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Original article

Pectins from various sources inhibit galectin-3-related cardiac fibrosis

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S U M M A R Y

Purpose of the study: A major challenge in cardiology remains in finding a therapy for cardiac fibrosis. Inhibition of galectin-3 with pectins attenuates fibrosis in animal models of heart failure. The purpose of this study is to identify pectins with the strongest galectin-3 inhibitory capacity. We evaluated the in vitro inhibitory capacity, identified potent pectins, and tested if this potency could be validated in a mouse model of myocardial fibrosis.
Methods: Various pectin fractions were screened in vitro. Modified rhubarb pectin (EMRP) was identified as the most potent inhibitor of galectin-3 and compared to the well-known modified citrus pectin (MCP). Our findings were validated in a mouse model of myocardial fibrosis, which was induced by angiotensin II (Ang II) infusion.
Results: Ang II infusion was associated with a 4−5-fold increase in fibrosis signal in the tissue of the left ventricle, compared to the control group (0•22±0•10 to 1•08±0•53%; $P < 0•001$). After treatment with rhubarb pectin, fibrosis was reduced by 57% vs. Ang II alone while this reduction was 30% with the well-known MCP (P = NS, $P < 0•05$). Treatment was associated with a reduced cardiac inflammatory response and preserved cardiac function.
Conclusion: The galectin-3 inhibitor natural rhubarb pectin has a superior inhibitory capacity over established pectins, substantially attenuates cardiac fibrosis, and preserves cardiac function in vivo. Bioactive pectins are natural sources of galectin-3 inhibitors and may be helpful in the prevention of heart failure or other diseases characterized by fibrosis.
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Introduction

Heart failure (HF) remains a global health burden, patients with HF suffer from their morbidity and have a poor prognosis [1]. The current challenge in heart failure treatment is to develop specific treatments for cardiac fibrosis [2].

Myocardial fibrosis is one of the most prominent adverse pathophysiological processes in heart failure [3,4], and is associated with systolic and diastolic dysfunction, arrhythmia and premature death [5]. Some contemporary agents such as the mineralocorticoid receptor antagonist (MRA) eplerenone reduce myocardial fibrosis formation by inhibition of the transforming growth factor beta (TGF-β1) pathway. Also, other renin-angiotensin-aldosterone system (RAAS) inhibitors and beta-blockers have an effect on fibrosis formation [6,7]. However, these effects are limited, and currently no treatment is available that specifically targets cardiac fibrosis. As a result, myocardial fibrosis is a prominent feature of progressive HF and a sign of poor prognosis [4,8,9].

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Research in Context

Evidence before this study

We performed a PubMed search using the terms “galectin-3 inhibitors”, “galectina-3 AND heart failure”, and found articles published between 1994 and 2020. Although several research papers and reviews about galectin-3 and its potential role as an onco-target exist, there is still a lack of published studies concerning galectin-3 inhibitors in the field of cardiology. In the heart, galectin-3 is a relevant factor in the development of cardiac fibrosis. Decreasing cardiac fibrosis in a heart failure model was shown to be possible in galectin-3 knock-out mice. There have been several attempts to find a potent galectin-3 inhibitor of cardiac fibrosis in vitro and in vivo, but so far, there is still a lack of high potency agents in the field of cardiology.

Added value of this study: Our translational study shows the advantage of our high-affinity galectin-3 inhibitors in vitro; and in vivo, one inhibitor was able to remarkably reduce cardiac fibrosis.

Implication of all available evidence: Future studies are needed before galectin-3 inhibitors can be applied in humans to prevent heart failure by specifically inhibiting the development of cardiac fibrosis.

In recent years, galectin-3 has evolved as an important mediator of cardiac fibrosis and a modifiable factor in HF. The physiology and pathophysiology of galectin-3 and its role as a biotarget has recently been reviewed into detail [10,11]. Specifically, the extracellular functions of galectin-3 are causally involved in cardiac fibrosis. For example, galectin-3 knockout mice are completely resilient to established pro-fibrotic perturbations, cardiac and vascular remodeling, and atherosclerosis is almost completely absent in these mice [12–14]. Therefore, it has been hypothesized that galectin-3 plays a central role in cardiac remodeling and elevated levels are associated with poor prognosis in HF [15], predict near-term HF rehospitalization [16], and predict decline in left ventricular ejection fraction after myocardial infarction [17]. In the general population, galectin-3 predicts new-onset of HF and mortality [18,19]. Galectin-3 is also involved in other organs, such as the kidney, liver and lungs, and inhibition of galectin-3 led to reduced fibrosis or damage in their tissues [20].

Attempts have been made to design galectin-3 targeted therapy. Specifically, synthetic galectin-3 inhibitors like GB0139 (formerly TD139) have been developed and this inhibitor has been shown to attenuate liver injury and progression of pulmonary fibrosis [21,22]. Currently, GB0139 is undergoing a Phase IIb clinical trial in patients with idiopathic pulmonary fibrosis [23]. Also, heparin-derived inhibitors of galectin-3 are currently being studied in cancer and other disease settings in vitro and in vivo [24]. More classical inhibitors are polysaccharides, which are recognized by the carbohydrate recognition domain (CRD) of galectin-3 [25]. N-acetyllactosamine is an example of a carbohydrate with a high affinity for the galectin-3 CRD, and has shown to prevent left ventricular (LV) dysfunction in heart failure prone rats and in mice [12].

