*	18 ± 2.7
1.5 mM	72 ± 5.5*	33 ± 7.3	68 ± 5.9*	0

Results expressed as percentage of viable cells in comparison with control (100%), and presented as mean ± SEM of three individual experiments in triplicate. *$P < 0.05$ compared to SNP.

Table 2 Cytotoxic effects of cyclamNO and SNP assessed by NR assay after 24 or 48 h of treatment

	NR (% of viable cells)			
	After 24 h		After 48 h	
	cyclamNO	SNP	cyclamNO	SNP
0.1 mM	97 ± 2.2	87 ± 5.2	97 ± 1.5*	59 ± 4.1
0.5 mM	89 ± 3.4	81 ± 7.3	83 ± 2.5*	44 ± 7.2
1.0 mM	82 ± 4.6	70 ± 6.8	75 ± 4.8*	15 ± 4.0
1.5 mM	68 ± 5.4*	57 ± 7.5	72 ± 1.7*	0

Results expressed as percentage of viable cells in comparison with control (100%), and presented as mean ± SEM of three individual experiments in triplicate. *P <0.05 compared to SNP.

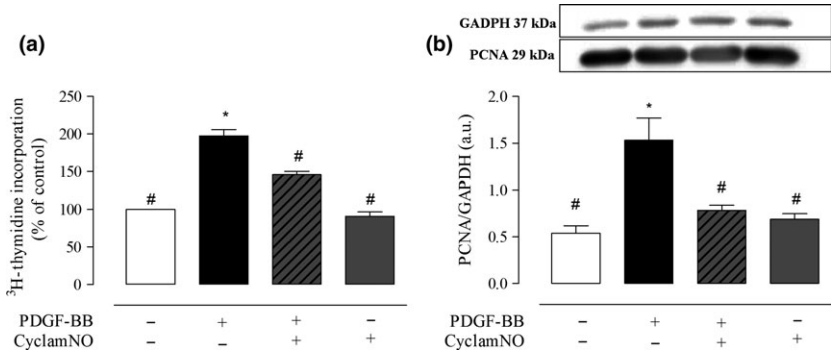

Figure 2 Platelet-derived growth factor type BB stimulated vascular smooth muscle cell proliferation in the presence of cyclamNO. (a) 3H-thymidine incorporation and (b) representative Western blotting for proliferating cell nuclear antigen expression 48 h after treatments with 0.1 mM of cyclamNO or 20 ng/ml of platelet-derived growth factor type BB. Values presented as mean ± SEM of three to five individual experiments in triplicate. $^*P < 0.05$ compared with control; $^\#P < 0.05$ compared to platelet-derived growth factor type BB.

