~~THE ANNEXIN-A1 MIMETIC~~The Annexin-A1 mimetic RTP-026 ~~PROMOTES ACUTE CARDIOPROTECTION THROUGH MODULATION OF IMMUNE CELL ACTIVATION~~promotes acute cardioprotection through modulation of immune cell activation

The corrections made in this section will be reviewed and approved by a journal production editor.

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~~ABSTRACT~~Abstract

***Aims*:** The cardio-protective and immuno-regulatory properties of RTP-026, a synthetic peptide that spans the Annexin-A1 (AnxA1) N-terminal region, were tested in rat acute myocardial infarction.

***Methods and ~~Results~~results*:** In vitro, selective activation of formyl-peptide receptor type 2 (FPR2) by RTP-026 occurred with apparent EC_{50} in the 10–30 nM range. With human primary cells, RTP-026 counteracted extension of neutrophil life-span and augmented phagocytosis of fluorescent *E.coli* by blood myeloid cells. An in vivo model of rat acute infarction was used to quantify tissue injury and phenotype immune cells in myocardium and blood. The rat left anterior descending coronary artery was occluded and then reopened for 2-hour or 24-hour reperfusion. For the 2-hour reperfusion protocol, RTP-026 (25–500 μg/kg; given i.v. at the start of reperfusion) significantly reduced infarct size by ~50 %, with maximal efficacy at 50 μg/kg. Analyses of cardiac immune cells showed that RTP-026 reduced neutrophil and classical monocyte recruitment to the damaged heart. In the blood, RTP-026 (50 μg/kg) attenuated activation of neutrophils and monocytes monitored through CD62L and CD54 expression. Modulation of vascular inflammation by RTP-026 was demonstrated by reduction in plasma levels of mediators like TNF-α, IL-1β, KC, PGE_2 and $PGF_{2\alpha}$. For the 24-hour reperfusion protocol, RTP-026 (30 μg/kg given i.v. at 0, 3 and 6 h reperfusion) reduced necrotic myocardium by ~40 %.

***Conclusions*:** RTP-026 modulate immune cell responses and decreases infarct size of the heart in preclinical settings. Tempering over-exuberant immune cell activation by RTP-026 is a suitable approach to translate the biology of AnxA1 for therapeutic purposes.

Chemical compounds studied in this article: Ac2–26 (Compound CID: 16138723); RTP-026 (Compound CID: not available); RTP-022 (Compound CID: not available); Glycylglycine (Compound CID: 11163)

Keywords:

Annexin-A1, Heart, Myocardial ischemia/reperfusion, Resolution of inflammation

Data ~~availability~~Availability

Data will be made available on request.

1 ~~INTRODUCTION~~Introduction

The resolution of inflammation identifies the second phase of the acute host response to injury and infection. Engagement of pro-resolving pathways and release of pro-resolving mediators is fundamental to ensure time and spatial control of the host response, assuring its termination without collateral tissue damage. The last two decades have witnessed an elucidation of the processes of resolution with identification of classes of pro-resolving mediators. The latter spans different classes of chemical structures, from lipids to peptides and proteins to autacoids [1,2]. It is also appreciated that these mediators are operative early on preventing an over-shooting of the inflammatory response and act through receptors which have been identified [3]. We have proposed that a new branch of pharmacology could be built on the biology of resolution, to enable the development of novel therapeutic entities to control on-going chronic inflammation [4], which burden a large set of diseases, from arthritis to atherosclerosis, from asthma to diabetes and metabolic diseases.

An important mediator is the protein Annexin A1 (AnxA1) which exerts pro-resolving properties in several contexts mainly through activation of a specific G-protein coupled receptor, termed formyl-peptide receptor type 2 or FPR2. FPR2 has been defined a master-receptor for resolution in view of the resolution-defective phenotype displayed by mice lacking this receptor [5]. One way to harness the properties of AnxA1 has been to identify pharmacophores within the 346 amino acids that form the protein: such approach has led to the identification of peptide ~~Ac2-26~~Ac2–26 [6]. This tool has been invaluable not only to establish the pharmacological potential of an AnxA1-based approach (reviewed recently [7]), but also to identify receptors for formylated peptides as downstream cellular targets for AnxA1 and its mimetics [8]. Intriguingly, while the whole protein displays higher selectivity for human FPR2 (over FPR1; [9]) the shorter peptide ~~Ac2-26~~Ac2–26 activates all three members of this family of human receptors [5,8].

In models of cardiovascular diseases AnxA1 protects against acute myocardial ischemia/reperfusion (I/R) injury as well as myocardial infarction that develops after permanent occlusion of the coronary artery [10,11]. In acute settings, the protection is linked to modulation of immune cell migration and reactivity [10]. In chronic settings, treatment with AnxA1 provokes a switch in cardiac macrophages towards a reparative phenotype [11]. Relevant herein is the series of studies which have shown peptide ~~Ac2-26~~Ac2–26 afforded cardio-protection in distinct experimental settings, a protection mediated by formyl peptide receptors as shown by several groups [12–15]. This wealth of studies, and others, have prompted the development of selective FPR2 agonists for protection against cardiac injury. Compugen identified a compound through predictive computational screenings, using FPR2 as a target: CGEN-855A attenuated tissue necrosis in experimental myocardial infarction [16]. More recently, Bristol Meyers Squibb described the cardio-protection of a small molecule selective for FPR2 [17], building on previous work conducted with compound 43 and compound 17a by Ritchie and collaborators [17,18].

A viable way to translate the tissue-protective properties of AnxA1 through FPR2 activation, is to identify peptides with better pharmacokinetic and pharmacodynamics properties. Long after the discovery of the properties of Ac2–26 [19], we have characterised peptides that span the whole AnxA1 N-terminal domain and modified them to be resistant to cleavage by serine proteases. One of these peptides, termed CR-AnxA1$_{2–50}$ (49 amino acid long) was obtained through cloning in Escherichia coli, displayed high selectivity for human FPR2 and protected against acute myocardial I/R injury in mice [20]. Peptide CR-AnxA1$_{~~2-48~~2–48}$ was designed by removing last two amino acids of CR-AnxA1$_{2–50}$ to augment resistance to serine proteases. CR-AnxA1$_{~~2-48~~2–48}$ retained the initial pharmacology of these peptides, it was equally potent at FPR2 and protected against acute myocardial injury after ischemia/reperfusion in mice [21]. In the present study, we describe the pharmacology of RTP-026, a peptide made by solid phase synthesis identical to CR-AnxA1$_{~~2-48~~2–48}$ on the AnxA1 N-terminal domain. The procedure of solid-phase peptide synthesis enables the efficient production of a substantial quantity of compounds, rendering peptides suitable for clinical use due to their ability to meet the high demand for large quantities and cost-effectiveness. As cloned proteins and peptides may undergo post-translational modifications once obtained in the *E.coli.*, testing whether RTP026, synthesised through solid phase, recapitulated some of the effects reported for CR-AnxA1$_{~~2-48~~2–48}$ was of significant importance. We used a combination of in vitro, in vivo and ex-vivo analyses to define the cardio-protection of RTP-026 in myocardial I/R injury while at the same time exploring its modulatory properties on immune cell activation and trafficking. Moreover, it was important to determine if RTP-026 displayed similar effects as the equivalent peptide produced by cloning (CR-AnxA1$_{~~2-48~~2–48}$):

preferential activation of FPR2 (over FPR1) and high affinity for the receptor (in the nanoM range). In some experiments, peptide Ac2–26 was used for comparative purposes since less potent and not selective for FPR2; in other experiments a peptide with the natural sequence for amino acid 2–50 of AnxA1, termed RTP-022, was also used.

2 ~~METHODS~~Methods

2.1 Synthesis and validation of RTP-026

RTP-026 is a synthetic linear 47-amino acid peptide, made by solid phase synthesis (Ala.Met.Val.Ser.Glu.Phe.Leu.Lys Gln.Ala.Trp.Phe.Ile.Glu.Asn.Glu.Glu.Gln.Glu.

Tyr.Val.Gln.Thr.**Leu**~~.Lys.Ser.Ser.Lys.Gly.Gly.Pro.Gly.Ser.Ala.Val.Ser.Pro.Tyr.Pro.Thr.Phe.Asn.Pro.Ser.Ser.Asp.Val-NH2~~. Lys.Ser.Ser.Lys.Gly.Gly.Pro.Gly.Ser.Ala.Val.Ser.Pro.Tyr.Pro.Thr.Phe.Asn.Pro.Ser.Ser.Asp.Val-NH2 acetate salt; MW=5180.67; Bachem). RTP-026 was screened for solubility in relevant vehicles to be used for acute i.v. dosing to animals, finding through visual inspection and HPLC-UV analyses to be soluble and stable in ~~1~~10 mM glycylglycine, pH 8.3 buffer at a concentration up to ~~5~~5 mg/ml. Solubility tests were performed at Bioneer A/S (Hørsholm, DK).

RTP-022 was also obtained by solid phase synthesis and corresponds to the natural sequence of amino acid ~~2 to 50~~ 2–50 of the AnxA1 N-terminal domain, as reported [20]. The natural sequence of RTP-022 (amino acid ~~2 to 50~~ 2–50 of the AnxA1 N-terminal region) has no modification ~~(Ala.Met.Val.Ser.Glu.Phe.Leu.Lys.Gln.Ala.Trp.Phe.Ile.Glu.Asn.Glu.Glu.Gln.Glu.Tyr.Val.Gln.Thr.~~ (Ala.Met.Val.Ser.Glu.Phe.Leu.Lys.Gln.Ala.Trp.Phe.Ile.Glu.Asn.Glu.Glu.Gln.Glu.Tyr.Val.Gln.Th~~Val~~**r.Val**~~.Lys.Ser.Ser.Lys.Gly.Gly.Pro.Gly.Ser.Ala.Val.Ser.Pro.Tyr.Pro.Thr.Phe.Asn.Pro.Ser.Ser.Asp.Val.Ala.Ala-NH2~~. Lys.Ser.Ser.Lys.Gly.Gly.Pro.Gly.Ser.Ala.Val.Ser.Pro.Tyr.Pro.Thr.Phe.Asn.Pro.Ser.Ser.Asp.Val.Ala.Ala-NH2 acetate salt; MW= 5308.8).