Furthermore, various types of pectins have shown to modulate the activity of galectin-3 as demonstrated in kidney injury, vascular fibrosis and in cancer [13,26,27]. Pectins are considered the most complex class of polysaccharides from plant origin and are reported to consist of different distinct structural elements [28]. The main elements of pectins are partly methyl esterified homogalacturonans consisting of long galacturonic acid sequences and rhamnogalacturonan I (RG-I), in which the backbone of alternating rhamnose- galacturonic acid dimers is substituted with side chains of arabinose and/or galactose [28,29]. Modified citrus pectin (MCP) is a specific pectin fraction that has been extensively studied as a ligand of galectin-3. MCP originates from an alkali-treated commercial citrus pectin, but the affinity of MCP for galectin-3 is low [30,31]. Therefore, our aim was to identify bioactive pectins with strong galectin-3 inhibiting properties, using an in vitro screening. We hypothesized that these modified pectins would exert stronger in vivo inhibition in a murine model of myocardial fibrosis and cardiac dysfunction.

Methods

Screening of pectins

Different complex pectins from fruits and vegetables were screened for galectin-3 inhibitory properties in two different in vitro assays: a chemotaxis assay and an enzyme-linked lectin assay. In the first screening round, we used pectins that were enzymatically modified by means of a technical pectinase preparation (Rapidase C600; DSM Food Specialties, Delft, the Netherlands) and available from previous studies to obtain enzymatically modified pectin (Supplemental figure 1; step 1 (as visualized for rhubarb)) [32]. In the next step, pectins were treated with a second enzyme to try to optimize effectivity: active pectin preparations were further tested by an experimental pectinase preparation from Aspergillus niger to obtain enzymatically further modified pectin (Supplemental figure 1; step 2) [33]. For the in vivo testing of Rhubarb pectin (EMRP), fresh rhubarb was obtained from the local market and treated with the pectinase preparation Rapidase C600 according to the protocol of Schols and Voragen [32]. EMRP was further purified using a centrifugal ultrafiltration unit having a 5000 Da cut-off membrane. Orange pectin was isolated following the same procedure using the pectinase preparation from Aspergillus niger [33]. MCP was also tested as this pectin already has been proven to be an effective galectin-3 inhibitor. Commercial sugar beet pectin and lemon pectin were obtained from CP Kelco (Lille Skensved, Denmark). Pectins were characterised for sugar composition using methods as described before [32].

Galectin-3 chemotaxis assay

Monocytes were isolated from heparinized buffy coat (Sanquin, Groningen, the Netherlands). First, the buffy coat was mixed 1:1 with phosphate-buffered saline (PBS), with 1 mM EDTA (pH 7•2). 25 mL of this mixture was brought onto 12•5 mL of Ficoll (1•073 g/mL; GE Healthcare Life Sciences, Eindhoven, the Netherlands). This was centrifuged during 30 min at 400 g at room temperature. The peripheral blood mononuclear cells (PBMCs) were collected with a Pasteur pipette. PBMCs were washed 3 times with PBS and centrifuged at 1200 rpm/ 177 g force (RCF) during 10 min at room temperature. The PBS was removed and the pellet was resuspended in 10 mL of RPMI 1640 medium with 25 mM HEPES and 2 mM L-Glutamine (Lonza, Switzerland), 10% fetal calf serum (FCS) and penicillin-streptomycin (100 IU/mL and 100 μg/mL); (Invitrogen, Breda, the Netherlands). Percoll solution was prepared with 9•25 mL 46% Percoll (GE Healthcare Life Sciences, Eindhoven, the Netherlands), 0•75 mL PBS and 54 mL of RPMI 1640 medium. The PBMC cells were counted and were diluted with medium to a concentration of $1–2 \times 10^6$ and were carefully loaded onto the Percoll layer in a 7•5 mL tube and centrifuged at 550 g during 30 min at room temperature. The monocytes formed a band in the gradient and were collected with a Pasteur pipette and diluted in 35 mL ice-cold PBS. This cell suspension was centrifuged at 280 g for 8 min at 4 °C, cells were resuspended with 35 mL of ice-cold PBS, centrifuged again and dissolved in 5 mL medium. Afterwards, counting cells were diluted in medium to 1×10^6 cells/mL.

For the chemotaxis assay, 96-wells, 3•2 mm diameter sites, 30-μL chemotaxis plates were used (Neuroprobe, USA). Galectin-3 was dissolved in medium at a concentration of 2 μM and was mixed with pectin (1–10 mg/mL), lactose (50mM-250 mM) or medium. This mixture was incubated for 30 min and gently shaken. 29 μL of this

suspension was inserted into the bottom well. The membrane was put onto the frame and 25 μL of the monocyte cell suspension was loaded on top of each well. The cover was put over the plate, and the cell migration plate was incubated for 4 h at 37 °C.

After incubation, the cell suspension on top of the filter was discarded and the filter was detached from the plate. Cotton buds were used to wipe non-migrated cells from the top of the filter. The top of the filter was very gently rinsed with PBS to remove any remaining cells. The filters were transferred to a shallow dish and fixated in 1% formalin for 10 min. The filter was gently rinsed with PBS and the membrane was cut out and put on a coverslip. Cells were mounted using Vectashield mounting medium with 4′, 6-diamino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, USA). Slides were analyzed with a fluorescence microscope (TissueGnostics TissueFAXS, Vienna, Austria) and images were analyzed (TissueQuest) to quantify the number of migrated cells.

