Identification of a pharmacological inhibitor of Epac1 that protects the heart against acute and chronic models of cardiac stress

Marion Laudette^{1,2}, Antonio Coluccia³, Yannis Sainte-Marie^{1,2}, Andrea Solari^{1,2}, Loubina Fazal^{1,2}, Pierre Sicard⁴, Romano Silvestri³, Jeanne Mialet-Perez^{1,2}, Sandrine Pons⁵, Bijan Ghaleh⁵, Jean-Paul Blondeau⁶, and Frank Lezoualc'h^{1,2*}

¹INSERM UMR-1048, Institut des Maladies Métaboliques et Cardiovasculaires, 1 avenue Jean Poulhès, 31432 Toulouse, France; ²Université de Toulouse—Paul Sabatier, 31432 Toulouse, France; ³Department of Drug Chemistry and Technologies, Sapienza University of Rome, Laboratory Affiliated to Istituto Pasteur Italia—Fondazione Cenci Bolognietti, I-00185 Roma, Italy; ⁴INSERM, CNRS, Université de Montpellier, PHYMEDEXP, IPAM, Montpellier, France; ⁵INSERM, U955, Equipe 03, F-94000 Créteil, France; and ⁶Université Paris-Sud, Faculté de Pharmacie, 92296 Châtenay-Malabry Cedex, France

Aims

Recent studies reported that cAMP-binding protein Epac1-deficient mice were protected against various forms of cardiac stress, suggesting that pharmacological inhibition of Epac1 could be beneficial for the treatment of cardiac diseases. To test this assumption, we characterized an Epac1-selective inhibitory compound and investigated its potential cardioprotective properties.

Methods and results

We used the Epac1-BRET (bioluminescence resonance energy transfer) for searching for non-cyclic nucleotide Epac1 modulators. A thieno[2,3-b]pyridine derivative, designated as AM-001 was identified as a non-competitive inhibitor of Epac1. AM-001 has no antagonist effect on Epac2 or protein kinase A activity. This small molecule prevents the activation of the Epac1 downstream effector Rap1 in cultured cells, in response to the Epac1 preferential agonist, 8-CPT-AM. In addition, we found that AM-001 inhibited Epac1-dependent deleterious effects such as cardiomyocyte hypertrophy and death. Importantly, AM-001-mediated inhibition of Epac1 reduces infarct size after mouse myocardial ischaemia/reperfusion injury. Finally, AM-001 attenuates cardiac hypertrophy, inflammation and fibrosis, and improves cardiac function during chronic β -adrenergic receptor activation with isoprenaline (ISO) in mice. At the molecular level, ISO increased Epac1-G protein-coupled receptor kinase 5 (GRK5) interaction and induced GRK5 nuclear import and histone deacetylase type 5 (HDAC5) nuclear export to promote the activity of the prohypertrophic transcription factor, myocyte enhancer factor 2 (MEF2). Inversely, AM-001 prevented the non-canonical action of GRK5 on HDAC5 cytoplasmic shuttle to down-regulate MEF2 transcriptional activity.

Conclusion

Our study represents a 'proof-of-concept' for the therapeutic effectiveness of inhibiting Epac1 activity in cardiac disease using small-molecule pharmacotherapy.

Keywords

Epac inhibitor • G protein-coupled receptor kinase • Cardiac disease • Fibrosis • Heart failure

1. Introduction

The biological actions of cyclic AMP (cAMP) are mediated by the protein kinase A (PKA), the cAMP-gated ion channels, and the more recently discovered exchange proteins directly activated by cAMP 1 and 2 (Epac1 and Epac2), which function as guanine-nucleotide exchange factors (GEFs) for the small G proteins, Rap1 and Rap2.¹ Epac proteins contain a

cAMP-binding domain and a catalytic region which interacts with Rap1 and facilitates the exchange of GTP for GDP. In heart, cAMP represents the strongest mechanism for increasing cardiac function in response to acute sympathetic stimulation of β -adrenergic receptor (β -AR).² In this setting, norepinephrine released from intracardiac nerve terminals activates β -ARs, which increase intracellular cAMP level to enhance PKA activity in cardiomyocytes. PKA regulates key excitation-contraction

coupling proteins to increase cardiac contraction and relaxation. Whereas acute stimulation of β -AR has beneficial effects on heart function, chronic activation of this pathway triggers pathological cardiac remodelling, which may ultimately lead to heart failure (HF).³

Based on the initial finding that Epac1 was up-regulated in various animal models of HF and in human failing hearts, recent studies aimed at determining the role of this cAMP-GEF in cardiac remodelling and HF. Consistent with its pro-hypertrophic action in neonatal and adult cardiomyocytes,^{4,5} genetic ablation of Epac1 prevented cardiac hypertrophy and fibrosis induced by sustained β -AR activation and improved cardiac function.⁶ Furthermore, Epac1 deletion provides cardioprotection against other forms of stress such as pressure overload-induced cardiac dysfunction and ischaemia–reperfusion (I/R) injury suggesting that pharmacological inhibition of Epac1 may slowdown the development of HF.^{7,8} However, some discrepancies have been reported in the literature as chronic β -AR activation-induced cardiac hypertrophy was attenuated in Epac1-deleted mice in one study,⁶ whereas in other studies genetic inhibition of Epac1 failed to attenuate myocardial growth in response to sustained β -AR activation or pressure overload.^{7,9} Therefore, to circumvent these discrepancies possibly due to the genetic background of the mice and to investigate Epac1 role in the development of cardiac diseases in other relevant animal models, it is necessary to develop selective pharmacological inhibitors of Epac1.

Among the rare selective Epac inhibitors, a tetrahydroquinoline analogue named CE3F4 was identified as a selective pharmacological inhibitor of Epac1 *in vitro*,^{10,11} but its low biodisponibility prevents its *in vivo* application. Another non-cyclic nucleotide small molecule designed as ESI-09 has been shown to have favourable pharmacological/toxicological profile when administrated *in vivo* to mice but this compound does not discriminate between Epac isoforms.^{12–14}

In this article, we identified and characterized a novel selective pharmacological inhibitor of Epac1 that we named AM-001. This small molecule is a thieno[2,3-b]pyridine derivative (3-amino-N-(4-fluorophenyl)-4-phenyl-6-(thiophen-2-yl)thieno[2,3-b]pyridine-2-carboxamide) which selectivity inhibits Epac1 catalytic activity. AM-001 displays cardioprotective properties against myocardial I/R injury and the detrimental effects of chronic β -AR activation.