Peptide ~~Ac2-26~~Ac2–26 was purchased from Tocris, Abingdon, UK (acetyl-Ala.Met.Val.Ser.Glu.Phe.Leu.Lys.Gln.Ala.Trp.Phe.Ile.Glu.Asn.Glu.Glu.Gln.Glu.Tyr.Val.Gln.Thr.Val.Lys; MW: 3089.46).

2.2 Human FPR2 activation in transfected HEK-293 cells

We cultured wild type (WT), human FPR2- and human FPR1-transfected HEK 293 cells. The transfection was stable as reported [9] and receptor expression confirmed by flow cytometry. Details are in Supplemental Material. Experiments were repeated three times with three technical replicates each.

2.3 Anti-survival effect on human neutrophils

Human samples were approved and obtained under the East London and the City research ethics committee 3 (Rec N.07/Q0605/29). The effect of RTP-026 was tested in the anti-survival test using serum amyloid A (SAA) as survival agent, as reported by Filép and colleagues [22]. Blood neutrophils were isolated using gradient centrifugation as detailed in Supplemental Material. Experiments were conducted with three to five distinct neutrophil preparations from different donors, using two technical replicates for each sample.

2.4 Whole blood phagocytosis assay

Human samples were approved and obtained under the East London and the City research ethics committee 3 (Rec N.07/Q0605/29). Details are available in Supplemental Material. Three distinct experiments were conducted with cells from different donors, using two technical replicates per sample.

2.5 Animals

The local 'Animal Use and Care Committee' approved animal experiments in accordance with the derivatives of both, the 'Home Office guidance on the Operation of Animals (Scientific Procedures) Act 1986', and the 'Guide for the Care and Use of Laboratory Animals' of the National Research Council. Ethics approval no. is PA672E0EE; Queen Mary Research ethics committee 3. This study was carried out on male Wistar rats (Charles River Laboratories, Harlow, UK) weighing 240–380 g, and Sprague Dawley rats (CardioMedex, Paris, France) weighing 175–200 g, both receiving a standard diet and water ad libitum~~*ad libitum.*~~. A non-recovery surgical procedure with 2-hour reperfusion was conducted on Wistar rats at Queen Mary University of London, while a recovery surgical procedure with 24-hour reperfusion was performed on Sprague Dawley rats at CardioMedex, Paris. The extent of area at risk was comparable across the rat strains. Details of power calculations and randomisation are available in Supplemental Material.

2.6 Rat myocardial ischemia/reperfusion injury

Myocardial ischemia/reperfusion was carried out as described previously [23]. Rats were anesthetized with thiopentone sodium (Intraval,120 mg/kg i.p. for induction, followed by 10 mg/kg i.v. for maintenance), a tracheostomy was performed and animals were allowed to artificially respire using a Harvard (room air; 70 strokes/min; tidal volume: 8–

10 ml/kg) throughout the experiment. Body temperature was maintained at 37 ± 1 °C with the aid of a rectal probe thermometer attached to a homeothermic blanket unit (Harvard Apparatus Ltd, Edenbridge, Kent, UK). The right carotid artery was cannulated and connected to a pressure transducer (Senso-Nor 844, Horten, Norway), to monitor mean arterial pressure and heart rate. The right jugular vein was cannulated to administrate maintenance anaesthesia and drugs. A parasternal thoracotomy was performed using electrocautery followed by retraction of the thymus and resection of the pericardium. A 6–0 silk suture was placed through the myocardium at the approximate level of the left anterior descending coronary artery (LAD). A piece of polyethylene tubing (Smiths Medical, Watford, UK) flared at one end, was placed over the suture to form a snare occluder. The coronary artery was occluded by tightening the occluder for 20 min, then the occlusion was released to allow reperfusion for 2 h under anaesthesia. Right before the reperfusion, vehicle or drugs were administrated via jugular vein. In a separate set of experiments performed by CardioMedex (Paris, France), reperfusion was run up to 24 h. After initial tests (not shown), the dose of 30 μg/kg i.v. RTP-026 was selected and administered to rats at time 0 (immediately after reopening of the coronary artery), then 3 and 6 h post-reperfusion. In this 24-hour reperfusion model, rats were anesthetized with 4 % isoflurane initially, followed by intubation, mechanical ventilation, and maintenance with 2–2.5 % isoflurane. The heart was exposed ~~via~~via left intercostal thoracotomy, and a 6–0 silk suture was placed around the LAD coronary artery to induce 30 min of ischemia, followed by 24 h of reperfusion. The chest was closed using 4.0 absorbable sutures. After anaesthesia was stopped, animals recovered on a heating pad. Upon spontaneous breathing, rats were extubated and monitored for pain or laboured breathing. Buprenorphine at 30 μg/kg was administered pre- and post-surgery, approximately 6 h apart, for analgesia.

2.7 Quantification of myocardial tissue injury

In both cases, at the end of the reperfusion period around ~~2~~2 ml of blood was taken from the carotid artery. LAD was re-occluded and ~~1.5~~1.5 ml of Evans Blue dye ~~(2%~~ (2 % wt/vol) was administered via the jugular vein to distinguish between perfused and nonperfused (area at risk; AAR) sections of the heart. The heart was excised and immersed in ice-cold ~~0.9%~~ 0.9 % saline to achieve cardioplegia and was sectioned into five to six slices of 1-mm to 2-mm using Rat Heart Slicer Matrix (Zivic Instruments, USA) to the level of the suture. After the right ventricular wall was removed, heart slices were stained in ~~1%~~ 1 % 2,3,5-Triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, UK) solution for ~~30~~30 min at ~~37°C~~37 °C with agitation (100 rotations/min). Stained slices were fixed in ~~4%~~ 4 % PFA for ~~48~~48 h before taking pictures. The following areas were measured using ImageJ: left ventricle area, ischemic area (non-blue area) and infarct area (in yellow colour). Area at risk (AAR) was expressed as a percentage of the left ventricle. The infarct size was expressed as a percentage of the AAR. Quantification was done by an operator blind to the treatments.

2.8 Myeloid cell recruitment and characterization in the heart with I/R injury

Hearts were collected after ~~2~~2 h reperfusion and residual blood cleared by retrograde perfusion with ~~5~~5 ml ice cold normal saline. Flow cytometry of left ventricular tissue was performed as detailed in Supplemental Material.

2.9 Alterations in blood cell numbers and profile in rats with I/R injury

After ~~2~~2 h reperfusion, blood samples were collected through carotid artery and immediately decanted into EDTA blood tubes (Sarstedt AG & Co., Nümbrecht, Germany). Approximately ~~0.1~~0.1 ml of blood was used for analyses of leukocytes. Flow cytometry of blood were carried out blindly and performed with protocols detailed in Supplemental Material.

2.10 Soluble mediator quantification in rat plasma

Cell-free plasma samples were analysed for presence of pro-inflammatory and pro-resolving mediators. For proteins and peptides, multiplex ELISA on cytokines and chemokines as well as growth factors, using Luminex kits (code 171-KTR2CK and RECYMAG65K) according to the manufacturer's instructions, were run by Labospace (Milan, Italy; ISO 9001:2015 registration). Altogether 31 mediators were analysed in the samples, running technical duplicates, once calibration and validation preliminary experiments were satisfactory. Lipid mediator profiling was conducted using LC-MS/MS-based lipid mediator profiling as described before [24]. Briefly samples were extracted using Solid Phase Extraction-based methodologies and lipid mediators identified and quantified using LC-MS-MS. Each lipid mediator was identified using established criteria, including: (i) matching retention time to synthetic or authentic standards and (ii) signal to noise ratio ≥5. Calibration curves were obtained for each mediator using synthetic compound mixtures that gave linear calibration curves with an r^2 values of 0.98–0.99.

In both cases, we report only data for selected protein or lipid mediators which were modulated by treatment of rats with RTP-026.

2.11 Statistics

All values described in the text and figures are presented as ~~mean ± standard~~ mean ± standard error of the mean (SEM) of n observations, where n represents the number of animals studied. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, California, USA). One-way ANOVA followed by Bonferroni's

post hoc test were used to compare intergroup differences. Comparing results were considered statistically significant when ~~P<0.05.~~P < 0.05. Partial Least Square Discriminant Analysis were performed using MetaboAnalyst. Here, features with a constant or single value across samples were deleted. Partial Least Square Discriminant Analysis was then performed following auto-scaling (mean-centred and divided by the standard deviation of each variable). PLS-DA is based on a linear multivariate model that identifies variables that contribute to class separation of observations on the basis of their variables (LM levels). During classification, observations were projected onto their respective class model. The score plot illustrates the systematic clusters among the observations (closer plots presenting higher similarity in the data matrix).