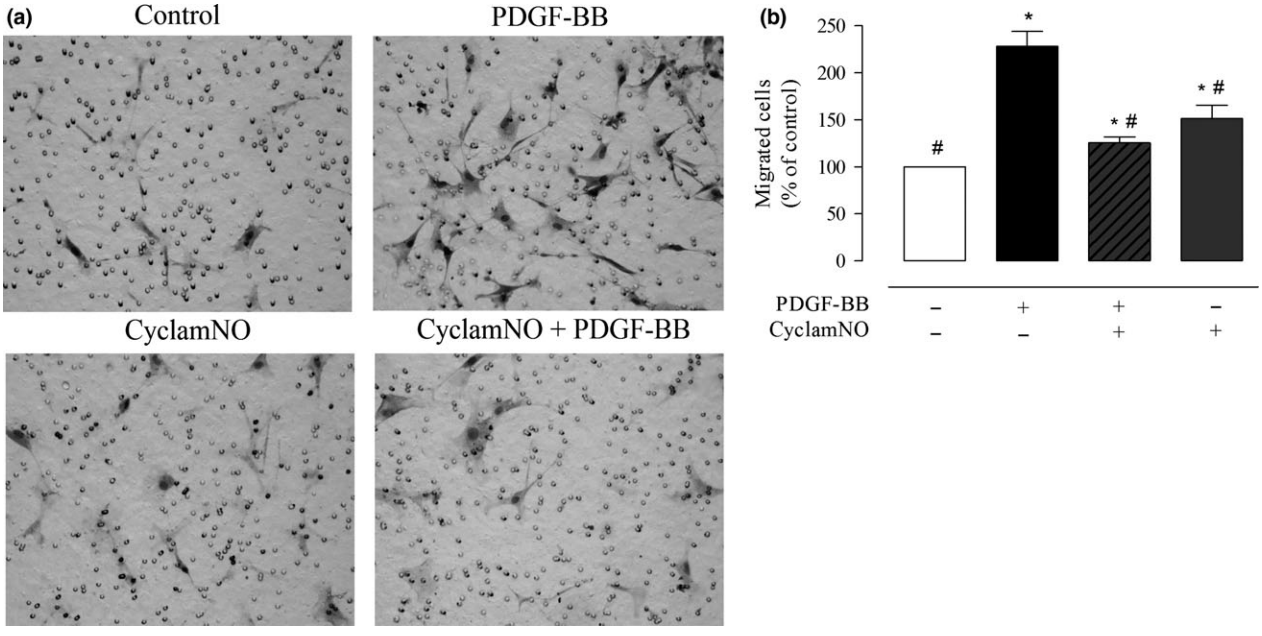

Figure 3 Platelet-derived growth factor type BB (20 ng/ml) induced vascular smooth muscle cell directional migration in the presence of cyclamNO (0.1 mM), as observed in representative images of the membrane inserts (a). Migrated cells were counted in full membranes (b). Values presented as mean ± SEM of three to five individual experiments in triplicate. $^*P < 0.05$ compared with control; $^\#P < 0.05$ compared to platelet-derived growth factor type BB. The magnification was 400×.

Non-directional migration was also evaluated by wound-healing assay (Figure 4a,b). In the presence of PDGF-BB, the migration to wound area was intense; a significant increase on the number of migrated cells (Figure 4b) was observed in relation to control (79.5 ± 4.4 and 26.3 ± 3.0 cells, respectively), promoting almost the complete closure after 16 h. In contrast, migration was almost completely prevented by the concomitant presence of cyclamNO in the cell medium (43.0 ± 4.5 cells). Incubation only with cyclamNO had no significant effect on cell migration in this assay (31.5 ± 4.0 cells).

Phenotypic switching prevention by cyclamNO

The phenotype switching to synthetic/proliferative was detected in PDGF-BB-treated VSMC (Figure 5), as evidenced by a remarkable reduction of ~60% (0.32 ± 0.04) in α-SMA protein expression in comparison with control cells (0.81 ± 0.08). Treatment with both PDGF-BB and cyclamNO resulted in expression close to control (0.67 ± 0.01), while treatment with cyclamNO only maintained the expression similar to control (0.83 ± 0.04).