2. Methods

2.1 Cell culture and transfection

All procedures were performed in accordance with the Guide for the care and use of laboratory animals and the veterinary committee has been informed of the cardiac myocyte isolation protocol used. Neonatal rats of 1–2 days old were euthanized by decapitation. Mice were euthanized by intraperitoneal injection of pentobarbital (300 mg/kg) and heparin (150 units). Primary culture of adult murine and rat neonatal cardiomyocytes (NRVM) was performed as previously described.^{4,8} Human Embryonic Kidney cells lines (HEK293) were maintained in minimal essential medium with FBS (10%) and penicillin-streptomycin (1%). All media, sera, and antibiotics used in cell culture were purchased from Invitrogen. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.2 Animals

All animal procedures were performed in accordance with Institutional Guidelines on Animal Experimentation and with a French Ministry of Agriculture license. Moreover, this investigation conformed to the

Guide for the Care and Use of Laboratory Animals published by the Directive 2010/63/EU of the European Parliament. Mice were housed in a pathogen-free facility, and all animal experiments were approved by the Animal Care and Use Committees of the University of Toulouse.

2.3 Myocardial I/R *in vivo*, assessment of area at risk and infarct size

The procedure of myocardial I/R and the assessment of area at risk and infarct size are already described in Ref.⁸ Mice (C57BL/6 or C57BL/6-SV129 background for Epac1 knock-out and control littermates) were anaesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated and ventilated mechanically. Anaesthesia was maintained throughout the surgical procedure with 1.5% isoflurane. Mice were treated with a bolus of AM-001 (10 mg/kg) or sterile saline solution 5 min before the reperfusion by intravenous injection 24 h after reperfusion, the area at risk and the infarct area were respectively determined by Evans blue and 2,3,5-triphenyltetrazolium chloride (TTC) staining. The dose of AM-001 was chosen on the basis of dose-ranging experiments in animals submitted to myocardial I/R. This dose was the optimal one that produced cardioprotection without immediate toxic effect and without precipitation of the compound.

2.4 Chronic ISO infusion

Osmotic mini-pumps (Alzet) were implanted subcutaneously in mice anaesthetized with isoflurane (1%). Pumps were filled with isoprenaline (ISO) or sterile saline and were set to deliver ISO at 60 mg/kg per day for 14 days. AM-001 treatment (10 mg/kg/d, i.p.) was diluted in 10% DMSO/20% Kolliphor/70% sterile saline). Mice were subsequently euthanized with barbiturate overdose (pentobarbital, 150 mg/kg, i.p.).

2.5 Echocardiography

Echocardiography was carried out on lightly anaesthetized (1 % isoflurane in air) mice placed on a heating pad. The left ventricular dimensions were obtained during TimeMovement mode acquisition from the parasternal short-axis view at the midventricular level using a Vevo2100 echograph and a 40 MHz transducer (M550, Visualsonics FUJIFILM). Images were analysed off line with VevoLab software (Visualsonics FUJIFILM). The operator was blind to the treatment group of the mice.

2.6 Determination of fibrosis

Hearts were transversely sectioned at 10 μ m thickness, fixed with 4% paraformaldehyde and stained with Sirius Red. Slides were scanned with NanoZoomer (Hamamatsu v1.2) and fibrosis was measured as positively stained area with Sirius Red and expressed as percent of total area, using ImageJ software (RSB).

2.7 Haematoxylin and eosin staining

Hearts were fixed in 4% paraformaldehyde and embedded in OCT compound (Tissue Tek, EMS). Longitudinal sections were performed at 10 μ m in thickness and stained with haematoxylin and eosin for histological examination.

2.8 Hepatotoxicity analysis

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in serum to determine the cytotoxicity, using specific kits purchased from ScienCell Research Laboratories following the manufacturer's instructions.

2.9 Bioluminescence resonance energy transfer (BRET) assay

BRET experiments were performed as previously described.¹⁰

2.10 Lactate dehydrogenase release and MTT assay

Lactate dehydrogenase (LDH) release in the cell culture medium and MTT assay were measured as previously described.¹⁵

2.11 Thermal shift assay

Thermal shift assay (TSA) was performed essentially as described previously,¹⁶ using a ViiA7 Thermofisher system. In brief, assays were assessed with a final protein concentration of 0.2 g/L in 96 well real time PCR plates. A 30 μ M Sp-007 was added to Epac1. Sypro Orange was used at 10 $\times$ concentration assuming the stock solution supplied by the manufacturer being 5000 $\times$. AM-001 was added at 1–60 μ M concentrations. Fluorescence traces were recorded with λ_{ex} = 560–590 nm and λ_{em} = 610–650 nm over a temperature range from 11°C to 80°C.

2.12 Data analysis

Data are expressed as mean $\pm$ SEM. Differences in quantitative variables were examined by one-way or two-way analysis of variance followed by *post hoc* test with Bonferroni correction using Graphpad Prism. Statistical significance was set to $P < 0.05$. Best fit values of EC₅₀ and IC₅₀ values and their 95% confidence intervals were computed by global non-linear regression according to a three-parameters dose–response model and compared on the basis of the extra sum-of square F test, using Graphpad Prism.

3. Results

3.1 AM-001 inhibits CAMYEL, an Epac1-based BRET sensor for cAMP

The CAMYEL BRET sensor is composed of Epac1 inserted between Renilla luciferase and citrine and has been successfully used to identify Epac inhibitors.^{10,17} Upon binding of cAMP, Epac1 undergoes conformational changes that result in a decrease of energy transfer due to luciferase moving away from citrine.¹⁰ Here, we screened a small in-house collection of 15 unrelated chemical compounds using CAMYEL (Supplementary material online, Figure S1A). Compounds #3 and #6 showed significant Epac1 inhibitory properties (Supplementary material online, Figure S1A). However, subsequent studies on compound 6 were discontinued due to poor dose–response relationship (Supplementary material online, Figure S1B). In contrast, compound #3, a thieno[2,3-b]pyridine derivative, named AM-001 (Figure 1A), appeared as a novel inhibitor of the cAMP-induced CAMYEL conformational change. AM-001 (20 μ M) inhibited the BRET variations induced by 100 μ M cAMP ($\approx$ 50% decrease) in the same order of magnitude of (R)-CE4F4 (20 μ M), an Epac1 inhibitor^{10,11} that was used as a standard in our experiments (Figure 1B). AM-001 decreased BRET maximal response in a concentration dependent-manner, but not the effective concentration of cAMP that gave half-maximal response (EC₅₀ = 30.7 μ M, 95% IC = 28.2 to 33.5 μ M) (Figure 1C). This is a major kinetic characteristic of a non-competitive inhibitor, which does not prevent substrate or activator from binding to the enzyme.¹⁸ Consistently, increasing concentrations of cAMP did not modify the half-maximal inhibitory concentration (IC₅₀) of AM-001 (53.7 μ M, 95% IC = 50.9 to 56.6 μ M) (Figure 1D).