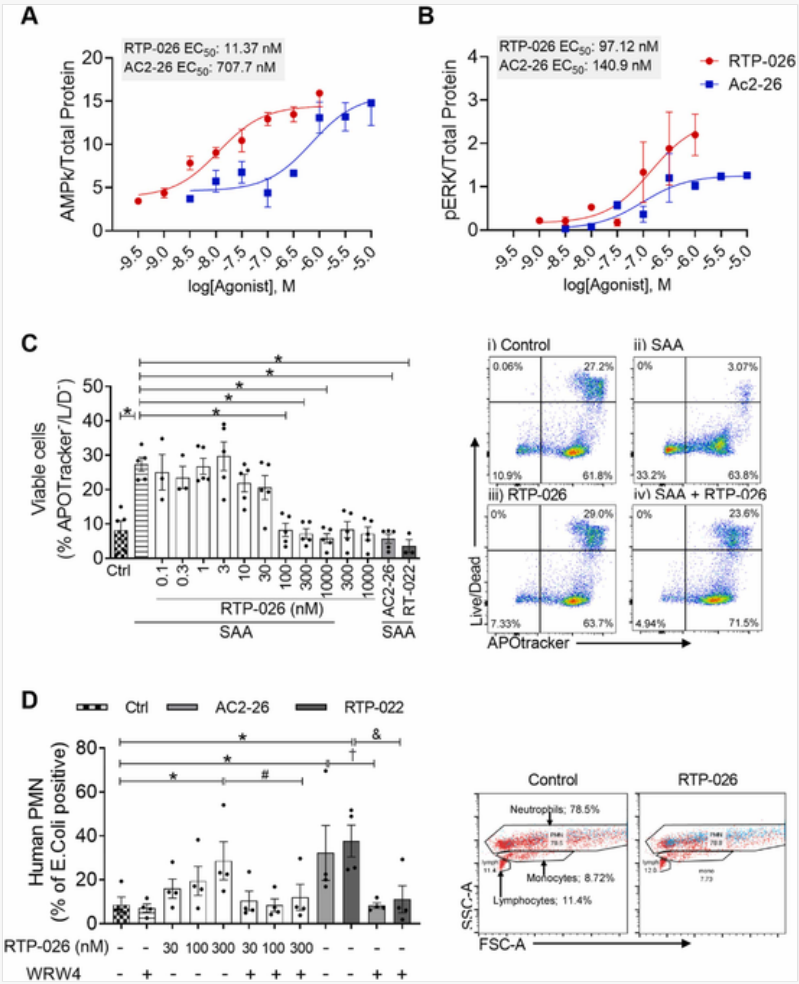
3 ~~RESULTS~~Results

3.1 Characterization of RTP-026 in vitro effects

We started this study by testing whether RTP-026 which was obtained by solid phase synthesis could retain the molecular pharmacology of the original peptide obtained through cloning in Escherichia coli [21]. HEK-293 cells transfected with either human FPR1, FPR2 or mock vector (Supplementary Fig. 1A), were incubated with different concentrations of RTP-026. The compound retained selectivity for FPR2 over FPR1 as shown by intracellular accumulation of phospho-ERK and phospho-AMPK (Supplementary Fig. 1B). When tested side by side with peptide ~~Ac2-26,~~Ac2–26, used as internal control, RTP-026 displayed higher potency on FPR2, both with phospho-AMPK (Fig. 1A) or phospho-ERK (Fig. 1B) as readouts. Calculated RTP-026 EC_{50} ranged between 10 and ~~100~~100 nM, while the EC_{50} for peptide ~~Ac2-26~~Ac2–26 was around ~~50%~~ 50 % higher. Such cellular responses were genuinely mediated by FPR2 activation as demonstrated by addition of the antagonist WRW_4 which abrogated RTP-026 signalling in FPR2-HEK293 cells (Supplementary Fig. 1B). To determine the pro-resolving nature of RTP-026 we selected two classical functional effects on immune cells, described for the natural protein AnxA1.

alt-text: Fig. 1

Fig. 1



~~Selective activation of human FPR2 and immune cells by RTP-026.~~Selective activation of human FPR2 and immune cells by RTP-026. HEK293 cells, purified neutrophils or whole blood samples were incubated with increasing concentration of RTP-026 (0.1–3000 nM). HEK293 cells were used to monitor pERK and AMPK phosphorylation (10 and 30 min; respectively), neutrophils to monitor

apoptosis (overnight incubation), whole blood to monitor phagocytosis of *E.Coli* Phrodo particles (10 min). (A,B) Concentration-response curves of AMPK (A) and ERK (B) phosphorylation upon stimulation with increasing concentrations of RTP-026 or Ac2–26. (C) Bar chart and representative scatter plots presenting viable neutrophils after overnight incubation with serum amyloid A (SAA) in presence or absence of Ac2–26 and RTP-022, used as control, or increasing concentrations of RTP-026. (D) Bar chart of % human neutrophils positive for *E.Coli* staining with representative dot plots. Data mean ± SEM of n = 3 or 4 independent experiments. (C) *P < 0.05 ~~vs.~~vs. Control + SAA group; (D) *P < 0.05 ~~vs.~~vs. Control, #P < 0.05 ~~vs.~~vs. 300 nM RTP026 group, †P < 0.05 ~~vs.~~vs. Ac2–26, &P < 0.05 ~~vs.~~vs. RTP-022 (one-way ANOVA followed by Bonferroni's test).

Firstly, we tested the anti-survival effect of RTP-026. Overnight incubation of human neutrophils led to a reduction of cell viability (to approximately ~~5–15%~~ 5–15 % of the initial cell input), an effect due to induction of early (Apotracker positive) and late apoptosis (Live/Dead and Apotracker double positive) events (Fig. 1C, representative panels i-iv). Such an effect on viability was significantly reduced by cell incubation with serum amyloid A (SAA) which led to marked survival (~3-fold over control settings). Addition of RTP-026 counteracted the pro-survival effect of SAA in a concentration-dependent fashion, with significant attenuations at ~~100–1000~~100–1000 nM (Fig. 1C; see panel iv for a representative scatter plot). In this set of experiments, we tested peptide ~~Ac2–26~~Ac2–26 and RTP-022 which equally antagonised the pro-survival effect of SAA once added at ~~1~~1 μM concentration (Fig. 1C).

Secondly, we tested the ability of RTP-026 to regulate bacteria phagocytosis [25]. The whole blood assay was ideal to monitor effects of RTP-026 on more than one immune cell type and also enabled analyses in blood samples from different species. Fig. 1D shows representative scatterplots, with the effect of ~~300~~300 nM RTP-026 on bacterial uptake in the neutrophil population, identified through its side and forward scatter characteristics. Bar graphs report the full dataset with the potentiating effects of RTP-026 (tested herein at ~~30–300~~30–300 nM range) and the likely engagement of FPR2 in this response, since the effects were abolished by the FPR2 antagonist WRW_4 ~~(10~~(10 μM). On the same graph, we report also the effects of peptide ~~Ac2–26~~Ac2–26 and RTP-022 tested at the single concentration of ~~1~~1 μM; their positive effects were antagonised by WRW_4 (Fig. 1D). The assay revealed similar effects of RTP-026 on human monocytes, together with the modulation afforded by the antagonist (Supplementary Fig. 2A). Similarly, the assay was run with rat blood: RTP-026 (tested at 100 and ~~300~~300 nM only here) augmented the phagocytosis capacity of both rat neutrophils and rat monocytes (Supplementary Fig. 2B).

Altogether these experiments provided confidence that RTP-026 was a potent and selective activator at human FPR2, endowed with pro-resolving properties of the parent protein AnxA1. On these bases, RTP-026 was tested in settings of acute myocardial I/R injury in the rat.

3.2 Characterization of RTP-026 in vivo effects on myocardial I/R injury

Occlusion of the coronary artery for ~~20~~20 min followed by 2-hour reperfusion, caused substantial infarct size. Representative images are shown in Fig. 2A. When vehicle was administered at the point of reperfusion, we could quantify the infarct size at the value of ~~61.06 ± 2.93%,~~ 61.06 ± 2.93 %, with AAR values of ~~53.20 ± 2.98%~~ 53.20 ± 2.98 % of the whole left ventricle (Fig. 2B,C). We used two vehicle groups to control for the effects of peptide ~~Ac2–26~~Ac2–26 and RTP-026. PBS was used as vehicle for ~~Ac2–26.~~Ac2–26. Glycylglycine was used as vehicle for RTP-026, as it kept the peptide in solution. The latter, given at reperfusion, afforded a significant reversion of the infarct size, with maximal efficacy at the dose of ~~50~~50 μg/kg, with values of infarct area changing from ~~63.38 ± 3.21%~~ 63.38 ± 3.21 % of the AAR in the vehicle to ~~25.78 ± 3.78%~~ 25.78 ± 3.78 % in the treatment group. At the highest dose tested of ~~500~~500 μg/kg and the lower dose of ~~25~~25 μg/kg, RTP-026 retained efficacy however less effective than the dose of ~~50~~50 μg/kg (Fig. 2C). Peptide ~~Ac2–26~~Ac2–26 dosed at ~~1~~1 mg/kg provided a protection against infarct size close to that measured with RTP-026 ~~500~~500 μg/kg and ~~25~~25 μg/kg (Fig. 2C). The cardio-protection promoted by RTP-026 or peptide ~~Ac2–26~~Ac2–26 was not consequent to changes on mean arterial blood pressure (Fig. 2D) or heart rate (Fig. 2 E).

