Cardiovascular Drugs and Therapy (2019) 33:687–692
https://doi.org/10.1007/s10557-019-06921-w

SHORT COMMUNICATION

A Novel Epigenetic Drug-Eluting Balloon Angioplasty Device: Evaluation in a Large Animal Model of Neointimal Hyperplasia

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Published online: 30 December 2019

Abstract

Purpose Drug-eluting balloon catheters (DEBc) coated with paclitaxel (PTX) have been associated with potential safety concerns. An efficacious but less toxic balloon coating may reduce these outcomes. We evaluated a novel DEBc, Epi-Solve, coated with metacept-3 (MCT-3), a member of the histone deacetylase inhibitor (HDACi) class of epigenetic agents, in a large animal model of neointimal hyperplasia (NIH).

Methods Plain balloon angioplasty (PABA) catheters were ultrasonically coated with MCT-3 to generate Epi-Solve DEBc. An ovine model of NIH formation was established utilising partial left common carotid artery (LCA) ligation. Twenty-eight days post neointima (NI) induction, PABA, Epi-Solve or PTX-coated DEBc were deployed at the site of induced NI formation. Twenty-eight days post-intervention, ligated vessels were evaluated for attenuation of NI formation, gene expression profiles and immunohistochemical analysis.

Results Epi-Solve DEBc demonstrated attenuation of NIH over no intervention and a trend to inhibition of NIH over PABA. Gene expression analysis and immunohistochemical studies identified significant anti-proliferative and anti-inflammatory signatures and reduced vascular endothelial cell activation compared to PABA.

Conclusions Epi-Solve is a novel HDACi-coated DEBc which demonstrates significant anti-proliferative and anti-inflammatory signatures and reduced vascular endothelial cell activation compared to PABA in an ovine model and may afford endothelial protection.

Keywords Epigenetic · Drug-eluting balloon angioplasty · Neointimal hyperplasia

Introduction

The advent of DEBc technology affords theoretical advantages over drug-eluting stents (DES) in NIH management, including homogenous drug delivery to vessel wall, immediate release (no agent in situ), reduced inflammation/thrombosis and anti-platelet requirements [1]. Improved outcomes together with cost-effectiveness of DEBc over DES may realise improved treatment options in NIH management [2–4].

PTX-coated DEBc are the most popular commercially available devices able to deliver this novel treatment modality. Recent reports suggest potential concerns relating to PTX DEBc including luminal obstruction and inflammation due to PTX-based particulate matter discharged at the time of deployment, and a recent meta-analysis generated a signal of unknown cause for increased all-cause mortality beyond 2 years in PTX-coated DEB and stents utilised in lower limb

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10557-019-06921-w) contains supplementary material, which is available to authorized users.

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revascularisation interventions prompting regulatory communications [5–7]. These observations suggest that notwithstanding the potentially superior clinical efficacy of DEBc, an opportunity exists to improve on current DEBc technology using novel coating molecules less likely to elicit potentially dangerous effects on target vessel responses and collateral deployment toxicity whilst retaining superior efficacy with regard to attenuation of NIH.

Our previous studies have identified MCT-3, a member of the HDACi epigenetic class of agents, with significant in vivo anti-NIH activity, low systemic toxicity [8, 9] and favourable physico-chemical properties predictive of bioavailability based on Lipinski's "rule of 5"[10], as a potential alternative DEBc coating. The current study evaluated the effect of an MCT-3-coated DEBc device, termed Epi-Solve, on NIH formation and investigated associated cellular and molecular mechanisms in a large animal model of NIH.