3.2 AM-001 inhibits Epac1 but not Epac2 catalytic activity *in vitro*

Next, we investigated the effects of AM-001 on Epac1 and Epac2 catalytic activities using the Epac GEF activity assay.¹⁰ The method is based on the Epac-stimulated dissociation of fluorescent b-GDP from recombinant Rap1 in the presence of an excess of non-fluorescent GDP. The initial velocity of the decay in fluorescence reflects exchange activity. First, we analysed if AM-001 physico-chemical properties were compatible with the Epac GEF assay. We found a linear correlation between AM-001 absorbance and concentration indicating that AM-001 was soluble up to 60 μ M in the exchange reaction buffer (Supplementary material online, Figure S2A). In addition, UV spectra of AM-001 at different concentrations showed that this compound did not absorb in the fluorescence excitation/emission window of bGDP (Supplementary material online, Figure S2B). Importantly, the emission fluorescence intensity of bGDP was not affected when AM-001 was used at concentrations up to 60 μ M (Supplementary material online, Figure S2C). Altogether, these data showed that AM-001 physico-chemical properties were compatible with the Epac GEF assay.

Measurement of initial velocities of exchange at various concentrations of Sp-8-CPT, a strong PDE-resistant Epac agonist¹⁹ gave EC₅₀ values of 7.5 $\pm$ 1.1 μ M and 5.6 $\pm$ 1.1 μ M for Epac1 and Epac2, respectively (Supplementary material online, Figures S3A–C). In addition, cAMP EC₅₀ values were 13.1 $\pm$ 1.1 μ M and 2.8 $\pm$ 1.1 μ M for Epac1 and Epac2, respectively (Supplementary material online, Figures S3A–C). Consequently, the apparent affinity of Epac1 was 1.7 times higher for Sp-8-CPT than for cAMP and the Vmax of Sp-8-CPT -activated Epac1 was 2.5-fold higher than the Vmax of cAMP activated Epac1 (Supplementary material online, Figure S3D). In contrast, the apparent affinity of Epac2 was 2 times higher for cAMP than for Sp-8-CPT and the Vmax of cAMP-activated Epac2 was 1.3-fold larger than the Vmax of Sp-8-CPT-activated Epac2 (Supplementary material online, Figure S3D). Therefore, a saturating concentration of Sp-8-CPT (30 μ M) was chosen for the subsequent inhibitory experiments. Both Epac isoforms induced a rapid decrease in fluorescence in the presence of 30 μ M Sp-8-CPT (Figure 2A and C). As previously reported,¹⁰ the exchange activity of Epac1 induced by the agonist (30 μ M Sp-8-CPT) was reduced by (R)-CE3F4 (20 μ M) (Figure 2A and B). AM-001 prevented Epac1 GEF activity (Figure 2A, B, and E), whereas it had no inhibitory effect on Epac2 GEF activity (Figure 2C–E). In the presence of a saturating concentration of Sp-8-CPT, the AM-001 IC₅₀ value was 48.5 $\pm$ 1.0 μ M for Epac1 but could not be quantified for Epac2 (IC₅₀ $\gg$ 1000 μ M) indicating that AM-001 was ineffective in suppressing Epac2 GEF activity (Figure 2D). Similar results (AM-001 IC₅₀ = 47.8 $\pm$ 1.1 μ M for Epac1 vs. IC₅₀ $\gg$ 1000 μ M for Epac2) were obtained using concentrations of Sp-8-CPT corresponding to the EC₅₀ for each Epac isoform (Supplementary material online, Figure S4). The fact that the IC₅₀ of inhibition of Epac1 by AM-001 was independent of the agonist concentration confirmed the non-competitive behaviour of AM-001 (Supplementary material online, Figure S5). Increasing Sp-8-CPT concentration from 1 to 100 μ M did not change the AM-001 IC₅₀ for Epac1 (Supplementary material online, Figure S5A). In addition, AM-001 decreased the maximal Epac1 GEF activity in a concentration dependent-manner, but not the EC₅₀ of Sp-8-CPT (Supplementary material online, Figure S5B). These results confirmed that AM-001 acted as an Epac1 non-competitive inhibitor, as already suggested by the BRET sensor experiments (Figure 1C and D).

AM-001 did not influence type I and type II PKA holoenzyme activation in the presence and absence of 10 μ M cAMP, further strengthening

Figure 1 AM-001 inhibits cAMP-induced Epac1-BRET sensor activation. (A) Structure of the Epac1 antagonist AM-001. (B) BRET assay. AM-001, (R)-CE3F4 (20 μ M), or vehicle were added to the cell extract before addition of cAMP (100 μ M), and BRET ratios (mean $\pm$ SEM from three wells) were measured and plotted as percent variations in BRET ratios relative to no-inhibitor control value. (C and D) BRET ratios were measured in triplicate in the presence of increasing concentrations of AM-001 or cAMP. Data were plotted as a function of the cAMP (C) or AM-001 (D) concentration. The curves obtained under each condition were analysed using Graphpad Prism, yielding the maximal BRET ratio responses. Values are mean $\pm$ SEM (triplicate; some error bars are masked by the symbols). **** p < 0.0001 vs. vehicle.

the specificity of this compound for Epac1 (Figure 3). Finally, using a thermal shift assay that allows monitoring of protein denaturation,²⁰ we observed that addition of AM-001 did not modify the melting temperature T_m (defined as the midpoints of thermo-unfolding) of Epac1, in the presence of increasing concentrations of AM-001, whether the agonist Sp-8-CPT was present or not (Supplementary material online, Figure S6), thereby excluding any non-specific protein denaturing action of AM-001.