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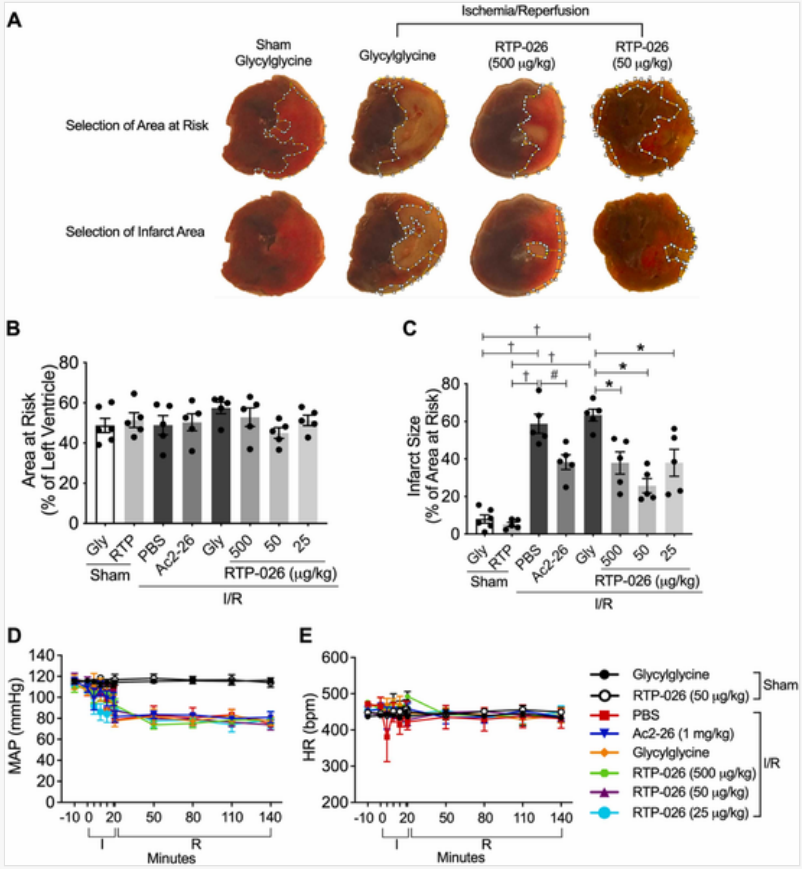
Fig. 2



~~RTP-026 reduces infarct size in rats subjected to myocardial ischemia/reperfusion.~~RTP-026 reduces infarct size in rats subjected to myocardial ischemia/reperfusion. Rats were treated i.v. with glycylglycine (0.8 ml/kg), RTP-026 (500, 50, or 25 µg/kg), Ac2–26 (1 mg/kg) or PBS (0.8 ml/kg) at the start of the reperfusion period. Tissues were analysed 2 h after reperfusion. Groups of sham-operated animals (sham) receiving glycylglycine or RTP-026 (50 µg/kg) were also evaluated. (A) Representative TTC staining images. Tissue in dark red colour: myocardium without ischemia; Tissue in bright red and yellow colour: myocardium underwent ischemia; Tissue in yellow colour: infarct myocardium. Top panel: selection of area at risk; bottom panel: selection of infarct area. The area at risk (B) and infarct size (C) were determined as described in Materials and Methods. (D) mean arterial pressure and (E) heart rate. Data are mean ± SEM. N = 5–6 rats per group. $^{\dagger}P < 0.05$ ~~vs.~~vs. sham groups; $^{\#}P < 0.05$ ~~vs~~vs. PBS group; $^{*}P < 0.05$ ~~vs.~~vs. glycylglycine group (one-way ANOVA followed by Bonferroni's test). I/R: ischemia/reperfusion; Gly: glycylglycine; MAP: mean arterial blood pressure; HR: heart rate.

These results prompted us to investigate cellular and molecular mechanisms which could underpin the pharmacological effects of RTP-026. We started by studying immune cell numbers in the hearts.

3.3 RTP-026 modulates immune cell recruitment into the heart with I/R injury

To establish if RTP-026 could modulate white blood cell trafficking to the heart, separate groups of rats were subjected to the I/R protocol and treated either with glycylglycine or RTP-026. For this set of experiments, we selected the most active dose of ~~50~~50 µg/kg RTP-026. Supplementary Fig. 3 presents the gating strategy used to characterise immune cells from hearts subjected to 2-hour reperfusion. These analyses revealed that treatment of rats with RTP-026 reduced ~~CD45+ cells~~ CD45 + cells by one third (Fig. 3A,B), with an equally significant reduction in myeloid cells, identified as CD45/CD11b double positive events (Fig. 3C,D). Further dissection using RP-1 immuno-reactivity to stain recruited neutrophils [26], allowed us to distinguish between neutrophils and monocyte/macrophage numbers in the rat heart (Fig. 3E). RTP-026 nearly abolished the recruitment of neutrophils (i.e. $CD45^{+}CD11b^{+}RP\text{-}1^{+}$ cells) as compared to vehicle-treated hearts (Fig. 3F) with much less effect on monocyte/macrophage (i.e. $CD45^{+}CD11b^{+}RP\text{-}1^{-}$) numbers (Fig. 3G). However, the monocyte/macrophage gating groups are composed of different cells, including classical monocytes, non-classical monocytes and macrophages. Using His48 and CD43 staining to identify classical and non-classical rat monocytes [27], we could now split this grouping (Fig. 3H). A significant reduction in the recruitment of $CD43^{-}His48^{high}$ classical monocytes was now observed for the RTP-026 treated group, with no effect on the numbers of $CD43^{+}His48l^{ow}$ non-classical monocyte (Fig. 3I,J).

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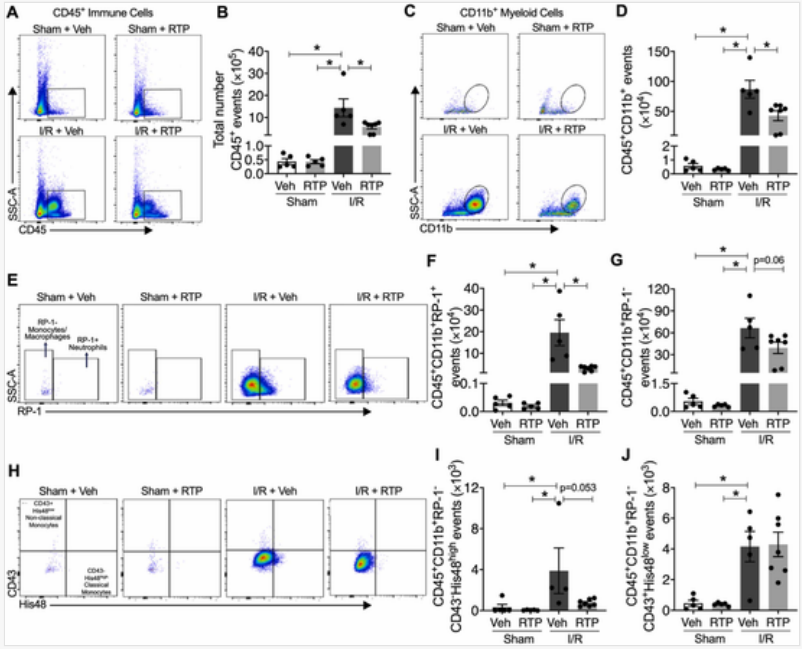
Fig. 3



~~RTP-026 reduces cardiac immune cells infiltration in rats subjected to myocardial ischemia/reperfusion.~~RTP-026 reduces cardiac immune cells infiltration in rats subjected to myocardial ischemia/reperfusion. Rats were treated i.v. with glycylglycine (vehicle; ~~0.8~~0.8 ml/kg), RTP-026 (~~50~~50 μg/kg) at the start of reperfusion period. Two hours after reperfusion, hearts were harvested and enzymatically digested, prior to assessment of cardiac immune cell phenotype by flow cytometry. Groups of sham-operated animals (sham) receiving glycylglycine or RTP-026 (~~50~~50 μg/kg) were also evaluated. (A) Representative dot plots showing cardiac leukocytes ($CD45^+$) in rats subjected to sham-operation or I/R injury receiving vehicle or RTP-026. (B) Cumulative data indicating total number of cardiac leukocytes. (C) Representative dot plots showing cardiac myeloid cells ($CD45^+CD11b^+$) in rats subjected to sham-operation or I/R injury and treated with vehicle or RTP-026. (D) Cumulative data indicating total number of cardiac myeloid cells. (E) Representative dot plots illustrating $CD45^+CD11b^+RP\text{-}1^+$ neutrophils and $CD45^+CD11b^+RP\text{-}1^-$ monocytes/macrophages in rats subjected to sham-operation or I/R injury receiving vehicle or RTP-026. Cumulative data indicating total number of (F) $CD45^+CD11b^+RP\text{-}1^+$ neutrophils and (G) $CD45^+CD11b^+RP\text{-}1^-$ monocytes/macrophages. (H) Representative dot plots illustrating $CD43^-His48^{high}$ classical monocytes and $CD43^+His48l^{ow}$ non-classical monocytes in rats subjected to sham-operation or I/R injury receiving vehicle or RTP-026. Cumulative data indicating total number of (I) $CD43^-His48^{high}$ classical monocytes and (J) $CD43^+His48^{low}$ non-classical monocytes. Data are ~~mean ± SEM. N=5–7~~ mean ± SEM. N = 5–7 rats per group. An outlier was excluded for classical monocytes analysis from I/R + vehicle group due to its extremely high value. ~~*P<0.05~~*$P < 0.05$ vs. I/R ~~+ vehicle~~ + vehicle group (one-way ANOVA followed by Bonferroni's test). I/R: ischemia reperfusion; Veh: vehicle; RTP: RTP-026.

When we consider cumulative numbers of the main populations we could identify in myocardial extracts, it emerges that myeloid cells ($CD45^+/CD11b^+$) represent approximately 60–70 % of the total immune cells recovered (compare Fig. 3D with B), and ~20 % of them are neutrophils (compare Fig. 3F ~~versus~~versus 3D); the remaining are monocytes or macrophages. Of the latter, the large majority are macrophages, whereas the recruitment of classical and non-classical monocytes seems to be equal at this time point, with similar numbers of $CD45^+/CD11^+/RP1^-/CD43^-/Hys48^{high}$ and $CD45^+/CD11^+/RP1^-/CD43^+/Hys48^{low}$ events, respectively (Fig. 3I,J).

These flow cytometry analyses on rat leukocytes reveal significant reduction in the recruitment of neutrophils and classical monocytes into the infarcted hearts of animals treated with the most active dose of RTP-026. Next, we studied if RTP-026 administration could regulate the phenotype of circulating cells.

3.4 RTP-026 modulates white blood cell phenotype in the circulation of rats with I/R injury

Ischemia/reperfusion of the heart provoked an approximate 5-fold increase in circulating polymorphonuclear leukocytes and a 3-fold increase in circulating monocytes, with minimal changes in lymphocyte numbers. Treatment of rats with ~~50~~50 μg/kg RTP-026 at reperfusion, did not affect these changes (Supplementary Fig. 4). The same held true for sham rats where the larger proportion of white blood cells was due to the lymphocyte population (Supplementary Fig. 4).