Methods

Production of DEBc

Epi-Solve DEBc were produced using PABA catheters with 40–150 mm long × 2.0–6.0 mm diameter balloons. Balloon surface area was calculated based on the area approximating cylinder sides ($2\pi rh$). The total quantity of MCT-3, previously synthesised in the laboratory [8, 9], required to coat each PABA at 3.0 μg/mm^2 (to approximate coating density of PTX-coated DEBc) was calculated and dissolved in 1.0 ml dimethyl sulfoxide (DMSO) then in 19.0 ml of a 50:50 mix of 100% ethanol and Ultravist® 370 (iopromide-based excipient) and delivered to the surface of an inflated balloon using a preprogrammed SonoTek Exactacoat ultrasonic spray coating system with an AccuMist ultrasonic nozzle. Subsequently balloons were deflated, air-dried overnight and packaged. Epi-Solve MCT-3 coating bioactivity was confirmed prior to deployment (Online Resource 1). PABA catheters were ultrasonically coated with a 50:50 mix of 100% ethanol and Ultravist® 370 under identical conditions to Epi-Solve DEBc to control for the excipient used to coat Epi-Solve DEBc. IN.PACT Admiral 130 cm long angioplasty catheters with 40–60 mm long × 5.0 mm diameter balloons coated with 3.5 μg/mm^2 PTX utilising a urea-based excipient were used in in vivo studies.

In vitro Studies

Cell Culture and Toxicity Studies

Human umbilical vein endothelial cells (HUVEC) maintained in Media-199 supplemented with penicillin/streptomycin, 20% FCS, 20 μg/ml endothelial cell growth factor and 20 μg/ml heparin and kept in a 5% CO_2 incubator at 37 C were used to compare MCT-3 and PTX cytotoxicity. 5.0 nmol/L–1.0 mmol/L (final concentration) of each agent was added to 1×10^5 HUVEC for 30 min–24 h. Cell viability was assessed using 0.4% trypan blue staining immediately after culture.

RNA Extraction, Reverse Transcription and Real-Time PCR

Extraction of mRNA from 10 μm thick sections from formalin-fixed paraffin embedded (FFPE) blocks of a 3.0–4.0 mm region of the LCA just proximal to ligation site and from the unligated RCA at the same level was undertaken utilising the Qiagen RNeasy FFPE RNA kit. Reverse transcription was performed using a 20 μl reaction mix containing dNTPs (100 mmol/L), MultiScribe Reverse Transcriptase (50 U/μl), RT buffer (10X), RNase Inhibitor (20 U/μl), nuclease-free water, ovine primers (Online Resource 2) and mRNA samples. Real-time PCR analysis was performed in duplicate using Sensifast SYBR No-Rox kit. Reaction volumes of 20 μl contained 2x Sensifast SYBR No-Rox mix, nuclease-free water and RT reaction product. Each PCR run also included wells of no template control. A melting point dissociation curve generated by the instrument confirmed that only a single product was present. Fluorescence data were quantitated using the threshold cycle (C_T) value. Data was normalised to actin and presented as the mean fold change of LCA:RCA mRNA ratios.

In vivo Studies

Female, adult Border Leicester crossbred sheep, weighing 50–60 kg, were utilised in the partial ligation method of flow-induced vascular remodelling [11] adapted for use in our ovine system.

Sheep were anaesthetised using 5 mg/kg of propofol, intubated and anaesthesia maintained with gaseous isoflurane at a flow rate of 2.5% with positive ventilation. A sterile surgical site was prepared on the left hand side of the neck. The LCA was exposed and partially ligated (30% luminal diameter reduction based on fluoroscopic assessment) with a silk suture. The incision was then closed and the animals recovered. Animals were assigned to one of 4 groups, treatment with PABA, Epi-Solve DEBc, PTX DEBc or control group (no intervention). Twenty-eight days' post partial ligation of the LCA, the treated groups had either a PABA, Epi-Solve or PTX DEBc positioned and fully inflated in the region of LCA ligation for 120 s, whilst the control group had no intervention. Following 28 days, the animals were euthanised and the ligated LCA and contralateral unligated right common carotid artery (RCA) collected for histopathological analysis. Routine

haematology and biochemistry analysis for each animal group at day 0 (pre-ligation), 4 (therapeutic intervention) and 8 weeks post-ligation (study termination) are provided in Online Resource 3.

Analysis of Carotid Artery Neointimal Hyperplasia

Carotid artery samples from (a) just proximal to the site of partial ligation of the LCA and (b) from the corresponding level from the contralateral unligated RCA were fixed with 10% neutral buffered formaldehyde solution for 48 h then embedded in paraffin. Carotid artery sections (4 μm thick) were stained with haematoxylin and eosin (H&E), imaged and scanned using an Olympus BX50 microscope and Aperio ScanScope AT Turbo scanner. Image J was utilised to measure the medial and neointimal areas of each section with morphometric analysis performed by a blinded investigator. The intimal and medial area of each group and their ratio were calculated by the following protocol: Intima area = inner elastic membrane surrounding area - lumen area; media area = outer elastic membrane surrounding area - inner elastic membrane surrounding area.