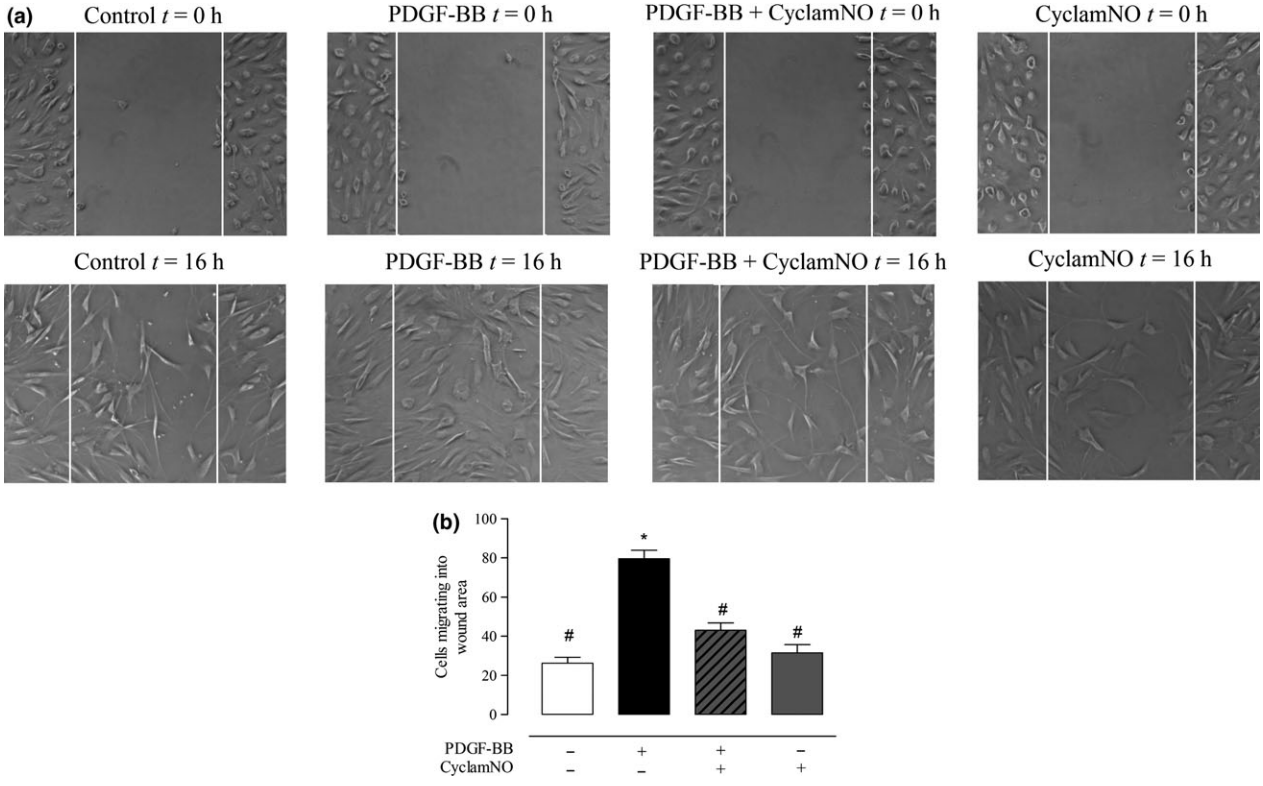

Figure 4 Platelet-derived growth factor type BB (20 ng/ml) induced non-directional migration in the presence of cyclamNO (0.1 mM). (a) Phase contrast images of the same representative field were taken 0 and 16 h after wounding. (b) The number of cells migrated into the wound zone were counted. Values presented as mean ± SEM of three to five individual experiments in triplicate. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared to platelet-derived growth factor type BB. The magnification was 200×.

Discussion

The cytotoxicity assays showed that cyclamNO is essentially non-toxic to VSMC. The low toxicity of ruthenium nitrosyls to host healthy cells has already been demonstrated and probably involves their ability to mimic iron ion and therefore bind to many biomolecules, such as albumin and the iron transport protein transferrin.[38,39,48] Remarkably, ruthenium is not accumulated in plasma or organs (liver and kidney) as observed for Wistar rats after intraperitoneal administration of [Ru(NO)(Hedta)].[49,50]

The IC_{50} value for cyclamNO in VSMC was not reached in the wide concentration range we used (0.1–1.5 mM). In studies with other cell lines, as fibroblasts, the IC_{50} value is only achieved at higher concentration levels of cyclamNO, superior to 2 mM, and it seems that it does not generate toxic metabolites.[22,24,34,38,39] In comparison, the SNP IC_{50} value in VSMC is nearly 0.5 mM.[47] and conceivably, at 1.5 mM, elimination of 100% of cell population after 48 h exposition was observed. The results of cytotoxicity assays were very useful to demonstrate the effects of this complex in this specific cell line, not shown before, and showed that the inhibitory effects of cyclamNO on VSMC are not simply due to cytotoxicity. All of this indicates that cyclamNO is definitely lowly toxic and can be safely applied. A concern regarding these concentrations of NO donors is that they are several orders of magnitude greater than concentrations required to affect vasoreactivity and blood pressure;[38,39,51] however, higher concentrations of exogenous NO are required to demonstrate inhibition of cellular functions in short-term *in vitro* experiments with cultured VSMC[15,52–55] as well as other cell types.[56] Nevertheless, these experiments may provide a basis for further investigations as *in vivo* experiments.