Enzyme-linked lectin assay (ELLA)

Galectin-3, dissolved in PBS, was fluorescently labeled using the DyLight 594 kit (Thermo Scientific, Waltham, USA). 5 μg of laminin-1 (R&D systems, Minneapolis, USA), dissolved in 100 μL PBS was added to each well in a black, 96-wells plate and was incubated overnight at 4 °C. After incubation, laminin-1 was removed, and the plate was washed with PBS. After removal of PBS, the wells were blocked with 200 μL of 2% bovine serum albumin (BSA) for 1 h at room temperature, while gently shaking. After 1 h, the BSA was removed and the wells were washed twice with PBS. After removal of PBS, fluorescent labeled galectin-3 at a concentration of 1 μg/μL was diluted 10x with PBS and from this mixture, 50 μL was added to an Eppendorf tube. 50 μL of (0•1−1•0 μg/μL) of pectin or 50 μL of PBS was added to the tube and incubated for 30 min. After incubation, this 100 μL was put in each well of the 96-wells plate and incubated overnight at 4 °C. The solution was removed, and each well was washed 2x with PBS. Absorbance at 280 nm and 595 nm was measured in the multimode microplate reader (Biotek Synergy H4, Winooski, USA) to determine the amount of bound fluorescent galectin-3.

At least 3 replications were performed for the in vitro experiments.

In vivo experiments

Animals

8−10 weeks old, male C57Bl/6J mice were obtained from Harlan (Horst, the Netherlands). All animals were drug and test naïve and were housed in the Central Animal Facility of the University Medical Center Groningen, which is a specific pathogen free (SPF) facility. All animals were single caged and had bedding material. The animal facility has a light-dark cycle of 12 h-12 h in a temperature-controlled environment. Animals had ad libitum access to chow and water, with or without addition of pectins. A power analyses was performed based on an estimated effect size of 15% of cardiac fibrosis as primary endpoint, a variation coefficient of 10%, an alpha of 0•05 and an expected drop out of 3 animals per group. 6 groups of 14 animals were requested. All experimental procedures were performed in accordance with the European Union guidelines for the care and use of animals. This animal experiment was approved by the Animal Ethical Committee of the University of Groningen (Groningen, the Netherlands), and approved as DEC 6661A.

Study design

Myocardial fibrosis was provoked by subcutaneous infusion of angiotensin II, which is an established model [34,35]. The control group received saline infusion via an osmotic minipump for 14 days. In these 2 groups, 3 types of diet were supplied: control, MCP treatment and enzymatically-modified rhubarb (EMRP) treatment. In total, 6 groups were studied: Ang II / control (n = 14), Ang II / MCP (n = 14), Ang II / EMRP (n = 14), Saline / control (n = 14), Saline / MCP (n = 14) and Saline / EMRP (n = 14). Pectins were dosed as 1% in drinking water, ad libitum. Cardiac function was determined with echocardiography and invasive hemodynamic measurements. Left ventricular (LV) tissues were used to perform immunohistochemical, expressional and biochemical analyses. Two independent replications of the experiment, with two weeks in between (n = 7/ group per replication) were performed (Supplemental figure 2).

Experimental procedures

Mice were subjected with either saline or Ang II (2•5 mg/kg/day) for 14 days. Ang II was dissolved in 0•9% NaCl and injected in a small osmotic pump (Alzet, Palo Alto, CA, USA, model 2004). Mice were anesthetized by inhalation of 2% isoflurane in O2. A small incision was made at the right flank wherein a subcutaneous pocket was created for pump implantation. The implantation of the osmotic pumps was performed by a single technician in order to minimize variation and all mice received a single dose of 3•0 mg/kg flunixin-meglumin to alleviate pain after the surgery. Animals were sacrificed by heart puncture under anesthesia and injection of 0•9% NaCl into the circulatory system via the apex of the heart. The experiments were conducted between 8am to 4pm. The experiments were performed in the Central Animal Facility of the University Medical Center Groningen. An independent technician implanted the osmotic pumps and allocated animals to treatment via block randomization. In the first independent experiment, half of the animals were studied and in the second independent experiment, the other half was done. All analyses were performed in a blinded fashion by a single technician.

Experimental outcomes

Echocardiography

M-mode and 2D transthoracic echocardiography (Vivid 7 equipped with 14-MHz linear array transducer; GE Healthcare, Chalfont St. Giles, UK) was performed to assess cardiac dimensional and functional parameters, one day before sacrifice. A single operator (RAdB), who was blinded for the status of the animals, performed all echocardiographic measurements. Mice were anesthetized with 2% isoflurane in O2 and were placed on a heating pad to obtain body temperature around 37 °C. The chest hair of the mice was removed via application of a topical depilation cream and washed thoroughly afterwards. Parasternal short axis views were obtained to ensure that M-mode recordings were recorded at LV mid-papillary level. M-mode tracings were used from three cine loops, to measure fractional shortening and LV dimensions.