To examine changes in elastin fibres, Verhoeff-Van Gieson (VVG) staining was performed on carotid artery sections. Stained sections were imaged and scanned using an Olympus BX50 microscope and Aperio ScanScope AT Turbo scanner. Image J was then used to measure the area of positively stained elastin in each section with morphometric analysis performed by a blinded investigator.

Immunohistochemical Analysis

ICAM-1 Expression

Carotid artery sections were dewaxed and incubated with a primary antibody, mouse anti-sheep CD-54 (1:100 dilution) at 4 °C overnight. Subsequently a secondary biotinylated anti-mouse antibody 1:200 was added for 30 min. After washing and incubation with avidin-biotin complex reagent, 3,3'-diaminobenzidine (DAB) reagent was applied to each section. Conversion of the DAB substrate to a coloured (brown) product was monitored, and slides were immersed in dH_2O to stop the reaction. Sections were dehydrated and coverslipped using DPX mounting media. Images were obtained using a light microscope by a blinded investigator.

CD31 Expression

Carotid artery sections were mounted on SuperFrost Plus slides and deparaffinised before performing antigen retrieval. Sections were blocked with 10% goat serum and incubated overnight at room temperature with CD31 rabbit polyclonal primary antibody (1:250 dilution). Immunoreactivity was detected using the Alexa Fluor® 488 goat anti-rabbit secondary antibody (1:500 dilution). Immunofluorescence-stained sections were observed under 20× magnification by a blinded investigator.

Statistical Analysis

Results were expressed as means ± standard error of the mean (SEM) and analysed using GraphPad Prism 5 software, using unpaired t-tests for two-group comparisons and one-way analysis of variance (ANOVA) followed by Tukey's post hoc ANOVA for three or more group comparisons. p-value of <0.05 was considered statistically significant.

Results

Significant NI was identified in the LCA from untreated (control) vessels and reduced in all treatment groups (Fig. 1a) with no NI identified in the RCA. Quantification analysis identified Epi-Solve DEBc as a potent inhibitor of NIH compared to no intervention with both Epi-Solve and PTX DEBc demonstrating a non-significant trend to greater attenuation of NI compared to PABA treatment (Fig. 1b).

Evaluation of the effects on gene expression signatures of non-coating (PABA) vs. coating (PTX-coated and Epi-Solve) was performed. Proliferative gene expression profiling demonstrated significant attenuation of Ki67 and PCNA expression together with significant augmentation of $p21^{CIP1/WAF1}$ expression in Epi-Solve and PTX DEBc-treated vessels over PABA treatment suggesting Epi-Solve treatment has potent anti-proliferative effects on the target blood vessel similar in magnitude to PTX DEBc (Fig. 1c). Epi-Solve and PTX DEBc-treated vessels also demonstrated significant attenuation of inflammatory gene signature markers IL-6 and MCP-1 (Fig. 1c).

MCT-3-treated HUVEC demonstrated significantly less cell death over PTX-treated cells over a range of concentrations and times (Fig. 2a). Significant attenuation of intercellular adhesion molecule-1 (ICAM-1) and plasminogen activator inhibitor type 1 (PAI-1) mRNA expression (Fig. 2B) together with reduced ICAM-1 and enhanced CD31 protein expression from Epi-Solve-treated animals was identified (Fig. 2C). Together these results suggest reduced endothelial activation post Epi-Solve deployment.