3.3 Specificity of AM-001 derivatives on Epac1 activation

Several commercially available analogues or derivatives of AM-001 (Supplementary material online, Figure S7A) were then studied for their inhibitory action on Epac1 activation, using CAMYEL (Supplementary material online, Figure S7B). Swapping the fluorine atom from the N-phenyl group to the 4-phenyl group (AM-005), as well as the absence of any fluorine atom on these phenyl groups (AM-004), had no significant effect on the inhibitory potency of the compounds (Supplementary material online, Figure S7B). The inhibitory activity of compounds lacking the 4-phenyl group (AM-009) or the N-phenyl group (AM-006) was strongly decreased but not abolished (Supplementary material online, Figure S7B). However, substitution of the N-phenyl group by a N-isopropyl group (AM-003) abolished the inhibitory effect (Supplementary material online, Figure S7B). The low activity of AM-007, AM-008 and AM-010 points to

the importance of the presence of the 6-thienyl group for inhibitory activity (Supplementary material online, Figure S7B). Finally no significant inhibitory activity was observed with AM-002 (Supplementary material online, Figure S7B), a compound in which the 2-carboxamide bond cannot freely rotate, and in which the primary amino group of AM-001 is involved in an additional thieno[3,2-d]pyrimidine scaffold. This preliminary structure-activity study showed that the phenyl groups and the 6-thienyl group were essential for AM-001 to exert its inhibitory activity. Therefore, our data indicated that there was chemical specificity within this family of compounds in their ability to reduce the cAMP-induced Epac1 conformational change.

3.4 AM-001 inhibits Epac1 signalling in cultured cells

AM-001 displayed no apparent cytotoxicity in various types of cultured cells (Supplementary material online, Figure S8). To confirm that AM-001 was an efficient Epac1 specific antagonist in cultured cells, we further tested its ability to block the activation of the Epac downstream effector, Rap1 in HEK293 cells (Figure 4). This cell line is commonly used for the evaluation of Epac pharmacological modulators on the cellular signalling of ectopically expressed Epac1 and Epac2 isoforms.^{10,21,22} The highly membrane-permeant Epac agonist 8-CPT-AM induced a robust activation of Rap1 in cells overexpressing Epac1 (Figure 4A). Consistent with

Figure 2 AM-001 inhibits Sp-8-CPT-induced Epac1 but not Epac2 activity. Epac1 (A) and Epac2 (C) activities were measured in the absence (■) or in the presence of 30 μ M Sp-8-CPT, either alone (●) or with 20 μ M (R)-CE3F4 (△) or with 20 μ M AM-001 (◇). Variations of relative fluorescence units (RFU) were studied as a function of time and fitted to single exponentials. Reported values are mean $\pm$ SEM (triplicate; some error bars are masked by the symbols). (B and D) Results were expressed as the initial velocity of Epac-catalysed GDP exchange, relative to the control values measured in the absence of inhibitor, which were set at 100%. (E) Initial velocities of nucleotide exchange induced by Epac1 (●) and Epac2B (□) were measured in triplicate in the presence of a saturating concentration of Sp-8-CPT (50 μ M) and increasing concentrations of AM-001. Results are expressed as the % of the initial velocity of GDP exchange measured in the absence of AM-001 from time course studies and are shown as mean $\pm$ SEM (some error bars are masked by the symbols). ** P < 0.01, **** P < 0.0001 vs. control value or indicated values.

the data obtained *in vitro* with Epac1 BRET sensor (Supplementary material online, Figure S7) and Epac1 exchange reaction (Supplementary material online, Figure S9), AM-001, but not the inactive analogues AM-002 and AM-003, prevented Epac1-induced Rap1 activation (Figure 4A–C). Next, we investigated the effect of 8-CPT-AM on the two Epac2 splice variants, Epac2A and Epac2B (Figure 4D–E). Since 8-CPT-AM is a preferential activator of Epac1 over Epac2,^{10,23} we increased its concentration up to 20 μ M to fully stimulate Epac2A (Supplementary material online, Figure S10A). In addition, we used a selective Epac2 agonist, S-220 to fully activate Epac2B (Supplementary material online, Figure S10B). In contrast to ESI-05, a previously characterized specific Epac2 inhibitor,²¹ AM-001 failed to inhibit Epac2A-induced Rap1 activation (Figure 4D). Moreover, AM-001 was ineffective to impede the increase in the amount of Rap1-GTP induced by S-220 in Epac2B-transfected cells (Figure 4E). Altogether, these data showed that AM-001 is efficient and specific in preventing Epac1-induced Rap1 activation in cultured cells.

3.5 AM-001 protects against I/R injury

We next examined whether AM-001 could affect the activity of Epac1 in physiologically relevant systems. Under hypoxia-reoxygenation (HX+R), WT cardiomyocytes exhibited an increase in membrane permeability assayed by LDH release (Figure 5A). AM-001 inhibited HX+R-induced WT cardiomyocyte death phenocopying Epac1 deletion (Figure 5A). Of note, AM-001 did not influence cell survival in Epac1 deleted (*Epac1*^{−/−}) cardiomyocytes placed in NX or HX+R conditions confirming the specificity of the compound towards Epac1 (Figure 5A). Finally, AM-001 inhibited the effect of the Epac1 agonist, 8-CPT-AM on cardiomyocyte death and hypertrophy (Figure 5B, Supplementary material online, Figure S11), highlighting the ability of AM-001 to counteract Epac1 detrimental effect.

We then investigated the therapeutic efficacy of an acute administration of AM-001 in a mouse model of acute myocardial I/R injury. A single bolus of AM-001 (8 mg/kg) or vehicle was injected intravenously 5 min

Figure 3 AM-001 does not influence PKA activity. (A and C) Time-course of decrease of NADH concentration measured at 340 nm in the presence of Types I and II PKA holoenzymes and the indicated compounds. A pharmacological inhibitor of PKA, H89 was used as a positive control. Similar results were obtained from three independent experiments. (B and D) Relative Type I and Type II PKA holoenzymes activity. The bar graph represents the mean $\pm$ SEM of three independent experiments. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ vs. indicated control condition, one way ANOVA, Bonferroni comparison test. Results are expressed as the percentage of control (vehicle).

before the reperfusion (Figure 5C). The ratio of infarct size to area-at-risk was significantly reduced in AM-001-treated animals ($36 \pm 3\%$) when compared with that in vehicle treated mice ($50 \pm 3\%$) (Figure 5D). Similar results were observed in *Epac1*^{-/-} mice subjected to I/R indicating that Epac1 pharmacological inhibition protected the heart against I/R injury in a similar order of magnitude as did Epac1 genetic ablation (Figure 5E). We did not observe any additional effect of AM-001 on the infarct size in *Epac1*^{-/-} mice compared with vehicle-treated *Epac1*^{-/-} mice (Figure 5E). Interestingly, although (R)-CE3F4 had a lower IC₅₀ than AM-001 in the BRET sensor assay, and had been shown to efficiently and selectively inhibit Epac1-dependent biological action *in vitro* in various cellular models,^{10,24} this inhibitor (8 mg/kg body) failed to reduce infarct size, suggesting that (R)-CE3F4 might have a poor bioavailability *in vivo* compared with AM-001 (Figure 5F). Altogether, these results demonstrate that acute injection of AM-001 is efficient to prevent myocardial reperfusion injury.