Hemodynamic measurements

Before sacrifice, mice were all subjected to invasive hemodynamic measurements (Millar instruments, Houston, USA), using an indwelling micromanometer-tipped pressure catheter that was introduced in the right carotid artery. A single, independent technician, who was blinded for treatment and diet, performed these measurements. After 5 min of stabilization, arterial pressure (in the ascending aorta) was recorded first. Afterwards, the catheter was advanced into the LV to record intracardiac pressures and heart rate. The catheter was finally removed, and the carotid artery was ligated.

Tissue procurement and immunohistochemistry

After the hemodynamic measurements, the thorax was opened, and the hearts were excised and rinsed in ice-cold PBS. The RV and the atria were dissected from the LV and all compartments were weighed. From the LV, mid-ventricular transverse sections were fixed in 4% paraformaldehyde before paraffin embedding, as described [36]. The remainder of the LV was snap frozen for RNA analysis.

Paraffin-embedded tissue was sliced into 4 μm sections. To measure fibrosis, Masson's trichrome staining was performed. Whole

stained sections were scanned with a high-throughput scanning system at room temperature (Nanozoomer 2•0-HT, Hamamatsu, Japan), and the amount of fibrosis was quantified for an entire section at 20x magnification (ScanScope, Aperio Technologies, Vista, USA), and expressed as the percentage of total area.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from snap frozen biopsies of the left ventricle using TRI reagent (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Quantity and purity of the RNA was determined using a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) at 230, 260 and 280 nm wavelength. For reverse transcription equal amounts of RNA were used for complementary DNA (cDNA) synthesis using the QuantiTect® Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's protocol. cDNA was used for quantification of gene expression by real-time polymerase chain reaction (RT-PCR).

Quantification of mRNA levels were performed (Bio-Rad CFX Manager 2•0) and mRNA levels were expressed as relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture and was normalized to the common reference gene Rplp0, encoding ribosomal phosphoprotein P0 [37]. This housekeeping gene is frequently used, and published regarding experiments performed in our laboratory, as a standard reference gene for cardiac tissue and as a heart failure reference gene [38,39]. Primer sequences that were used for quantitative PCR analyses are displayed in supplemental Table 1.

Statistical methods

All data are presented as means ± standard errors of the mean (SEM). Mice were analyzed in 2 separate subgroups comparing effect of infusion (Ang II vs saline) and effect of treatment (control vs MCP vs EMRP). Statistical analysis among groups was performed using analysis of variance (ANOVA) using Dunnett's post-hoc test (to compare with a control group) if data were normally distributed, or with the Kruskall-Wallis test followed by Mann-Whitney U test in case of skewed distribution. All results were tested at the $P < 0•05$ level of significance. Statistical analyses were performed using GraphPad Prism (La Jolla, USA).

Results

In vitro screening of pectins

Chemotaxis assay

Before the screening of pectins, we confirmed whether monocyte migration was present in the chemotaxis assay, as shown in Fig. 1A. After $n = 9$ rounds of screening, a selection of enzymatically modified pectins were tested in a chemotaxis assay to establish galectin-3 inhibitory properties (Fig. 1B, Supplemental figure 1 (step 1)). The best performing pectins were selected and enzymatically modified to investigate whether further enzymatic modification would result in increased inhibitory properties (Supplemental figure 1 (step 2)). These enzymatically modified pectins were further tested in a chemotaxis assay (Fig. 1C). As a control, lactose and MCP were included, which are established galectin-3 inhibitors. Lactose and MCP showed substantial inhibition of monocyte chemotaxis (23•8% (± 3•4) and 30•1% (± 10•1), all $P < 0•001$ compared to positive control set as 100%; respectively). Surprisingly, in the chemotaxis assay, the various enzyme extracted pectin fractions, representing branched pectin structures, showed substantial inhibition. The Enzymatically-Modified Rhubarb Pectin (EMRP) and Enzymatically Further Modified Rhubarb Pectin (EFMRP) rich in homogalacturonan as well rhamnogalacturonan I parts (supplementary Table 2) showed strong inhibition of galectin-3 induced monocyte migration (15•1% (± 5•4) and 19•8% (± 4•6) respectively, both $P < 0•001$), compared to positive control set as 100%, respectively (Fig. 1C)). Commercial sugar beet pectin (SUBP) and citrus pectin (CP) were hardly active in the assay.

Enzyme-linked lectin assay

To validate our initial observations, our modified pectins were applied in an independent in vitro assay, an enzyme-linked lectin assay (ELLA), in which the direct, physical binding with galectin-3 was studied. After the administration of lactose and MCP to galectin-3, the absorbance of fluorescently labeled galectin-3 was lower, and EMRP and EFMRP show similar effects compared to these established galectin-3 inhibitors ((20•6% (± 6•8) and 23.2% (± 3•2) both $P < 0•001$ compared to positive control, which was set as 100% (Fig. 1D)). Further modification of EMRP by a second enzyme did not result in increased inhibitory capacities.