The presence of cyclamNO significantly inhibited PDGF-BB stimulation, according to the percentage of cells with 3H-thymidine-incorporated and PCNA protein expression. These results revealed the potential use of cyclamNO to inhibit VSMC proliferation, even in the presence of potent proliferative stimuli. Transwell and wound-healing assays were used to directly investigate the VSMC migration induced by PDGF-BB. Compared to PDGF-BB alone, cyclamNO prevented migration significantly alone and even in the presence of PDGF-BB in the transwell assays.

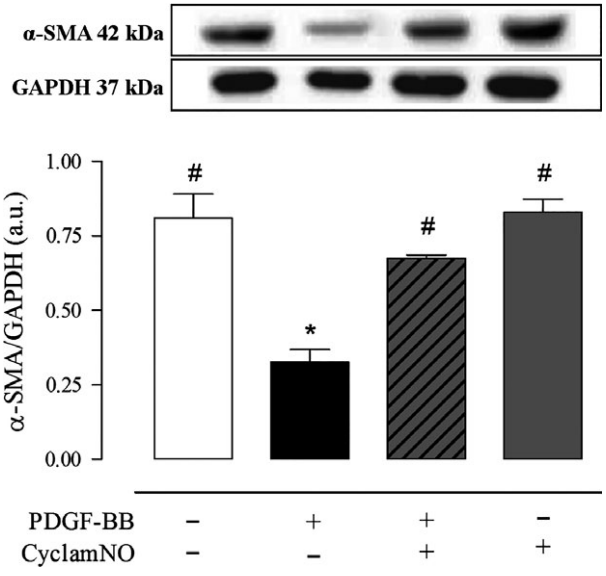

Figure 5 Representative Western blotting for alpha smooth muscle actin expression in vascular smooth muscle cells after 48-h exposition to 0.1 mM of cyclamNO or 20 ng/ml of platelet-derived growth factor type BB. Values are presented as mean ± SEM of three to four individual experiments in triplicate.*$P < 0.05$ compared to control; #$P < 0.05$ compared to platelet-derived growth factor type BB.

According to the wound-healing assays, the presence of cyclamNO in the cell medium also reduces significantly the cells migration.

Vascular smooth muscle cell proliferation plays an important role in several vascular diseases. Consequently, inhibition of VSMC differentiation represents a potentially therapeutic strategy.[2] The present study demonstrated that the ruthenium nitrosyl complex cyclamNO inhibited the effects of PDGF-BB on VSMC phenotype transition. Our results suggest that cyclamNO promotes the maintenance of VSMC phenotype, and, consequently, the contractile function.

The VSMC are highly specialized cells whose primary function is contraction, regulating blood pressure and flow distribution by alterations in the diameter of the vessels and, therefore, exhibit elevated expression of contractile proteins, ion channels and signalling molecules required for their major function; however, changes in local environmental can influence the state of differentiation of the VSMC, which undergo profound and reversible changes, favouring a synthetic/proliferative phenotype.[2] High levels of PDGF-BB were observed under conditions of vascular remodelling, as in regions of atherosclerotic plaques and restenosis.[57] This growth factor is one of the most potent suppressors of VSMC contractile genes.[58] Through the activation of platelet-derived growth factor receptor β (PDGF-Rβ), PDGF-BB initiates a complex signalling cascade, resulting in a pro-remodelling state and stimulating late proliferation through its release from activated platelets continuously in chronically injured areas.[59–62] Within 48 h of PDGF-BB exposition, VSMC shifts to a synthetic phenotype, as revealed by downregulation of the VSMC-differentiated phenotype marker α-SMA. However, in the presence of cyclamNO in addition to PDGF-BB, this alteration was almost fully prevented, and, therefore, maintaining the VSMC primary function, that is contraction.