To define the activation state of rat circulating polymorphonuclear leukocytes and monocytes, cell surface expression of CD62L, CD54 (ICAM-1) and CD11b were used. Fig. 4 reports these datasets. At 2-hour reperfusion, the large majority of circulating polymorphonuclear leukocytes had lost CD62L expression (~~~90%~~ (~90 % reduction): Fig. 4A shows representative histograms, whereas Fig. 4B and ~~4~~C focus on $CD62L^{high}$ polymorphonuclear leukocytes, which are the non-activated neutrophils. RTP-026 significantly reverted the marked polymorphonuclear leukocyte activation both in terms of proportion of cells with high CD62L expression (Fig. 4B) and their absolute number (Fig. 4C). Intriguingly, also in sham rat blood there was a degree of CD62L shedding so that the proportion of $CD62L^{high}$ polymorphonuclear leukocytes was around ~~40%~~ 40 % of the total: this was incremented by ~~50%~~ 50 % with RTP-026 treatment to yield approximately ~~60%~~ 60 % of the total numbers of cells (Fig. 4B).

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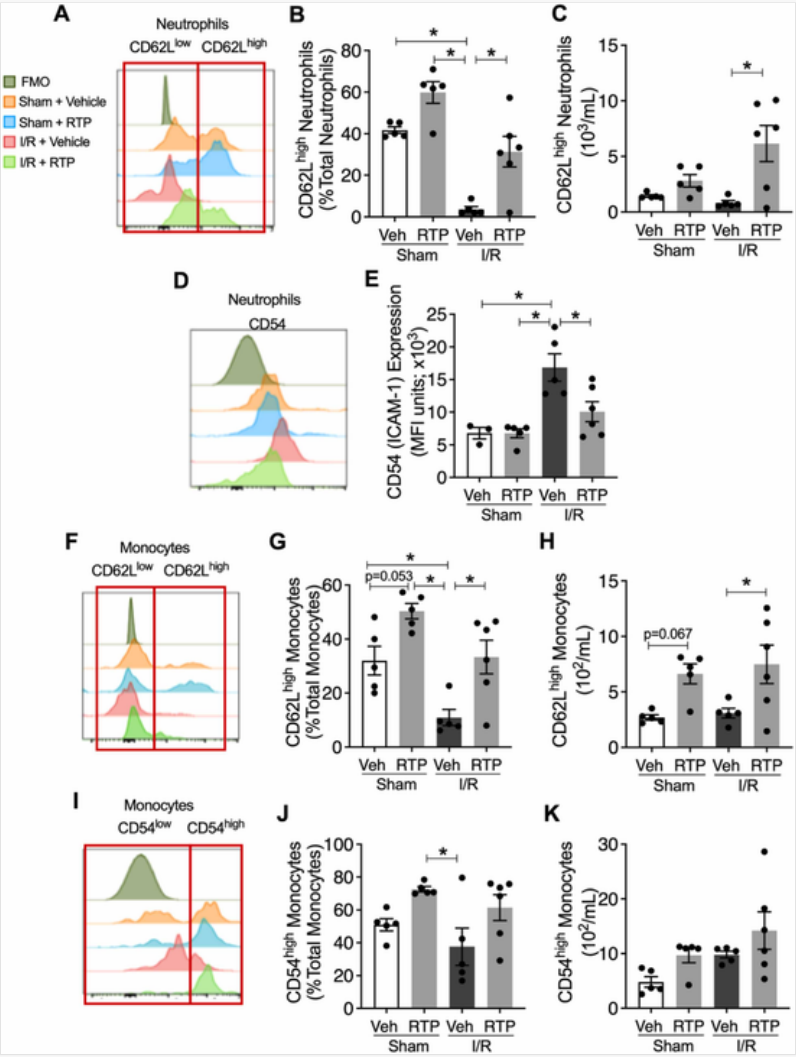
Fig. 4



~~RTP-026 reduces circulating neutrophil and monocyte activation in rats subjected to myocardial ischemia/reperfusion.~~RTP-026 reduces circulating neutrophil and monocyte activation in rats subjected to myocardial ischemia/reperfusion. Rats were treated i.v. with glycylglycine (vehicle; ~~0.8~~0.8 ml/kg), RTP-026 (~~50~~(50 μg/kg) at the start of reperfusion period. Two hours after reperfusion, blood was collected and CD62L and CD54 cell surface expression was assessed by Attune NxT Flow Cytometer. Neutrophil and monocyte activation was monitored with antibodies recognizing adhesion molecules (A-C, F-H) CD62L and (D, E, I-K) CD54. Groups of sham-operated animals (sham) receiving glycylglycine or RTP-026 (~~50~~(50 μg/kg) were also evaluated. (A) Histogram illustrating CD62L^{high} and CD62L^{low} neutrophils. Cumulative data indicating (B) percentage and (C) total number of CD62L^{high} neutrophils. (D) Histogram illustrating CD54 expression on neutrophils. (E) Cumulative data indicating median fluorescence intensity (MFI) units of CD54 expression on neutrophils. (F) Histogram illustrating CD62L^{high} and CD62L^{low} monocytes. Cumulative data indicating (G) percentage and (H) total number of CD62L^{high} h monocytes. (I) Histogram illustrating CD54^{high} and CD54^{low} monocytes. Cumulative data indicating (J) percentage and (K) total number of CD54^{high} monocytes. Data are mean ~~± SEM. N = 5–6~~ ± SEM. N = 5–6 per group. ~~*P<0.05~~*P < 0.05 vs. I/R ~~+ vehicle~~ + vehicle group (one-way ANOVA followed by Bonferroni's test). I/R: ischemia reperfusion; Veh: vehicle; RTP: RTP-026.

A more clear-cut outcome was obtained when CD54 expression was measured: here all polymorphonuclear leukocytes expressed this adhesion molecule. We could quantify changes in cellular expression levels through the specific MFI units: these were elevated ~~2~~2 h after reperfusion in vehicle-treated rats while nearly back to sham levels in the rats treated with RTP-026 (Fig. 4D, ~~4~~E).

Equivalent though not identical changes due to I/R injury and the modulation afforded by RTP-026 were quantified for blood monocytes: the compound preserved at least in part the proportion of CD62L^{high} monocytes and attenuated the split of ~~CD54+ cells~~ CD54 + cells in this population (Fig. 4~~F–4K).~~F–K). When we quantified CD11b we could not detect major changes in our experimental settings, either due to the infarction protocol or to animal treatment with RTP-026 (Supplementary Fig. 5).

Altogether, whole blood analyses in the infarcted rat samples suggested that RTP-026 could selectively modulate the degree of leukocyte activation in the circulation, without impacting on total cell numbers. Moreover,

polymorphonuclear leukocyte, and to a lesser extent monocyte, phenotypes could be significantly regulated by the compound. Next, we deemed important to investigate whether these changes in markers of cell activation could be secondary to modulation of inflammatory mediators in the circulation.

3.5 RTP-026 affords selective regulation of pro-inflammatory mediators

Several analytes were quantified in the plasma samples generated from the rats used for the I/R experiments presented in Fig. 2. Three groups of samples from rats treated with RTP-026 were tested with multiplex ELISA, observing a selective modulation of a small number of mediators. The three major protein mediators to be affected by RTP-026 were TNF-α, IL-1β and KC. Fig. 5 presents these data, showing that 2-hour reperfusion elevated circulating levels of these three mediators, with a most remarkable increase for the CXC motif chemokine KC. Of the samples generated from rats treated with RTP-026, significant reduction was attained with the dose of ~~50~~50 μg/kg on all three mediators, whereas the higher dose of ~~500~~500 μg/kg attenuated only the elevation of IL-1β (Fig. 5B). Other mediators which were augmented in the vehicle I/R group were vascular endothelial growth factor and fractalkine, but these were not reduced by RTP-026 (Fig. 5D,~~5~~ E). Several other mediators were quantified and shown to change neither following I/R or after RTP-026 administration: many are not shown, with data for MIP-1α, IL-6, IL-10 and eotaxin being presented in Fig. 5 ~~F–5I.~~F–I.

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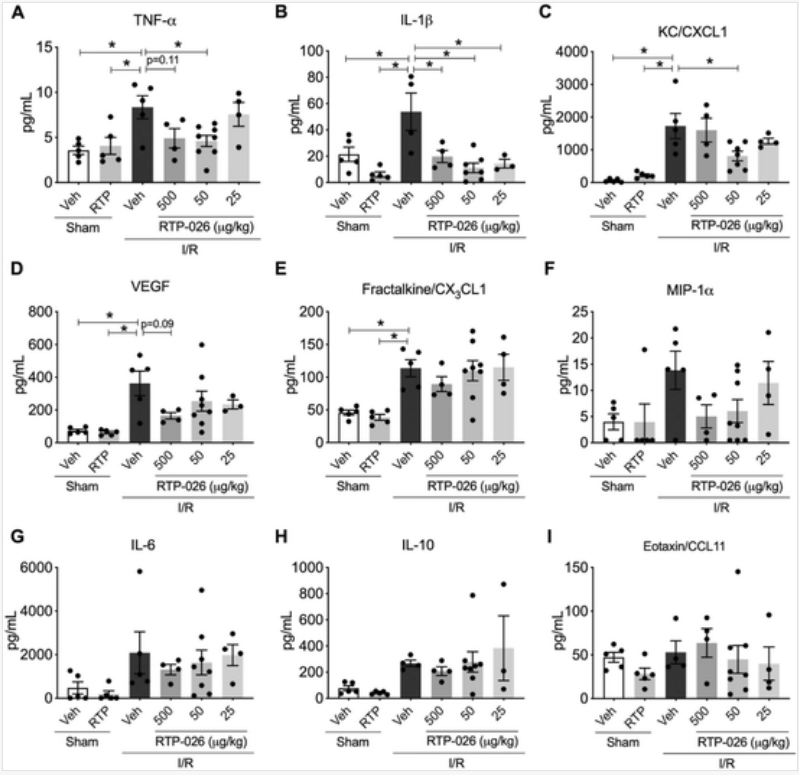
Fig. 5



~~RTP-026 reduces pro-inflammatory mediators in the circulation of rats subjected to myocardial ischemia/reperfusion.~~RTP-026 reduces pro-inflammatory mediators in the circulation of rats subjected to myocardial ischemia/reperfusion. Rats were treated i.v. with glycylglycine (vehicle; ~~0.8~~0.8 ml/kg), RTP-026 (500, 50 or ~~25~~25 μg/kg) at the start of reperfusion period. Two hours after reperfusion, plasma samples were obtained and aliquots run through the Luminex platform. Groups of sham-operated animals (sham) receiving glycylglycine or RTP-026 (~~50~~(50 μg/kg) were also evaluated. Concentrations of (A) TNF-α, (B) IL-1β, (C) KC/CXCL1, (D) VEGF, (E) Fractalkine, (F) MIP-1α, (G) IL-6, (H) IL-10 and (I) Eotaxin/CCL11. Data are ~~mean ± SEM. N = 4–8~~ mean ± SEM. N = 4–8 rats per group. Outliers with extremely high values were excluded. ~~*P<0.05~~*P < 0.05 vs. I/R + vehicle group (one-way ANOVA followed by Bonferroni's test). I/R: ischemia reperfusion; Veh: vehicle; RTP: RTP-026.