In vivo validation of pectins in a heart failure model

To validate our findings in vivo, we prepared a larger batch of the pectin with the overall best performer in the in vitro screens, which was EMRP, in an in vivo experiment, taking along the pectin MCP as the "gold standard". After Ang II infusion, a significant increase of systolic pressure (SBP) and diastolic pressure (DBP) was observed (SBP: 101•7 ± 7•6 to 126•5 ± 10•0 mmHg; $P < 0•001$ and 71•0 ± 7•8 to 89•5 ± 6•6 mmHg; $P < 0•001$ respectively). Treatment with MCP or EMRP did not attenuate the elevation in blood pressure (Fig. 2A/B). After Ang II infusion, LV weight (mg, corrected for tibia length (mm), *1000) was elevated (from 6•5 ± 0•3 to 8•2 ± 1•0 mg/mm; $P < 0•001$). Again, treatment with either pectin did not affect this (Fig. 2C). Furthermore, Ang II induced an upregulation of the markers ANP (∼ 4-fold increase; $P < 0•001$), Acta1 (∼ 4-fold increase; $P < 0•001$) (Fig. 2D/E), and also galectin-3/ LGALS3 (∼ 1•6-fold increase; $P < 0•01$)(Fig. 2F).

Cardiac fibrosis is attenuated by EMRP

Masson's trichrome staining was performed to quantify myocardial fibrosis (Fig. 3). Ang II infusion was associated with a 4–5-fold increase in signal in the LV tissue, compared to the saline treated control group (0•22 ± 0•10 to 1•08 ± 0•53%; $P < 0•001$). MCP and EMRP treatment reduced cardiac fibrosis by 30% (P = NS) and 57% ($P < 0•05$), respectively.

Attenuation of fibrosis is accompanied with reduced leukocyte infiltration

To assess the inflammatory response of leukocytes, a CD45 staining was performed (Fig. 4A/B). Ang II infusion caused an increase in CD45+ cells in the LV tissue from 10•7 ± 4•1 to 33•6 ± 16•0 cells/mm^2; $P < 0•001$. Both treatment with MCP as well as EMRP significantly reduced CD45+ cells (−56%; $P < 0•05$ and −57%; $P < 0•05$, respectively). Furthermore, EMRP reduced gene expression of pro-inflammatory genes including Il6, Il10 and Cd68 (−47%; $P < 0•05$, −50%; $P < 0•05$, −17%; $P < 0•05$) (Fig. 4C-E).

EMRP treatment results in improvement of functional parameters

Intracardiac pressures, especially LVESP and LVEDP, were increased after Ang II infusion (from 98•1 ± 10•4 to 115•0 ± 11•8 mmHg; $P < 0•01$ and from 8•9 ± 4•4 to 17•3 ± 6•5 mmHg; $P < 0•01$), respectively, but treatment with pectins did not significantly change these pressures. Functional changes in cardiac function were assessed by echocardiography and invasive hemodynamic measurements (Fig. 5). Ang II induced a reduction of fractional shortening (from 41•0 ± 5•0 to 33•0 ± 4•1%; $P < 0•01$), however, EMRP treatment prevented this impairment in fractional shortening (39•7 ± 4•6; $P < 0•05$ compared to Ang II control).