3.6 AM-001 reduces pathological cardiac remodelling during chronic β -AR activation

We next sought to determine whether AM-001 could reduce pathological cardiac remodelling during chronic β -AR activation. To this end, WT C57BL/6 mice were treated with either the non-selective β -AR agonist, isoproterenol (ISO), or vehicle for 14 days in the presence of AM-001 (from day 3 to day 14) or its vehicle (Figure 6A). Liver and renal tissue

samples from AM-001 treated mice did not display any apparent histological alterations as revealed with the haematoxylin and eosin (H&E) staining (Figure 6B). In addition, plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) that are indicative of hepatotoxicity, were equivalent in each group (Figure 6B). Yet, cardiac function, the ratio of left ventricular weight (LVW) to tibia length (LVW/TL) and heart rate were similar at baseline in vehicle and AM-001 treated mice (Supplementary material online, Table S1, Figure 6C, Supplementary material online, Figure S12). Therefore, chronic injection of AM-001 had no toxic effects in animals and did not alter basal cardiac function under the experimental conditions used.

Chronic infusion of ISO resulted in a significant increase in LVW/TL ratios compared with control vehicle (Figure 6C). Conversely, injection of AM-001 3 days after the beginning of the adrenergic stress decreased this parameter (Figure 6C). In addition, fibrosis and infiltration of inflammatory cells were decreased in AM-001-treated mice (Figures 6D and E). AM-001 failed to block TGF- β 1 induced-expression of α -smooth muscle actin (α -SMA, marker of myofibroblasts) in neonatal rat cardiac fibroblasts suggesting that Epac1 inhibition did not influence fibroblast-to-myofibroblast conversion (Supplementary material online, Figure S13A). However, the proliferative ability of cardiac fibroblasts was significantly decreased with AM-001 (Supplementary material online, Figure S13B) and this could account for the decreased fibrosis observed in mice treated with AM-001. Of particular importance, after 14 days of ISO infusion the effect of AM-001 was associated with an improved cardiac

Figure 4 AM-001 prevents Epac1-induced Rap1 activation in cultured cells. Amounts of Rap1-GTP ($n = 5$ in each group, mean $\pm$ SEM) were determined in HEK293 cells transfected with Epac1 (A–C), Epac2A (D), or Epac2B (E). Cells were preincubated or not with AM-001 (A, D, E), AM-002 (B), or AM-003 (C) (20 μ M, 30 min) and were then treated or not with 8-CPT-AM (10 μ M for Epac1 activation and 20 μ M for Epac2A activation) or S-220 (50 μ M for Epac2B activation) for 10 min. ESI-05 was used as positive control of Epac2A inhibition. Representative immunoblots are shown. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, vs. indicated values, one way ANOVA, Bonferroni comparison test.

function, as demonstrated by the higher fractional shortening (FS) in AM-001 treated mice than in vehicle treated mice (Supplementary material online, Table S1, Figure 6F). Consistently, this better contractility observed in the presence of AM-001 during ISO treatment correlated with a decrease in left ventricular end-systolic internal diameter (LVSDs) and left ventricular end-diastolic internal diameter (LVSDd) (Supplementary material online, Table S1, Figure 6F).

3.7 AM-001 prevents the non-canonical action of G-protein receptor kinase 5

Since GRK2 and G-protein receptor kinase 5 (GRK5) are important GPCR regulation molecules and are also capable of regulating cardiac hypertrophic responses, we investigated whether the prohypertrophic cAMP-GEF, Epac1 could influence GRKs signalling following chronic activation of β -AR. ISO treatment increased GRK2 and GRK5 expression (Figure 7A, Supplementary material online, Figure S14). Interestingly, the up-regulation of GRK5, but not GRK2 was significantly reduced in AM-001 treated animals suggesting that Epac1 specifically targets GRK5

(Figure 7A, Supplementary material online, Figure S14). Mechanistically, GRK2 and GRK5 constitutively interacted with Epac1 in non-stimulated condition in primary cardiomyocytes (Figure 7B). ISO increased Epac1-GRK5 complex formation while it decreased Epac1-GRK2 interaction (Figure 7B). In addition, AM-001 prevented ISO-induced Epac1/GRK5 complex formation, but not Epac1/GRK2 interaction indicating a specific role of Epac1 in GRK5 signalling (Figure 7B).

Previous studies have shown that GRK5 can act as a class II HDAC kinase to phosphorylate histone deacetylase-5 (HDAC5), thereby relieving the activity of the prohypertrophic transcription factor, myocyte enhancer factor 2 (MEF2).²⁵ We found that ISO regulated the nuclear import and export of GRK5 and HDAC5, respectively (Figures 7C and D). Accordingly, knock down of GRK5 with specific siRNA (Si-GRK5) prevented ISO-induced HDAC5 cytoplasmic accumulation (Supplementary material online, Figure S15A and B), MEF2 transcriptional activity (Figures 7F) and ANF hypertrophic marker (Supplementary material online, Figure S15C). Of particular importance, we demonstrated that GRK5 was a downstream effector of Epac1 since Si-GRK5 blocked the stimulating effect of 8-CPT-AM (Epac1 agonist) on MEF2 and ANF (Supplementary

Figure 5 AM-001 is cardioprotective against I/R injury. Isolated adult WT (A and B) or *Epac1*^{-/-} (A) cardiomyocytes were pretreated or not with AM-001 (20 μ M, 30 min), stimulated or not with 8-CPT-AM (10 μ M, 30 min), and subjected to normoxia (NX) or hypoxia-reoxygenation (HX+R) treatment. Cell viability was determined by LDH release in under NX or HX+R conditions ($n = 6-9$). (C) Schematic time scale and experimental strategy to evaluate the effect of AM-001 on I/R injury. (D-F) Quantification of the area at risk (AAR) expressed as percentage of left ventricle size and infarct size expressed as percentage of AAR. Representative cardiac cross-sections stained with Evans blue and TTC of WT (D, F) mice pretreated or not with AM-001 (D) or R-CE3F4 (F) and subjected to I/R are shown. Statistical significance was determined by two-way ANOVA followed by Bonferroni post-test or *t*-test. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$, vs. indicated values.

material online, Figure S15D). Inhibiting Epac1 with AM-001 mimicked the effect of Si-GRK5 on MEF2 and ANF transcriptional activation (Figure 7F, Supplementary material online, Figure S15C). Altogether these data indicate that Epac1 activation induces GRK5 nuclear import and HDAC5 nuclear export to promote cardiomyocyte hypertrophy (Figure 7G).