The same approach was used for lipid mediator analysis, this time testing plasma samples from rats treated with RTP-026 500 μg/kg or 50 μg/kg. Fig. 6A displays the principal component analysis based on the plasma levels of more than 60 bioactive mediators, including the pro-inflammatory prostaglandins, ionotropic leukotrienes and the pro-resolving and tissue protective specialized pro-resolving lipid mediators (Table S3). This analysis demonstrated that circulating lipid mediator concentrations were different among the three groups, not only for I/R ~~versus~~versus sham, but also for RTP-026, with the 50 μg/kg dose group being the most different from the other clusters (Fig. 6A). Assessment of the Variable Importance in Projection (VIP) score, which identifies those mediators that contribute to the observed separation between the different clusters, for the lipid mediators identified demonstrated that RTP-026 dose-dependently upregulated the expression of several specialized pro-resolving lipid mediator including Resolvin D6 (RvD6) and Maresin 2 (MaR2; Fig. 6B). We also quantified a downregulation in the levels of a number of inflammatory eicosanoids including prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\ \alpha}$) (Fig. 6C–E). Thromboxane B_2 (TxB_2) levels were decreased following RTP-026 treatment, although values did not reach statistical significance (Fig. 6C). In line with the data presented in Fig. 5, the active dose of 50 μg/kg RTP-026 produced the most significant changes for lipid mediator levels.

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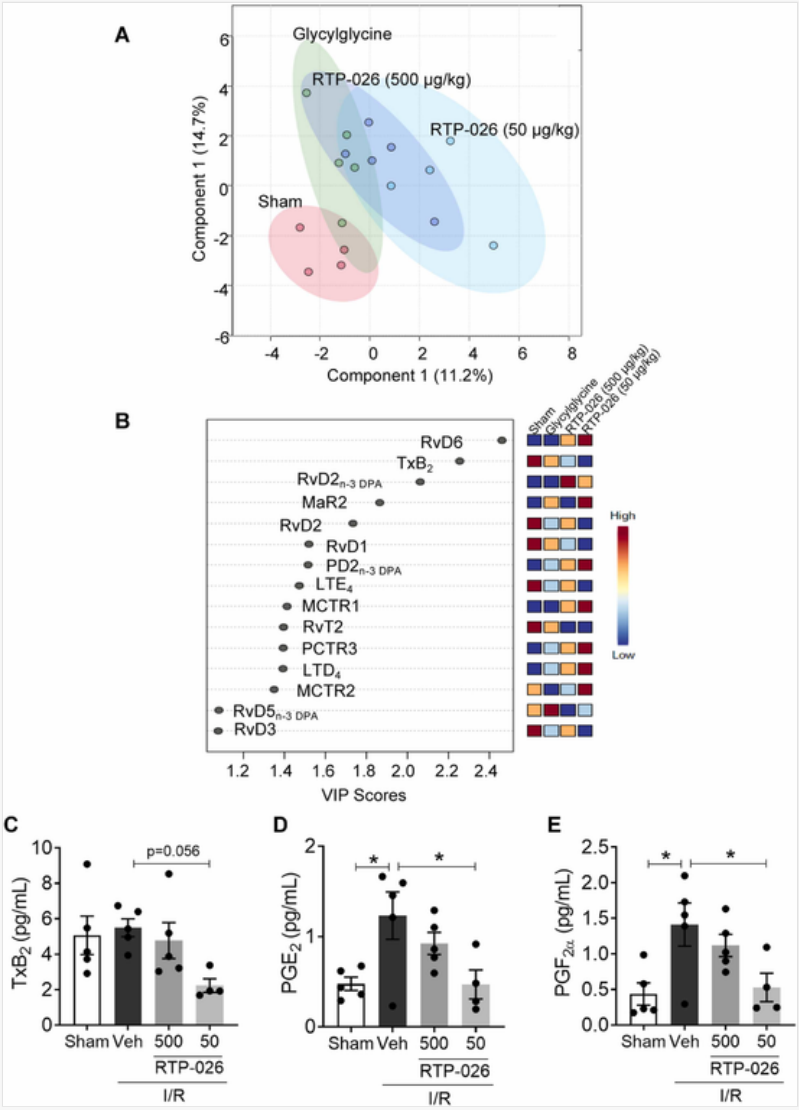
Fig. 6


A
Glycylglycine
RTP-026 (500 µg/kg)
RTP-026 (50 µg/kg)
Sham
Component 1 (14.7%)
Component 1 (11.2%)
B
RvD6
TxB2
RvD2n-3 DPA
MaR2
RvD2
RvD1
PD2n-3 DPA
LTE4
MCTR1
RvT2
PCTR3
LTD4
MCTR2
RvD5n-3 DPA
RvD3
VIP Scores
Sham
Glycylglycine
RTP-026 (500 µg/kg)
RTP-026 (50 µg/kg)
High
Low
C
TxB2 (pg/mL)
p=0.056
Sham Veh 500 50
RTP-026
I/R
D
PGE2 (pg/mL)
*
*
Sham Veh 500 50
RTP-026
I/R
E
PGF2α (pg/mL)
*
*
Sham Veh 500 50
RTP-026
I/R

~~RTP-026 modulates lipid mediator concentrations in the circulation of rats subjected to myocardial ischemia/reperfusion.~~RTP-026 modulates lipid mediator concentrations in the circulation of rats subjected to myocardial ischemia/reperfusion. Rats were treated i.v. with glycylglycine (vehicle; 0.8 ml/kg), RTP-026 (500 or 50 μg/kg) at the start of reperfusion period. Two hours after reperfusion, blood was collected and mediators in the plasma were identified and quantified using LC-MS/MS based lipid mediator profiling. A group of sham-operated animals (sham) receiving glycylglycine was also evaluated. (A,B) Lipid mediator concentrations among the four groups were then interrogated using Partial Least Square Discriminant Analysis. (A) Scores Plot, with each dot representing profiles from each mouse (B) Variable Importance in Projection (VIP) score plot depicting the relative levels of each of the mediators across the indicated four experimental groups. (C,E) Cumulative levels for select lipid mediators including (C) Thromboxane B_2 (TxB_2), (D) Prostaglandin E_2 (PGE_2) and (E) Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Data are mean ± SEM of N = 4–5 rats per group. *P < 0.05 ~~vs.~~vs. I/R + vehicle group (one-way ANOVA followed by Bonferroni's test). I/R: ischemia reperfusion; Veh: vehicle.

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Collectively these data indicate that pharmacologically active doses of RTP-026 can inhibit systemic production of pro-inflammatory mediators, including those known to activate neutrophils and monocytes like TNF-α, KC and PGE_2.

3.6 RTP-026 in vivo effects on myocardial I/R injury at 24-hour post-reperfusion

Having demonstrated that RTP-026 afforded cardio-protection in acute settings of reperfusion-mediated injury, it was important to assess the translational potential of this compound in settings which better mimic the clinical scenario. To this end, a protocol with a 24-hour post-reperfusion endpoint was run (Fig. 7A). Initial experiments were conducted by testing RTP-026 at a dose range of 10, 30 or ~~100~~100 μg/kg (not shown). On these bases, the ~~30~~30 μg/kg dose was selected together with a delivery regimen of i.v. injection at time 0, 3- and 6-hours post-reperfusion. At ~~24~~24 h post-reperfusion, rats treated with RTP-026 displayed similar areas at risk as those treated with the vehicle, however a significant attenuation of tissue necrosis was quantified ~~(~40%~~ (~40 % reduction, Fig. 7B, ~~7~~C). Additionally, RTP-026 treatment reduced plasma troponin I, a marker for cardiac injury (Fig. 7D). No difference was detected in plasma PGE_2 or $PGF_{2\alpha}$ levels amongst the experimental groups (Supplementary Fig. 7).