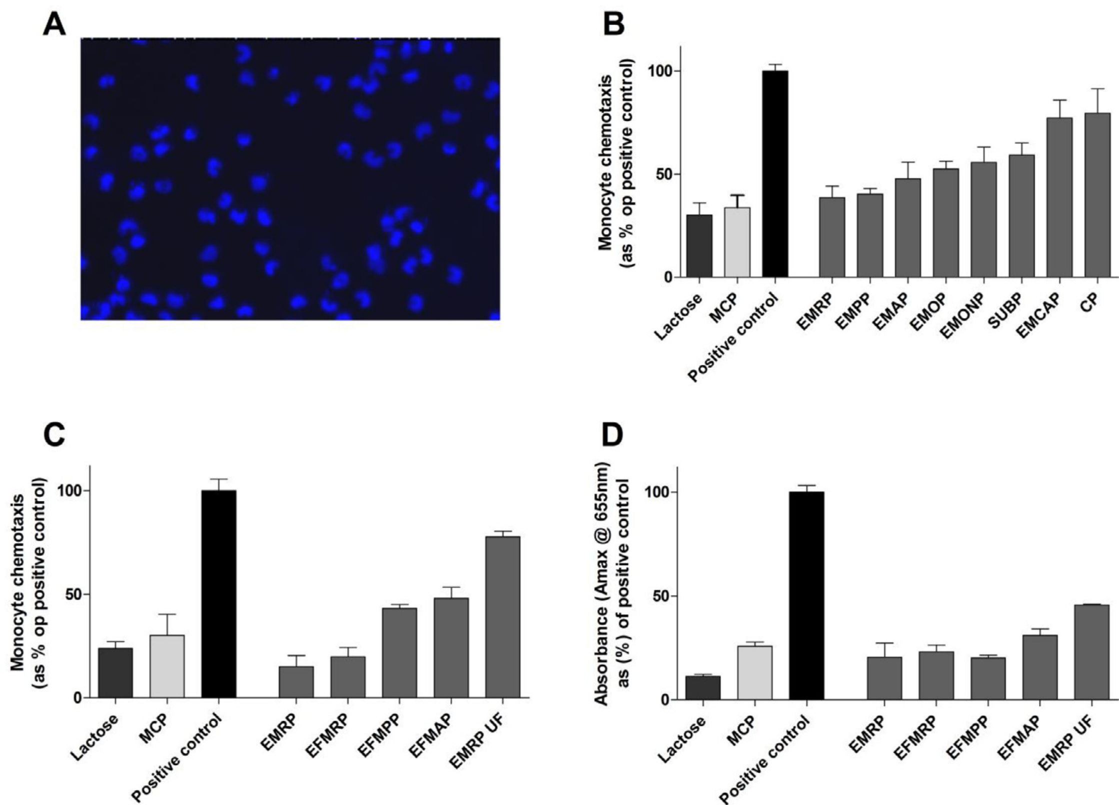

Fig. 1. Overview of screening of pectins in chemotaxis assay and enzyme-linked lectin assay (ELLA) Monocytes were stained with DAPI to count the number of migrated cells (A). Enzymatically modified pectins were screened in a chemotaxis assay (B). Enzymatically further modified pectins were tested in a chemotaxis assay (C) and an enzyme-linked lectin assay (D). Well-known galectin-3 inhibitors, lactose and modified citrus pectin (MCP), were used as control to inhibit galectin-3/LGALS3 activity. *N* = 4. All data are presented as means ± standard errors of the mean (SEM).

Abbreviations: EMRP: Enzymatically modified rhubarb pectin; EMPP: Enzymatically modified pear pectin; EMAP: Enzymatically modified apple pectin; EMOP: Enzymatically modified orange pectin; EMOnP: Enzymatically modified onion pectin; SUBP: Sugar beet pectin; EMCaP: Enzymatically modified cauliflower pectin; CP: Citrus pectin; EFMRP: Enzymatically further modified rhubarb pectin; EFMPP: Enzymatically further modified pear pectin; EFMAP: Enzymatically further modified apple pectin; EMRPUF: Enzymatically modified rhubarb pectin (ultrafiltered).

Discussion

We demonstrate that using two different in vitro assays, it is feasible to identify bioactive pectins from various fruits and vegetables with varying galectin-3 inhibitory properties. Enzyme extracted pectins from various sources like apple, pear and orange representing mainly the highly branched rhamnogalacturonan I segments of pectin were highly active in our bioassays. We identified EMRP, having

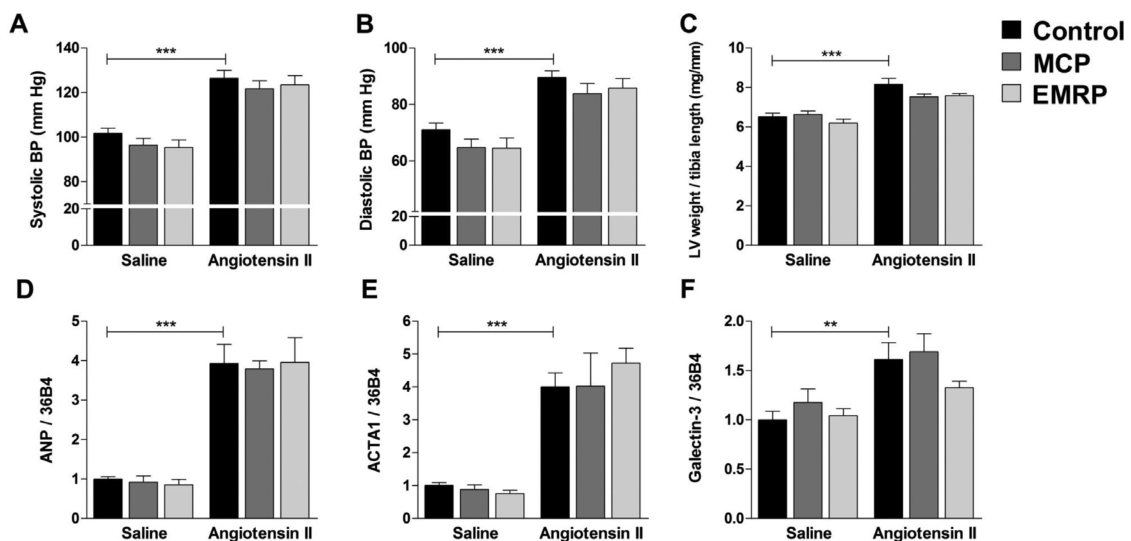

Fig. 2. Validation of the animal model: Angiotensin (Ang) II infusion induces elevated systolic and diastolic blood pressure (A, B), which is accompanied with increased left ventricular (LV) weight (corrected for tibia length) (C) and an increase in cardiac expression of markers of pressure overload (D, E). Also, expression of cardiac galectin-3/LGALS3 was increased after Ang II infusion (F). ***: $P < 0•001$ compared to saline control. **: $P < 0•01$ compared to saline control. *N* = 9−14. All data are presented as means ± standard errors of the mean (SEM).