4. Discussion

The lack of Epac1 specific antagonists with *in vivo* applications has greatly limited our ability to discern the biological functions of this family of proteins and understand their role in numerous diseases. A thieno[2,3-*b*]pyridine derivative, named AM-001, with potential Epac inhibitory properties was identified in our in-house chemical collection. Several

other thieno[2,3-*b*]pyridine derivatives were previously reported for their potential in anticancer therapy,²⁶ but AM-001 is a novel thieno[2,3-*b*]pyridine analogue that specifically inhibits Epac1-induced Rap1 activation.

AM-001 decreased the BRET maximal response as well as the V_{max} of Epac1-catalysed Rap1-GDP exchange, without affecting the EC_{50} for Epac1 agonists, so that an excess of agonist did not relieve the inhibition promoted by AM-001. Therefore, AM-001 did not appear to exert its antagonism by directly competing with cAMP or cAMP analogues for binding to Epac1. These data suggest that AM-001 is a noncompetitive antagonist relative to Epac1 agonists, with inhibition working both at low as well as at high agonist concentrations. This noncompetitive mode of inhibition may represent a pharmacological advantage over competitive inhibitors, which are mostly active at low agonist concentrations. Noncompetitive inhibition may be of particular therapeutical relevance

Figure 6 AM-001 attenuates myocardial remodelling during chronic β -AR activation. (A) Schematic timeline to study AM-001 effect during chronic β -AR activation with ISO. (B) Representative haematoxylin and eosin stained liver and kidney sections of mice treated with either vehicle or AM-001 during 14 days. Scale bar, 100 μ m (top panels). AST and ALT activities in the serum of mice treated under the indicated conditions (bottom, left panel) ($n = 7$ per group). Comparison of FS in animals between Day 1 and Day 14 in the presence or absence of AM-001 (bottom, right panel) ($n = 12$ per group). (C) LVW/TL ratios of mice after 14 days of treatment with vehicle or AM-001. (D, E) Mouse heart sections stained with either sirius red (fibrosis) or CD45 (inflammatory cells). Scale bar, 100 μ m. Bar graphs show the quantification of fibrosis or CD45+ cells ($n = 6-8$ per group). Arrows show CD45+ cells. (F) Comparison of FS, LVIDs and LVIDd in ISO treated animals between Day 1 and Day 14 in the presence or absence of AM-001 ($n = 18-19$ per group). Statistical significance was determined by two-way ANOVA followed by Bonferroni post-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. indicated values. ns, not significant.

in pathophysiological conditions with an increase in level of catecholamines resulting in overstimulation of the cAMP-adenylyl cyclase system in cardiomyocytes as observed in chronic heart failure.

Interaction of Epac1 with Rap1 is not necessary for AM-001 to express its antagonistic effect. Indeed, the CAMYEL biosensor construct detects Epac1 intramolecular conformational changes triggered by cAMP in the absence of Rap. The use of this method shows that AM-001 acts primarily by blocking the Epac1 conformational changes induced by agonists, which is likely the cause of its inhibitory effects in our subsequent experiments.

AM-001 does not only exert its GEF inhibitory action in cell-free assays *in vitro*, since it also blocked efficiently intracellular Epac1-mediated Rap1 activation in cultured HEK293 cells ectopically

expressing Epac isoforms. In contrast to its effect on Epac1, AM-001 is devoid of antagonist action on Epac2 in the cell-free GEF assay. Similarly, AM-001 failed as well to inhibit Epac2A- and Epac2B-induced Rap1 activation in cultured cells. Its absence of effect on PKA further demonstrates its specificity towards Epac1 among the intracellular cAMP effectors.

Among the thieno[2,3-*b*]pyridine derivatives studied, none had an improved activity towards Epac1 compared with AM-001. The preliminary structure-activity study showed that compounds having a N-fluorophenyl group, a 4-phenyl group, and a 6-thienyl group seem to be the most efficient molecules to block Epac1 activation. The influence of the 3-amino group remains to be fully investigated. As it is, this study demonstrates chemical ligand specificity among AM-001 analogues. MTT

Figure 7 AM-001 prevents the non-canonical action of GRK5 on cardiomyocyte hypertrophy. (A) Representative immunoblots of Epac1, GRK2, and GRK5 in mouse myocardial samples. (B–E) NRVMs were preincubated or not with AM-001 (20 μ M, 30 min) and were then treated or not with ISO (10 μ M, 10 min). (B) Representative immunoblots of 3 independent immunoprecipitation (IP) experiments against Epac1. IgG and GAPDH were used as control for IP. Input, cell lysates. (C) Representative immunoblots performed on nuclear and cytosolic fractions, or total lysates. Bar graphs represent the quantification of nuclear GRK5 (D) or HDAC5 (E) expression, and cytosolic/nuclear GRK5 (D) or HDAC5 (E) quantification ratio ($n = 4$). (F) NRVMs transfected with the luciferase (Luc) reporter plasmid driven by three MEF2 binding sites (MEF2-Luc) and Si-CT or Si-GRK5 were pre-treated or not with AM-001 (20 μ M, 1 h) and were stimulated with ISO (10 μ M, 6 h) ($n = 4$). Then, Luc activity was measured. \$\$\$ P < 0.01 vs. Si-CT Vehicle-ISO, *** P < 0.01, **** P < 0.001 vs. Si-CT Vehicle + ISO. (G) A model showing the effect of Epac1-GRK5 signalling in cardiomyocyte hypertrophy. Upon β -AR stimulation, Epac1 interacts with GRK5 and the Epac1-GRK5 complex translocates to the nucleus. There, GRK5 phosphorylates HDAC5 leading to its nuclear export and thereby relieving MEF2 transcriptional activity. AM-001 prevents GRK5 nuclear translocation and its prohypertrophic effect. Statistical significance was determined by two-way ANOVA followed by Bonferroni post-test. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. indicated values. ns, not significant.

analysis *in vitro* and H&E staining analysis, as well as AST and ALT levels *in vivo* indicated that AM-001, at the doses used in our study, was not toxic to cells and animals.