Discussion

The current study evaluated the anti-NIH effect together with cellular and molecular mechanisms of a novel DEBc, Epi-Solve, coated with the HDACi MCT-3, in a large animal

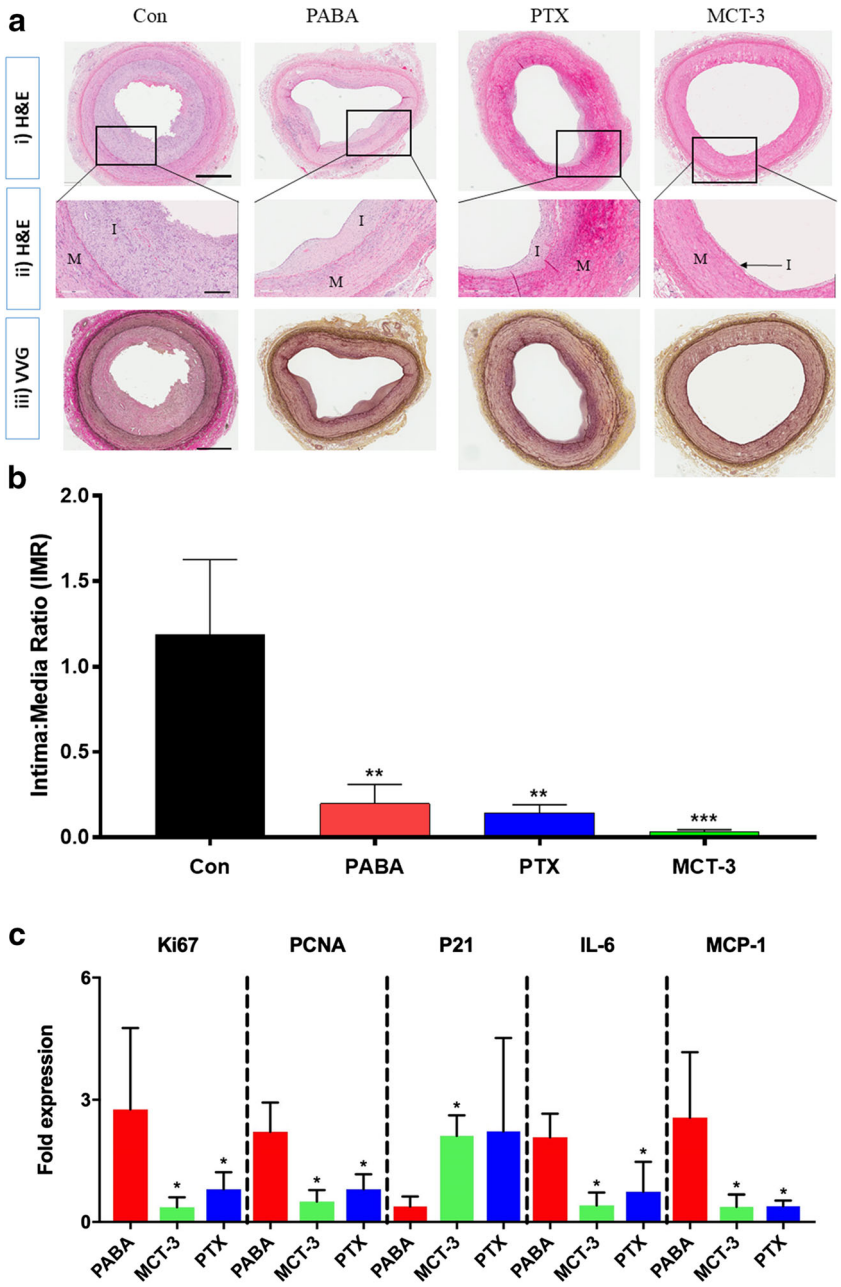

Fig. 1 **a and b** Epi-Solve DEBc attenuates LCA neointimal hyperplasia **a** Con = untreated, PABA = PABA, PTX = PTX DEBc and MCT-3 = Epi-Solve DEBc. (i) 20× magnification, scale bar = 1.0 mm. (ii) 80× magnification of area outlined in (i), scale bar = 300 μm. (iii) 20× magnification VVG staining, scale bar = 1.0 mm. I = neointima, M = media. **b** Con = untreated, PABA = PABA, PTX = PTX DEBc, MCT-3 = Epi-Solve DEBc. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Con, $n = 4–8$. **c** Epi-Solve DEBc has anti-proliferative and anti-inflammatory gene expression signatures in vivo Ki67, PCNA, $p21^{WAF1/CIP1}$, IL-6 and MCP-1 mRNA expression. PABA = PABA, MCT-3 = Epi-Solve DEBc and PTX = PTX DEBc. $^{*}p < 0.05$ vs. PABA, $n = 4–8$

model of NIH. Specific effects of HDACi in the vasculature have been well documented and include attenuation of NIH together with augmentation of vascular endothelial cell survival via reduction of pro-inflammatory cytokine expression [9, 12, 13].