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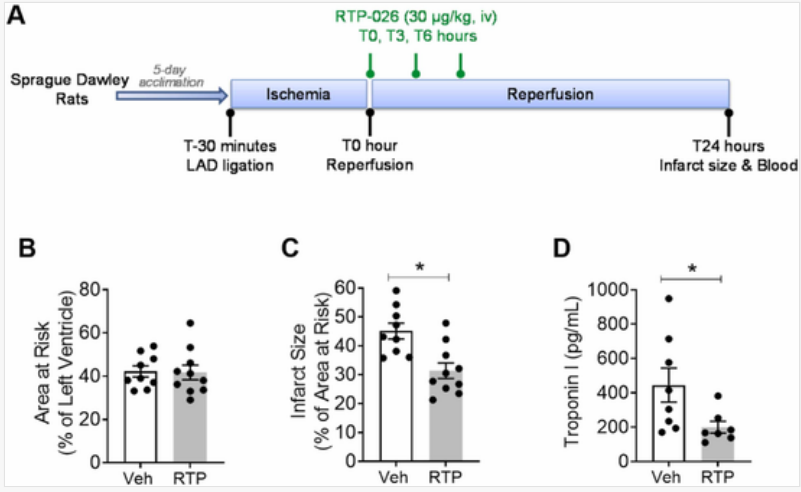
Fig. 7



~~RTP-026 reduces 24-hour post-reperfusion infarct size.~~RTP-026 reduces 24-hour post-reperfusion infarct size. (A) Schematic indicating treatment and experimental regime. Rats were treated i.v. with glycylglycine (vehicle; 0.8 ml/kg) or RTP-026 (30 μg/kg) at reperfusion (time 0), as well as 3 h and 6 h later. Tissues and plasma samples were analysed 24 h after reperfusion. The area at risk (panel B), infarct size (panel C) and plasma Troponin I levels (panel C) were determined as described in Materials and Methods. Data are mean ± SEM of N = 9–10 rats per group. *P < 0.05 ~~vs.~~vs. vehicle group.

4 ~~DISCUSSION~~Discussion

We describe the molecular and whole animal pharmacology of RTP-026 with a focus on acute cardio-protection against I/R injury. These results indicate that RTP-026 is a genuine AnxA1 mimetic with protective effects in settings of heart injury. A series of cellular analyses shows that RTP-026 regulates the influx of neutrophils and classical monocytes into the cardiac tissue, probably secondary to a modulation of their activation status in the circulation. The modulation of neutrophils and monocytes in the blood may be due, at least in part, to an inhibition of the synthesis and/or release of pro-inflammatory mediators. Altogether this study supports the development of RTP-026 as a novel therapy for myocardial I/R injury.

RTP-026 is an AnxA1-derived peptide that spans the whole N-terminal region of the protein. Obtained by solid phase synthesis, this study was undertaken firstly to determine if RTP-026 retained the pharmacology of the peptide produced by cloning, CR-AnxA1~~2–48~~2–48; secondly, to unveil molecular and cellular mechanisms of RTP-026 in an acute myocardial I/R injury model.

The in vitro data produced with HEK293 cells indicate that RTP-026 is a selective human FPR2 activator. In fact, RTP-026 recapitulates the selectivity of the whole protein AnxA1 for human FPR2. In our original studies [9,28], AnxA1 had an apparent affinity around 10–30 nM for the receptor, and RTP-026 apparent EC_{50} was coherent with these concentrations, ranging from 11 to 90 nM in the cell signalling assays. It is difficult to make direct comparisons across studies which made use of different cells [28] or different protocols, for instance using radio-labelled ligand displacement in the original study [9] ~~versus~~versus post-receptor signalling readouts (here). It is important to note that RTP-026 retained high selectivity for FPR2 over FPR1. When tested side to side with the non-selective peptide Ac2–26, RTP-026 was also three- to ten-fold more potent. This higher potency can be explained with its higher number of amino acids, 47 in total, against the 25 amino acids of peptide Ac2–26. We propose the longer sequence of the N-terminal domain implicates that RTP-026 may acquire or maintain the active conformation that interacts with the active pocket of human FPR2.

Studies with mutant receptors where the different domains of human FPR1 and human FPR2 were swapped [29] indicated that AnxA1 whole protein binds to the N-terminal domain of FPR2 (amino acid ~~1–39)~~1–39) as well as to the second extracellular loop (amino acid ~~146–240).~~146–240). The crystal structure of FPR2 has been recently elucidated, reporting specific interaction sites for short molecules like formylated peptides or small molecules [30,31]. Resolution of the crystal structure of CGEN-855A (another FPR2 peptide agonist) bound to the receptor, residues R^{201} and R^{205} formed a hydrogen bond or sustained a polar interaction, respectively [32]; these two amino acids fall into the sequence ~~146–240~~146–240 of FPR2 that is required for AnxA1-mediated receptor activation [29]. We speculate that due to its length, RTP-026 may interact with both receptor domains engaged by AnxA1; this underpins receptor selectivity and downstream pharmacology.

Pro-resolving mediators promote specific actions which favour the timely resolution of acute inflammation, while controlling it also in space to avoid over-spilling and secondary damage [33]. AnxA1 is a protein endowed with all the canonical pro-resolving properties, these being i) reduction in neutrophil recruitment; ii) promotion of non-phlogistic recruitment of monocytes; iii) reduction of neutrophil life span and induction of apoptosis; iv) promotion of efferocytosis; v) augmentation of bacteria phagocytosis and vi) switch in target cell phenotype (e.g. M2-like macrophages) [34]. For RTP-026 to be considered a genuine AnxA1 mimetic, it was crucial to retain at least some of these pro-resolving actions. Like the parent protein, RTP-26 potentiated phagocytosis of bacteria and impacted on the lifespan of polymorphonuclear leukocytes, promoting apoptosis. These effects are likely to be secondary to FPR2 activation because i) they occurred at concentrations in line with those estimated in the experiments of signalling and, more functionally, ii) the FPR2 antagonist WRW_4 abrogated the activating effects of RTP-026 on cell phagocytosis and apoptosis.

When tested in the I/R injury in the rat, RTP-026 was most active at the dose of ~~50~~50 µg/kg, resulting in a reduction in the death of myocardium that was slightly more effective than ~~1~~1 mg/kg peptide ~~Ac2-26.~~Ac2–26. On molar bases, this indicates more than 40-fold increase in potency for RTP-026. This difference in potency emerged also from the molecular pharmacology analyses, with higher affinity for human FPR2 as well as selectivity for FPR2 over FPR1. The cardio-protection afforded by RTP-026 was not secondary to changes in blood pressure and heart rate, indicating a direct effect on the heart. Studies conducted with AnxA1, peptide ~~Ac2-26~~Ac2–26 and, more recently, FPR2 agonists, as reviewed in the Introduction, support this possibility.

Patients undergoing heart attack receive a mixture of therapeutic approaches including antithrombins, aspirin, thienopyridines and glycoprotein IIb/IIIa inhibitors. Nonetheless, is somehow surprising that there is no current therapy that targets the inflammatory response typical of reperfusion injury. The role of inflammation in cardiovascular pathologies is well demonstrated in preclinical settings [35], with recent trials revealing its importance also in man. A ground-breaking proof-of-concept, randomised, placebo-controlled clinical study, the CANTOS study published in 2017 [36], tested the effect of an anti-IL-1β antibody (Canakinumab) to demonstrate therapeutic efficacy in patients with prior myocardial infarction by reducing cardiovascular mortality and non-fatal stroke. Importantly, Canakinumab in combination with traditional statin treatment, decreased the number of adverse cardiovascular events independent of lipid lowering effect, with greatest efficacy in patients with highest inflammatory state [36]. Unfortunately, anti-IL-1β therapy also led to broad immunosuppression with an increase in fatal infections, which is not tolerated in these patients who are typically elderly, frail and suffering from a range of co-morbidities.

Resolution pharmacology postulates that pro-resolving therapies will not be burdened by immuno-suppression. This was observed here in the context of the acute inflammatory response to I/R, with RTP-026 reducing yet not abrogating recruitment to the heart of immune cells like neutrophils and classical (inflammatory) monocytes. Preclinical studies emphasize the importance of modulating immune cell recruitment, rather than complete abolition. For instance, total removal of neutrophils is detrimental in settings of myocardial infarction induced by permanent ligation of the coronary

artery, in part consequent to a reduced recruitment of classical monocytes to the damaged heart and downstream lack of macrophage polarization towards a reparative phenotype [37]. In these settings of chronic heart injury, Soehnlein and colleagues reported the protective effect of human recombinant AnxA1 through polarization of macrophages towards a pro-angiogenic phenotype being able to release VEGF-A [11], which would form new blood vessels to compensate for hypoxia and ischemia. Of relevance, in rat models, serum VEFG-A levels are positively linked to increased microvessel density in the infarcted area, indicating its role in cardiac remodelling and angiogenesis [38]. In our experiments of acute heart I/R injury, RTP-026 inhibited neutrophil recruitment to the infarcted heart by ~90% ~90 % but the recruitment of classical monocytes was only affected by ~50%. ~50 %. Most recent data with small molecule FPR2 agonists [17], point again to the ability of promoting a reparative macrophage within the myocardium as the cellular endpoint to reduce organ damage and long-lasting functional defects. A hypothetical mechanism could be that RTP-026 avoids excess recruitment of white blood cells to the infarcted heart, allowing a sufficient number of neutrophils and, predominantly, classical monocytes to then incite polarization of the macrophage, the latter being resident as well as differentiated from the recruited classical monocytes. Within the time frame of our experimental protocol, we could not monitor macrophage numbers and phenotypes, but this could be the aim of future studies where mechanisms are followed up to day 7 or day 14 post-infarction. Relevantly, these cellular paths have been described following administration of AnxA1 in models of muscle injury [39] and joint arthritis [40], both of them characterised by proper resolution and tissue repair.