Moreover, it is known that the phenotype transition induced by PDGF-BB also facilitates VSMC proliferation and migratory activity.[58] Here, we demonstrated that the presence of cyclamNO on cell medium was effective in attenuating both events. Generally, these properties are attributed to NO direct interaction with sGC enzyme to produce cGMP and activate GMP-dependent protein kinases, which can phosphorylate several intracellular proteins and inhibit migration stimulated by chemoattractants, including PDGF-BB and angiotensin II.[15,63,64] Previous studies have already shown large inhibition of VSMC phenotype through the administration of several NO donors, including organic nitrates, S-nitrosoglutathione, NONOates, S-nitroso-N-acetyl-DL-penicillamine and SNP,[16,52,65,66] despite their dissimilarities regarding the mechanism of release, cytotoxicity and duration of effects.

Vascular effects of cyclamNO have been reported. In one previous study published by our group,[39] the mechanism of action of cyclamNO *in vivo* had been characterized. Briefly, the intravenous injection of cyclamNO produced a dose-dependent (5–100 nmol/kg) reduction on the mean arterial blood pressure (MAP) in hypertensive rats induced by intravenous infusion of phenylephrine. Previous administration of the soluble guanylate cyclase (sGC) inhibitor, methylene blue (83 nmol/kg/min), or the NO scavenger, carboxy-PTIO ($6x10^2$ to $6x10^3$ nmol/kg/min), almost abolished the MAP reduction induced by cyclamNO, showing that this effect was due to sGC stimulation to lead to cGMP accumulation induced by NO release from cyclamNO. The *trans*-[RuCl(tfms)(cyclam)](tfms) (tfms = trifluoromethanesulfonate) complex, named cyclam-tfms, is a precursor for cyclamNO by reacting with NO or nitrite, and whose cation belongs, as one of the forms of the catalyst, to the catalytic nitrite/NO conversion cycle[29,36] did not interfere in the blood pressure values. Furthermore, *trans*-$[Ru(NO)Cl(cyclam)](PF_6)_2$ sustains the duration of these effects for longer period than ruthenium nitrosyls as *trans*-$[Ru(NH_3)_4(L)(NO)]^{3+}$ (L = isn, py, inaH) under the same conditions,[39] and also much longer, almost 20 times the duration of the effect provided by equimolar SNP.[38] Recently, cyclamNO showed vascular relaxing effects in aortic rings from normotensive male Wistar rats precontracted with noradrenaline assigned to NO release.[51] In the present case, the low cytotoxicity, the inhibition of

proliferation and migration and the maintenance of the contractile phenotype marker expression of VSMC are consistent with the possible release of NO from cyclamNO after reduction, although these effects could be attributed to the complex itself.

Conclusions

The ruthenium nitrosyl complex *trans*-[Ru(NO)Cl(cyclam)] $(PF_6)_2$, cyclamNO, attenuated PDGF-BB-induced VSMC proliferation and migration, and maintained contractile phenotype marker expression, with lower cytotoxicity, especially when compared to the clinically used NO donor SNP, without its side effects, and maintaining cell viability even at high concentrations. The results indicate that the inhibitory effects of cyclamNO on VSMC are not simply due to cytotoxicity, and they are also consistent with the possible release of NO from cyclamNO after reduction, although these effects could be attributed to the complex itself.

Altogether, our results indicate that cyclamNO may be an interesting alternative as NO-prodrug for the prevention of the development of IH and a potential strategy for the development of cardiovascular devices, such as stents, with the large advantage that released NO can inhibit VSMC besides it can stimulate endothelial cell growth and survival, facilitating the re-endothelialization process after injury, reducing thrombogenicity and platelet activation,[67] resulting in an improvement of the safety and efficacy of these devices.

Two features of cyclamNO outstand: it may be embedded in matrices, as in coatings of surgical stainless steel for stents,[35] and it is able to enter in a catalytic nitrite to NO conversion cycle. These features combined may constitute a system that conceivably may be capable to provide low but relatively long-lasting NO production and, therefore, a long-lasting effect, improving the benefits of targeted NO delivery. Future directions will include the evaluation of the complex behaviour embedded in a surgical stainless steel matrix, and this device effects in vascular cells lines.

Declarations

Conflict of interest

The authors declare no conflict of interests associated with this publication, and there has been no financial support for this work that could have influenced its outcome. The manuscript has been read and approved by all named authors, and there are no other persons who satisfied the criteria for authorship but are not listed.

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