Epi-Solve DEBc may inhibit NIH and reduce vascular endothelial cell activation and death compared to PTX-coated DEBc. Gene expression studies suggest significant, Epi-Solve-mediated, anti-proliferative and anti-inflammatory signatures compared to PABA intervention identifying the HDACi MCT-3 as a balloon coating with a potentially anti-NIH and enhanced re-endothelialisation profile. Comparison with PABA intervention, a known modulator of significant proliferative and inflammatory effects in the target vessel [14, 15], was performed to evaluate the effect of balloon coating (either PTX or MCT-3) vs. no coating (PABA) on blood vessel gene expression and cellular profiles. A trend to inhibition of NIH when comparing PABA intervention vs. Epi-Solve and PTX DEB'c, whilst not significant, and a recognised limitation of the study to be addressed in future definitive studies, is nonetheless supportive of the potential translation of the observed gene expression and cellular effects of the MCT-3-coated Epi-Solve DEBc into attenuation of NI and enhanced endothelial protection.

Whilst porcine models have been the predominant preclinical restenosis model represented in the literature, ovine

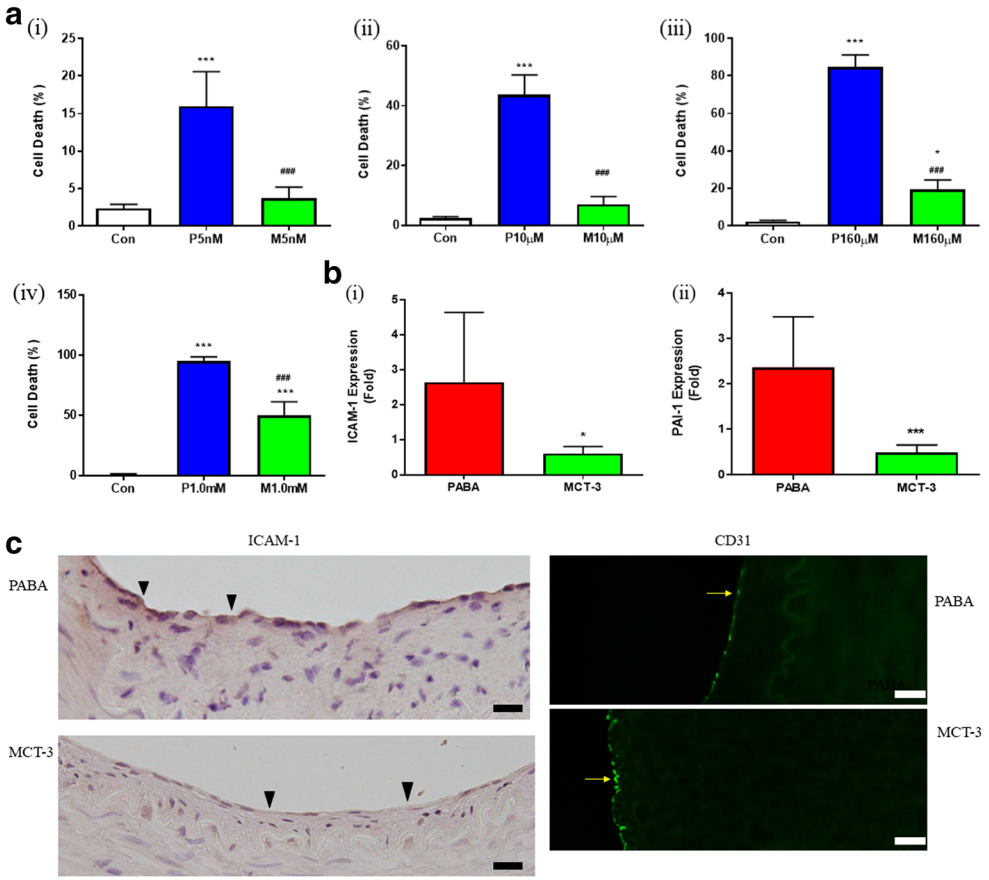

Fig 2 **a-c** MCT-3 and Epi-Solve effects on vascular endothelial cells in vitro and in vivo. **a** In vitro effects of MCT-3 and PTX on cytotoxicity of HUVEC. (i) Con = Untreated, P5nM = 5.0 nmol/L PTX treatment for 24 h, M5 nM = 5.0 nmol/L MCT-3 treatment for 24 h. ***$p < 0.001$ vs. Control, $^{###}p < 0.001$ vs. P5nM, $n = 3–4$. (ii) Con = Untreated, P10μM = 10.0 μmol/L PTX treatment for 24 h, M10 μM = 10.0 μm/L MCT-3 treatment for 24 h. ***$p < 0.001$ vs. Control, $^{###}p < 0.001$ vs. P10μM, $n = 3–4$. (iii) Con = Untreated, P160μM = 160.0 μmol/L PTX treatment for 30 min, M160 μM = 160.0 μmol/L MCT-3 treatment for 30 min.*$p < 0.05$ vs. Control, ***$p < 0.001$ vs. Control, $^{###}p < 0.001$ vs. P160μM, $n = 3–4$. (iv) Con = Untreated, P1.0mM = 1.0 mmol/L PTX treatment for 24 h, M1.0mM = 1.0 mm/L MCT-3 treatment for 24 h. ***$p < 0.001$ vs. Control, $^{###}p < 0.001$ vs. P1.0mM, $n = 3–4$. **b** ICAM-1**(i)** and PAI-1**(ii)** mRNA expression with PABA = PABA or MCT-3 = Epi-Solve DEBc. *$p < 0.05$, ***$p < 0.001$ vs. PABA, unpaired t-test, $n = 4–8$. **c** ICAM-1immunohistochemistry and CD31 immunofluorescence with PABA = PABA and MCT-3 = Epi-Solve DEBc. Scale bar = 50 μm. Arrow-heads = vascular endothelium.