RTP-026 may affect the recruitment of white blood cells to the heart in multiple ways, by impacting on cardiac specific processes of inflammation onset, and/or by affecting the inflammatory response in the vasculature. As the compound was administered intravenously, we addressed the second option. In line with its modulatory properties and as such distinct from classical anti-inflammatory therapies which come with immuno-suppression (e.g. CANTOS study discussed above), RTP-026 inhibited neutrophil and monocyte reactivity with an attenuation of the cell surface changes in CD62L and CD54. CD62L (L-selectin)(L-selectin) is an adhesion molecule rapidly released through the actions of a protease, termed sheddase [41]: the mechanism is highly sensitive so that CD62L shedding is one of the first responses observed in activated neutrophils. It was interesting to note that even in sham rats, circulating neutrophils had lost CD62L from their surface to a degree, a response susceptible to the inhibitory action of RTP-026. As predicted, the shedding was more marked post-I/R, yet even in these more extreme conditions, RTP-026 significantly attenuated this response. It should be noted that CD62L is important for neutrophil and monocyte rolling, a pre-requisite to cell adhesion and extravasation [42,43]. Therefore, there is an apparent contrast between the ex-vivo data on immune cells in the heart and the CD62L analyses in circulating cells, as preservation of CD62L by the compound should lead to a better recruitment post-I/R [44,45]. Two further points; i) other adhesion molecules can sustain immune cell extravasation [46,47], and ii) the association between preservation of CD62L on circulating neutrophil and cardio-protection after I/R has been described for other pharmacological agents [48]. In any case, we reason important the different nature of the assays and the actual information they provided: we used CD62L as a marker for RTP-026 ability to modulate the activation of circulating neutrophils and monocytes; this effect was mirrored, in part, by the modulation of CD54 cell surface levels, and was somewhat selective, because no changes in total CD11b expression were quantified (we did not quantify active conformation CD11b) [49]. Finally, the effects of RTP-026 on immune cell recruitment into the heart, and activation within the vasculature, were not secondary to changes in the number of circulating white blood cells indicating that, at least within the 2-hour timeframe, the bone marrow is an unlikely target for the compound.

The actions of RTP-026 treatment of rat white blood cell activation were paralleled by a modulation of inflammatory mediators. The effect of the compound was selective with only three protein mediators been reduced: TNF-α, IL-1β and KC (or CXCL1). The latter two were the most affected with >70% ≥70 % reduction on net values. Similar observations were made for pro-inflammatory lipid mediators with significant reduction for PGE_2 and $PGF_{2\alpha}$. We note that with the exception of IL-1β, all these mediators of inflammation are produced by immune cells and act onto immune cells. KC is produced by immune cells as well as endothelial cells and is a potent chemoattractant for neutrophils [50]. A crosstalk between TNF-α and KC has been reported with the chemokine targeting mast cells and macrophages to set up the inflammatory response post-immunization and challenge with methylated bovine serum albumin [51]. As far as it concerns PGE_2 applying a protocol of rat myocardial I/R injury similar to ours, an antagonist to the PGE_2 type 3 receptor (EP3) afforded significant cardio-protection [52]. The EP3 antagonist had to be infused throughout the reperfusion period, whereas in our experiments, RTP-026 was effective as a single dosage at reperfusion in the 2-hour protocol. Thisese favourable pharmacokineticsencouraging pharmacological effects justified the testing of RTP-026 in a 24-hour I/R protocol, with administration stopping at 6-hour post-reperfusion. Again, the peptide was effective in reducing cardiac tissue death to a degree that was comparable to its modulation of the 2-hour reperfusion injury. We speculate that the mechanisms discussed here may be relevant to the protection afforded by RTP-026 at 2424 h reperfusion and, possibly, even at longer time points post-injury. This would not be surprising for a pro-resolving agonist, as discussed recently [53].

4.1 Limitations of the study

CR-AnxA1~~2-48~~2–48 was developed to be resistant to proteinase by replacing Valine with Leucine in position 24 and by removing the two C-terminal Alanines, in position 49 and 50 [21]. However, the property of resistance to proteinase has not been tested in RTP026, even though it has the same amino acid sequence as CR-AnxA1~~2-48~~2–48. Therefore, further study is warranted.

Additionally, the 2-hour and 24-hour reperfusion models were conducted with different ischemic times in different rat strains to obtain the most optimal cardiac damage while staying within the authorized severity limit (less than a ~~10%~~ 10 % mortality rate) for each model. We have observed differences in infarct sizes in these two models conducted by two independent research centres. This difference could be attributed to 1) variability in surgical procedures; 2) occlusion of LAD coronary artery at different levels as reflected by some differences in the AAR; 3) rat strain differences in susceptibility to cardiac damage [54]. However, it is essential to note that the rats used in each research centre were comparable among their own groups undergoing the same surgical procedures. It is noteworthy that despite conducting these two I/R models in two independent research centres with different rat strains, the therapeutic effects of RTP026 were consistently robust against myocardial I/R injury, which has significantly enhanced confidence in its effectiveness.

5 ~~CONCLUSION~~Conclusion

We describe here the molecular and in vivo pharmacology of RTP-026 and provide evidence for mechanism behind the cardio-protection against I/R injury. Further development of this compound will enable the exploitation of the large wealth of information on the pro-resolving protein AnxA1, possibly taking us a step closer to resolution pharmacology, that is the development of new medicines which can incite reparative and regulatory processes within the patient, offering a fresh and novel approach to the clinical management of complex diseases, including myocardial I/R injury, without causing immuno-suppression.

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CRediT authorship contribution statement

Jianmin Chen – planned experiments, performed experiments, analysed data, contributed to manuscript preparation. **Silvia Oggero** - planned experiments, performed experiments, analysed data, contributed to manuscript preparation. **Chiara Cecconello** - performed experiments, analysed data, **Jesmond Dalli** - performed experiments, analysed data, contributed to manuscript preparation. **Hedayatullah Hayat** - performed experiments, analysed data. **Ahmad Hjiej Andaloussi** - analysed data**. Samra Sanni** – analysed data, contributed to manuscript preparation. **Thomas ~~NE~~ EN Jonassen –** planned project, contributed to manuscript preparation. **Mauro Perretti** – planned project, wrote the ~~manuscript~~manuscript.

Declaration of Competing Interest

Mauro Perretti declares to be a shareholder of Antibe Therapeutics and ResoTher Pharma A/S ~~ApS~~ and a director of William Harvey Research Limited; a scientific advisory board member for Antibe Therapeutics and SynAct Pharma Aps. Mauro Perretti and Jesmond Dalli are a co-inventor on a patent related to AnxA1 pro-resolving peptides (European Patent 3533457 B1). Thomas ~~NE~~ EN Jonassen is co-founder and shareholder of ResoTher Pharma A/S [Instruction: This should be A/S instead of Aps.]~~Aps~~. Samra Sanni holds managerial position at ResoTher Pharma[Instruction: This should be A/S instead of Aps.] A/S ~~Aps~~. ResoTher Pharma A/S ~~Ap~~s owns intellectual property rights on RTP-026.

~~ACKNOWLEDGEMENTS~~Acknowledgement

~~N/A~~N/A. ~~conflict of interestMP declares to be a shareholder of Antibe Therapeutics and ResoTher Pharma ApS and a director of William Harvey Research Limited; a scientific advisory board member for Antibe Therapeutics and SynAct Pharma Aps. MP and JD are a co-inventors on a patent related to AnxA1 pro-resolving peptides (European Patent 3533457 B1). TNEJ is co-founder and shareholder of ResoTher Pharma Aps. SS holds managerial position at ResoTher Pharma Aps. ResoTher Pharma Aps owns intellectual property rights on RTP-026.~~

Appendix A Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2023.107005.

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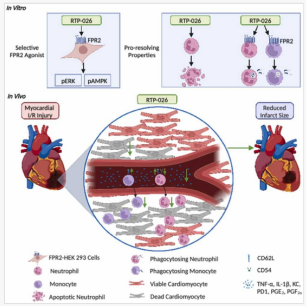
Graphical ~~abstract~~Abstract

Images may appear blurred during proofing as they have been optimized for fast web viewing. A high quality version will be used in the final publication. Click on the image to view the original version.

alt-text: ga1



~~RTP-026 promotes acute cardio-protection through formyl peptide receptor type 2 modulation of immune cell activation.~~RTP-026 promotes acute cardio-protection through formyl peptide receptor type 2 modulation of immune cell activation. In vitro, RTP-026 selectively activates FPR2 indicated by intracellular accumulation of phospho-ERK and phospho-AMPK in FPR2-HEK-293 cells.

RTP-026 possesses pro-resolving properties indicated by its ability to 1) counteract extension of neutrophil life-span induced by serum amyloid A; 2) augment phagocytosis of fluorescent *E.coli* by both human and rat monocytes and neutrophils. In vivo, myocardial ischaemia/reperfusion (I/R) injury is induced by occlusion of the rat left anterior descending coronary artery for 20 min followed by 2-hour reperfusion. In the blood, RTP-026 reduces the levels of soluble mediators, namely TNF-a, IL-1b, KC, protectin D1 (PD1), prostaglandin E_2 (PGE_2) and $PGF_{2a.}$ As a consequence, it reduces activation of neutrophils and monocytes with increased CD62L and decreased CD54 expression. This leads to reduced recruitment of neutrophils and classical monocytes to the damaged heart, which eventually contributes to reduced infarct size.

~~HIGHLIGHTS~~Highlights

- Peptide RTP-026 obtained by solid phase synthesis retains pro-resolving ~~properties~~properties.
- RTP-026 displays pro-apoptotic effects on human ~~neutrophils~~neutrophils.
- RTP-026 augments phagocytosis of *E.coli* by human myeloid cells.
- RTP-026 reduces infarct size in preclinical models of myocardial ~~ischemia/reperfusion~~ischemia/reperfusion.
- RTP-026 tempers immune cell activation in circulation and recruitment to the ~~heart~~heart.

Appendix A Supplementary material

Multimedia Component 1

Supplementary Figure 1

Supplementary material

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Queries and Answers

Q1

Query: Please review the **given names** (no colouring) **and surnames** (highlighted in teal colouring) to make sure that we have identified them correctly and that they are presented in the desired order. Carefully verify the spelling of all authors' names as well. If changes are needed, please provide the edits in the author section.

Answer: Reviewed

Q2

Query: Your article is being processed as a regular item to be included in a **regular issue**. Please confirm if this is correct or if your article should be published in a special issue using the responses below.

Answer: Regular issue

Q3

Query: Correctly acknowledging the primary funders and grant IDs of your research is important to ensure compliance with funder policies. Please make sure that funders are mentioned accordingly. After you have finalized your review (by accepting the current state or providing necessary changes), please click 'Reviewed' below to resolve the query.

Answer: OK