Early coronary reperfusion has been established as the best therapeutic strategy to limit infarct size and improve prognosis. However, the process of reperfusion can itself induce cardiomyocyte death, known as myocardial I/R injury, for which there is still no effective therapy.²⁷ Our finding on the cardioprotective effect of AM-001 (administered just before the reperfusion) in a preclinical model of myocardial I/R injury, highlights the therapeutic potential of acute Epac1 inhibition in cardiac ischaemia. Importantly, AM-001 recapitulates the beneficial effect of

Epac1 genetic ablation in I/R, since similar decrease in infarct size was observed in AM-001 treated mice and *Epac1*^{-/-} mice. Furthermore, AM-001 had no additional effect on the infarct size in *Epac1*^{-/-} mice compared with vehicle treated *Epac1*^{-/-} mice. These data further emphasize the specific effect of AM-001 on Epac1. The mechanisms by which Epac1 inhibition affords cardioprotection involves a decrease in ROS production, apoptosis and mitochondrial Ca²⁺ overload.⁸ However, since Epac1 has been recently shown to influence platelet aggregation,²⁸ one cannot exclude that a potential decrease of platelet aggregation with AM-001 could reduce no reflow phenomenon which would end up with decreased myocardial infarct size.

Our data also showed that AM-001 was beneficial in chronic treatment in a mouse model of cardiac remodelling induced by sustained activation of β -ARs. Indeed, when injected 2 days after the onset of the stress, AM-001 attenuated the increase in cardiac mass and fibrosis induced by sustained β -AR activation. This is consistent with previous genetic studies showing that Epac1 null mice were protected against β -AR-induced cardiac remodelling.^{6,7} A very interesting finding from our study was that AM-001-treated mice displayed an improved cardiac contractile function in response to chronic activation by β -AR. Importantly, cardiac functional improvement observed with AM-001 was similar to our previous data obtained with Epac1^{-/-} mice.⁶ In addition, our data are consistent with the preserved inotropic reserve (tested by dobutamine induced increase in contractility) observed in Epac1 deleted mice subjected to thoracic aortic constriction.⁷ Because several studies have demonstrated that acute activation of endogenous Epac modulates Ca²⁺ handling such as an increase in sarcoplasmic reticulum Ca²⁺ leak in quiescent myocytes,^{7,9,24} one could speculate that Epac1 inhibition via AM-001 represents a new means of normalizing Ca²⁺ cycling in the setting of chronic β -AR-induced cardiac remodelling.

Recent data indicate that GRK5 is a key regulator of pathological cardiac hypertrophy with this role being independent of any GRK activity on receptors.^{25,29} Specifically, GRK5 triggers the nuclear export of HDAC5 and the de-repression of the prohypertrophic transcription factor MEF2 to promote pathological cardiac remodelling.^{25,29} Our results showing that AM-001 prevents the nuclear translocation of GRK5 to induce the nuclear accumulation of HDAC5 indicates that Epac1 exerts epigenetic regulation to facilitate the development of cardiac hypertrophy (Figures 7G). Of particular interest, β 1-AR has been shown to induce the recruitment of β -arrestin-Epac1 complex at the plasma membrane, whereby it activates a pro-hypertrophic signalling involving CaMKII and HDAC4 nuclear export.³⁰ It is proposed that GRK (probably GRK2)-mediated β 1-AR phosphorylation results in β -arrestin recruitment, which induces stable interaction with CaMKII and Epac1 at the plasma membrane.³¹ Thus, GRKs seem to play different roles in Epac1 prohypertrophic signalling.

In summary, our study has identified a novel thieno[2,3-b]pyridine analogue as a specific pharmacological inhibitor of Epac1 that functions both *in vitro* and *in vivo*. Our data indicate that this compound may not only be a valuable pharmacological tool to explore physiological and pathological processes related to signalling pathways that are regulated by Epac1, but also has a great potential to be developed as a novel molecular therapeutic for relevant human diseases. Finally, our data strongly support the concept that Epac1 can serve as a target for the development of new therapies for the treatment of cardiac diseases.

Acknowledgements

We acknowledge members of the UMS006 facilities, Xavier Sudre and his staff for animal housing and Carine Pestourie, Marie-Laure Boizeau at *in vivo* imaging service (ENI) (UMS 006/INSERM/UPS, Anexplo/Genotoul Plateform). We acknowledge the support from Histology and APC platforms I2MC (Lucie Fontaine and Alexandre Lucas) and from GeT TQ platform (Frederic Martins). We also thank Delphine Courilleau (Paris-Sud University) for advice on

measurements of Epac catalytic activities and Julien Viaud for the critical reading of the manuscript.

Conflict of interest: none declared.

Funding

This work was supported by the Institut National de la Santé et de la Recherche Médicale, Agence Nationale de la Recherche, Fondation pour la Recherche Médicale (Programme 'Equipes FRM 2016', DEQ20160334892 to F.L.), Fondation de France (00066331), and Fédération Française de Cardiologie. M.L. was recipient of a PhD training grant from Université de Toulouse.