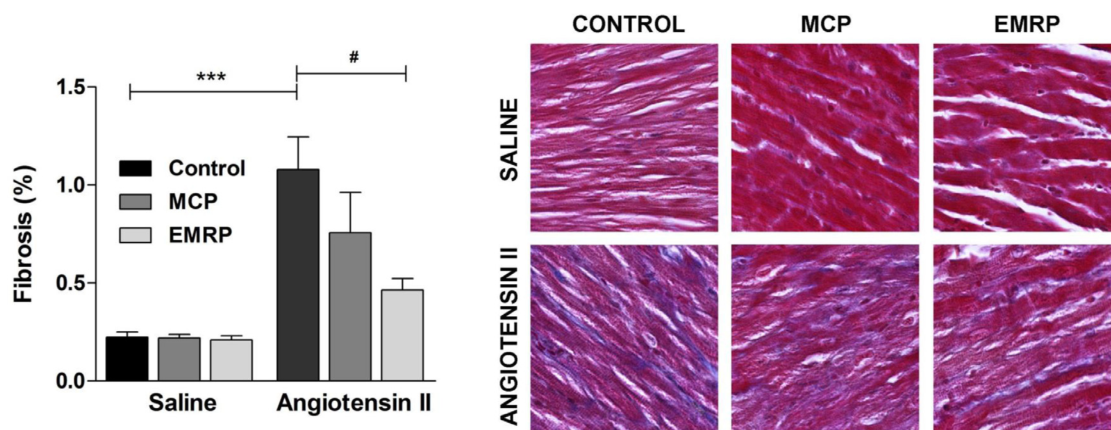

Fig. 3. Treatment with pectins reduces myocardial interstitial fibrosis. Angiotensin (Ang) II infusion induces a 4−5-fold increase in fibrosis deposition in the left ventricle (LV)., measured with masson's trichrome stain. Modified citrus pectin (MCP) did not show a significant inhibition of Ang II-induced fibrosis, but after enzymatically modified rhubarb pectin (EMRP) treatment, cardiac fibrosis was prevented. ***: $P < 0•001$ compared to saline control. #: $P < 0•05$ compared to Ang II control. $N = 9−14$. All data are presented as means ± standard errors of the mean (SEM).

both homogalacturonan and rhamnogalacturonan segments as a potent pectic galectin-3 inhibitor, more potent than the "archetypical" MCP, which is an established pectin galectin-3 inhibitor [26,40−42]. In a murine model of myocardial fibrosis, we showed that EMRP, a pectin with a relatively high galactose content in the rhamnogalacturonan region, was able to prevent Ang II-induced myocardial fibrosis and cardiac dysfunction. The EMRP-derived reduction in cardiac fibrosis was accompanied with a reduction in the inflammatory response. Together, our data suggest that specific pectic fragments from food sources exert inhibitory effects on galectin-3. However, due to the variety of fruits and vegetables from which an active pectin preparation could be isolated, the sugar composition, and consequently the precise structure of the pectins, is quite variable. Given these characteristics, we postulate that the intake of specific pectins or pectic fragments from the pectic rhamnogalacturonan I structural element being decorated with galactose side chains may be associated with certain health benefits via the inhibition of galectin-3.

Biomarkers are primarily used for diagnosis or risk prediction. However, some biomarkers are also biotargets, and may also serve as therapeutic targets [43]. In recent years, also galectin-3 was identified as a modifiable risk factor in HF, which can be targeted by several compounds, especially carbohydrate compounds, including N-acetyllactosamine residues [20,44], which bind to the CRD of galectin-3. Binding results in a conformational change of the galectin-3 molecule and thereby the saccharide ligand acts as an "on/off" switch for the biological function of galectin-3 [45]. Pectins and other galectin-3 ligands may use this switch to modify the activity of galectin-3.