models of restenosis/intimal proliferation are readily identifiable [16]. Indeed similarities of the ovine coagulation and fibrinolytic systems to humans, more so than other species, are of considerable significance for a representative animal model of restenosis [17], and when considering the oversizing of 30–50%, porcine vessels require to generate comparable amounts of restenosis [18]; the ovine partial ligation model we have utilised in our study has distinct advantages and is potentially more representative of the human restenosis phenotype.

Excipients utilised for balloon-coating iopromide (Epi-Solve DEBc) and urea (PTX DEBc) are both regarded as hydrophilic "spacers" enhancing the solubility of lipophilic agents [19] and identified as providing similar drug loss rates during passage and inflation, similar vessel wall uptake of balloon-coating agent and similar efficacy with regard to inhibition of neointimal area at the same balloon-coating agent concentration [20] suggesting effects observed in our study are likely to be mediated by the balloon drug coatings rather than differences in excipients utilised.

Given a combination of anti-proliferative and anti-inflammatory effects together with potential for vascular endothelial protection, HDACi may represent a potentially novel DEB coating with inherent advantages over PTX coatings whilst still demonstrating potent anti-NIH effects. Additional, definitive studies confirming anti-NIH effects together with evaluation of the impact of excipient, efficiency of balloon-based drug delivery, quantification of residual balloon-associated drug post-deployment and formal pharmacokinetic studies will inform on the potential clinical utility of the Epi-Solve DEBc.

Acknowledgements Edmund WC Khong of Eastern Health Clinical School, Monash University, Australia: for Data interpretation; Dr David Jones of Bio21 Institute, The University of Melbourne, Australia for SonoTek spray coater; National Health and Medical Research Council of Australia Grant 1113681; National Heart Foundation of Australia,

Future leader fellowship ID:102068 Australian Research Council, Grant LE100100147; Medtronic Australasia Pty and Abbott Australasia Pty Lt, PABA; and IN.PACT Admiral DEBc.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in studies involving animals were in accordance with the ethical standards of the Baker Heart and Diabetes Institute Animal Ethics Committee, Melbourne, Australia, Approval number 1513767.1.

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