References

- Robichaux WG 3rd, Cheng X. Intracellular cAMP sensor EPAC: physiology, pathophysiology, and therapeutics development. *Physiol Rev* 2018;**98**:919–1053.
- Bers DM. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol* 2008;**70**:23–49.
- El-Armouche A, Eschenhagen T. β -adrenergic stimulation and myocardial function in the failing heart. *Heart Fail Rev* 2009;**14**:225–241.
- Morel E, Marcantoni A, Gastineau M, Birkedal R, Rochais F, Garnier A, Lompre AM, Vandecasteele G, Lezoualc'h F. cAMP-binding protein Epac induces cardiomyocyte hypertrophy. *Circ Res* 2005;**97**:1296–1304.
- Métrich M, Lucas A, Gastineau M, Samuel J-L, Heymes C, Morel E, Lezoualc'h F. Epac mediates β -adrenergic receptor-induced cardiomyocyte hypertrophy. *Circ Res* 2008;**102**:959–965.
- Laurent A-C, Bissierier M, Lucas A, Tortosa F, Roumieux M, De Régibus A, Swiader A, Sainte-Marie Y, Heymes C, Vindis C, Lezoualc'h F. Exchange protein directly activated by cAMP 1 promotes autophagy during cardiomyocyte hypertrophy. *Cardiovasc Res* 2015;**105**:55–64.
- Okumura S, Fujita T, Cai W, Jin M, Namekata I, Mototani Y, Jin H, Ohnuki Y, Tsuneoka Y, Kurotani R, Suita K, Kawakami Y, Hamaguchi S, Abe T, Kiyonari H, Tsunematsu T, Bai Y, Suzuki S, Hidaka Y, Umemura M, Ichikawa Y, Yokoyama U, Sato M, Ishikawa F, Izumi-Nakaseko H, Adachi-Akahane S, Tanaka H, Ishikawa Y. Epac1-dependent phospholamban phosphorylation mediates the cardiac response to stresses. *J Clin Invest* 2014;**124**:2785–2801.
- Fazal L, Laudette M, Paula-Gomes S, Pons S, Conte C, Tortosa F, Sicard P, Sainte-Marie Y, Bissierier M, Lairez O, Lucas A, Roy J, Ghaleh B, Fauconnier J, Miale-Perez J, Lezoualc'h F. Multifunctional mitochondrial Epac1 controls myocardial cell death. *Circ Res* 2017;**120**:645–657.
- Pereira L, Cheng H, Lao DH, Na L, van Oort RJ, Brown JH, Wehrens XH, Chen J, Bers DM. Epac2 mediates cardiac β 1-adrenergic-dependent sarcoplasmic reticulum Ca²⁺ leak and arrhythmia. *Circulation* 2013;**127**:913–922.
- Courilleau D, Bissierier M, Jullian JC, Lucas A, Bouyssou P, Fischmeister R, Blondeau JP, Lezoualc'h F. Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. *J Biol Chem* 2012;**287**:44192–44202.
- Courilleau D, Bouyssou P, Fischmeister R, Lezoualc'h F, Blondeau JP. The (R)-enantiomer of CE3F4 is a preferential inhibitor of human exchange protein directly activated by cyclic AMP isoform 1 (Epac1). *Biochem Biophys Res Commun* 2013;**440**:443–448.
- Almahariq M, Tsalkova T, Mei FC, Chen H, Zhou J, Sastry SK, Schwede F, Cheng X. A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion. *Mol Pharmacol* 2013;**83**:122–128.
- Almahariq M, Chao C, Mei FC, Hellmich MR, Patrikeev I, Motamedi M, Cheng X. Pharmacological inhibition and genetic knockdown of exchange protein directly activated by cAMP 1 reduce pancreatic cancer metastasis *in vivo*. *Mol Pharmacol* 2015;**87**:142–149.
- Gong B, Shelite T, Mei FC, Ha T, Hu Y, Xu G, Chang Q, Wakamiya M, Ksiazek TG, Boor PJ, Bouyer DH, Popov VL, Chen J, Walker DH, Cheng X. Exchange protein directly activated by cAMP plays a critical role in bacterial invasion during fatal rickettsioses. *Proc Natl Acad Sci USA* 2013;**110**:19615–19620.
- Santin Y, Sicard P, Vigneron F, Guilbeau-Frugier C, Dautaur M, Lairez O, Couderc B, Manni D, Korolchuk VI, Lezoualc'h F, Parini A, Miale-Perez J. Oxidative stress by monoamine oxidase-A impairs transcription factor EB activation and autophagosome clearance, leading to cardiomyocyte necrosis and heart failure. *Antioxid Redox Signal* 2016;**25**:10–27.
- Rehmann H. Epac-inhibitors: facts and artefacts. *Sci Rep* 2013;**3**:3032.
- Jiang LI, Collins J, Davis R, Lin KM, DeCamp D, Roach T, Hsueh R, Rebres RA, Ross EM, Taussig R, Fraser I, Sternweis PC. Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *J Biol Chem* 2007;**282**:10576–10584.

18. Copeland RA. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*, 2nd ed. Weinheim, Germany: Wiley-VCH; 2000.
19. Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Døskeland SO, Blank JL, Bos JL. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol* 2002;**11**:901–906.
20. Pantoliano MW, Petrella EC, Kwasnoski JD, Lobanov VS, Myslik J, Graf E, Carver T, Asel E, Springer BA, Lane P, Salemme FR. High-density miniaturized thermal shift assays as a general strategy for drug discovery. *J Biomol Screen* 2001;**6**:429–440.
21. Tsalkova T, Mei FC, Li S, Chepurny OG, Leech CA, Liu T, Holz GG, Woods VL, Cheng X. Isoform-specific antagonists of exchange proteins directly activated by cAMP. *Proc Natl Acad Sci USA* 2012;**109**:18613–18618.
22. Zhu Y, Mei F, Luo P, Cheng X. A cell-based, quantitative and isoform-specific assay for exchange proteins directly activated by cAMP. *Sci Rep* 2017;**7**:6200.
23. Schwede F, Bertinetti D, Langerijs CN, Hadders MA, Wienk H, Ellenbroek JH, de Koning EJ, Bos JL, Herberg FW, Genieser HG, Janssen RA, Rehmann H. Structure-guided design of selective Epac1 and Epac2 agonists. *PLoS Biol* 2015;**13**:e1002038.
24. Lezoualc'h F, Fazal L, Laudette M, Conte C. Cyclic AMP sensor EPAC proteins and their role in cardiovascular function and disease. *Circ Res* 2016;**118**:881–897.
25. Hullmann J, Traynham CJ, Coleman RC, Koch WJ. The expanding GRK interactome: implications in cardiovascular disease and potential for therapeutic development. *Pharmacol Res* 2016;**110**:52–64.
26. Eurtivong C, Reynisdóttir I, Kuczma S, Furkert DP, Brimble MA, Reynisson J. Identification of anticancer agents based on the thieno[2, 3-b]pyridine and 1H-pyrazole molecular scaffolds. *Bioorg Med Chem* 2016;**24**:3521–3526.
27. Heusch G. Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning. *Circ Res* 2015;**116**:674–699.
28. Nygaard G, Herfindal L, Asrud KS, Bjørnstad R, Kopperud RK, Oveland E, Berven FS, Myhren L, Hoivik EA, Lunde THF, Bakke M, Døskeland SO, Selheim F. Epac1-deficient mice have bleeding phenotype and thrombocytes with decreased GPIIb/IIIa expression. *Sci Rep* 2017;**7**:8725.
29. Traynham CJ, Hullmann J, Koch WJ. Canonical and non-canonical actions of GRK5 in the heart. *J Mol Cell Cardiol* 2016;**92**:196–202.
30. Berthouze-Duquesnes M, Lucas A, Saulière A, Sin YY, Laurent A-C, Galés C, Baillie G, Lezoualc'h F. Specific interactions between Epac1, β -arrestin2 and PDE4D5 regulate β -adrenergic receptor subtype differential effects on cardiac hypertrophic signaling. *Cell Signal* 2013;**25**:970–980.
31. Mangmool S, Shukla AK, Rockman HA. β -arrestin-dependent activation of Ca^{2+} /calmodulin kinase II after $\beta(1)$ -adrenergic receptor stimulation. *J Cell Biol* 2010;**189**:573–587.