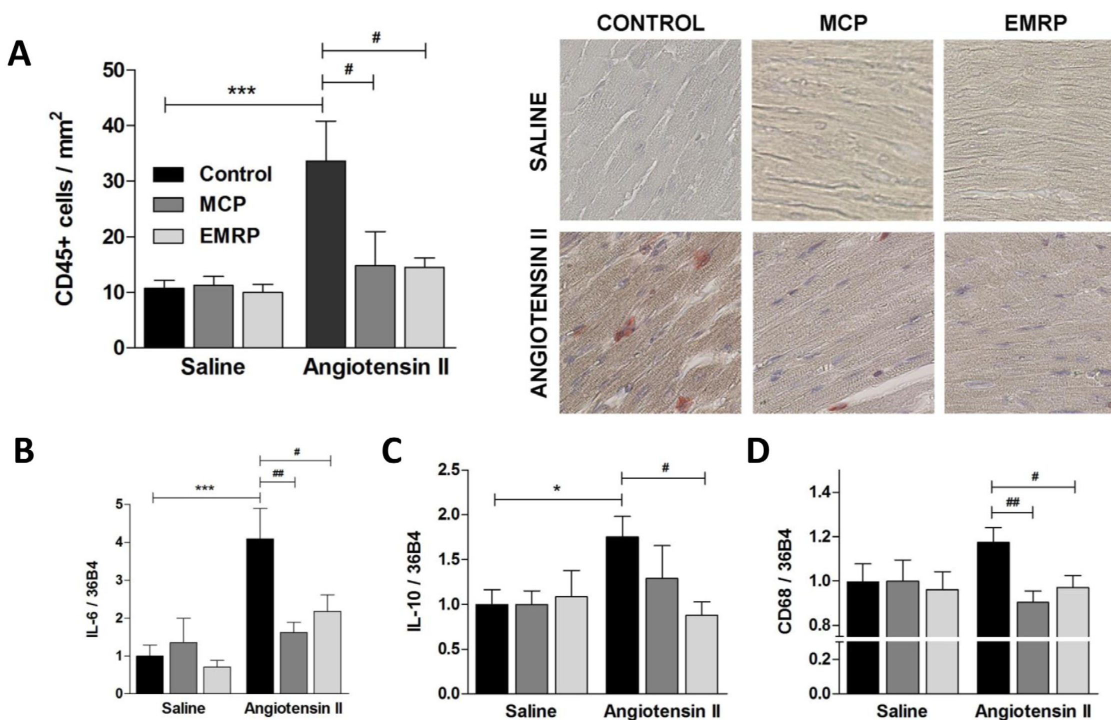

Fig. 4. Pectins reduce the inflammatory response. After Ang II infusion, an increase in CD45+ cells was observed in the LV tissue, measured with the CD45+ stain (A). Both modified citrus pectin (MCP) and enzymatically modified rhubarb pectin (EMRP) attenuated the increase of CD45+ cells. MCP and EMRP also attenuated the expression quantified with RT-qPCR of other inflammatory markers as Il6 (B), Il10 (C) and Cd68 (D) expressed as fold change. ***: $P < 0•001$ compared to saline control, *: $P < 0•05$ compared to saline control, ##: $P < 0•01$ compared to Ang II control, #: $P < 0•05$ compared to Ang II control. $N = 9−14$. All data are presented as means ± standard errors of the mean (SEM).

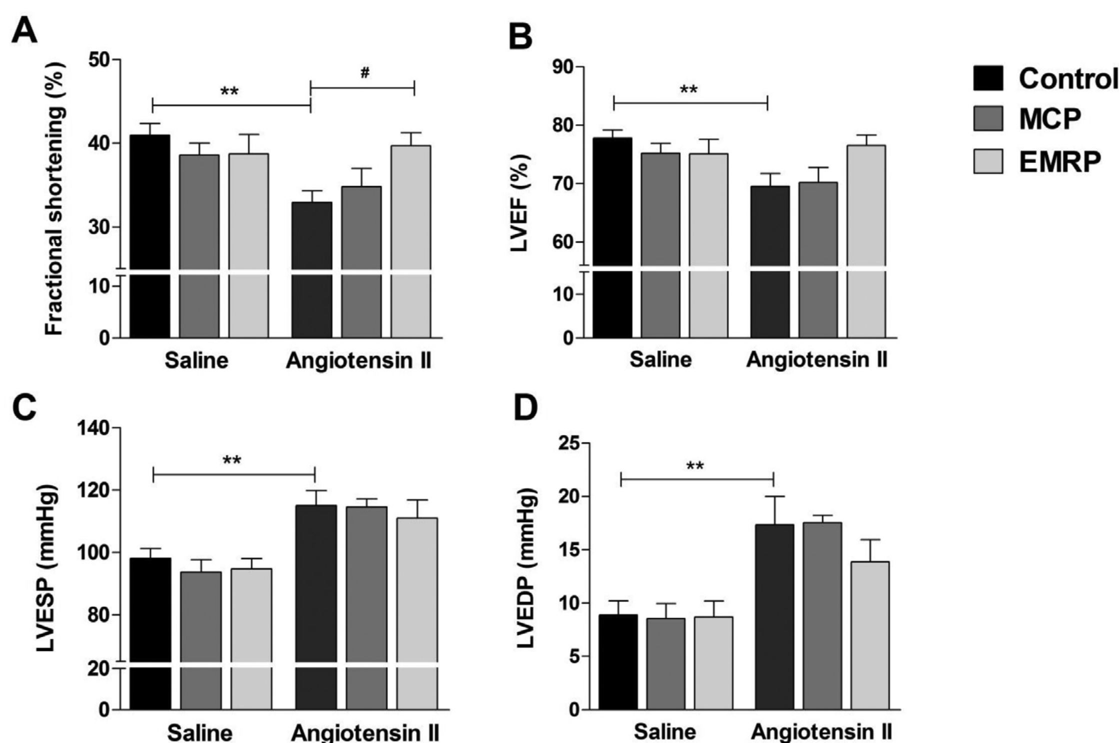

Fig. 5. Treatment with rhubarb pectin improves fractional shortening. Angiotensin (Ang) II infusion reduced fractional shortening (A) and left ventricular ejection fraction (LVEF) (B). Enzymatically modified rhubarb pectin (EMRP) prevented this effect for fractional shortening (A) and borderline (but non-significantly) for LVEF (B). Also, LV end-systolic (C) and end-diastolic pressure (D) was increased after Ang II infusion, but these pressures were not decreased after pectin treatment. **: $P < 0•01$ compared to saline control, #: $P < 0•05$ compared to Ang II control. $N = 9-14$. All data are presented as means ± standard errors of the mean (SEM).

Pectins are complex polysaccharides and structural components of cell walls, consisting of a backbone containing branches which are called "hairy regions" [46]. As a soluble fiber, they have been associated with several cardiovascular health benefits [47,48], also via inhibition of galectin-3 [49].

With our in vitro screening assays, we further explored previously mentioned galectin-3 induced actions [50,51]. MCP is the best studied pectin so far and it has been reported that MCP blocks galectin-3 induced effects, thereby preventing acute kidney injury, acute liver injury and pulmonary fibrosis [13,26,42]. Furthermore, it blocks aldosterone-induced renal and cardiac fibrosis [52]. However, these effects were limited because MCP is a mixture of different molecules, with a relatively low affinity for galectin-3 [30,31]. To identify pectins with higher affinity for galectin-3 we performed an in vitro screening with enzymatically modified pectins. Ultimately, other pectins can inhibit galectin-3 activity, and pectins with superior activity over MCP were identified. Recent studies have shown that subfractions of MCP are more active than original MCP [49]. Furthermore, it was shown that pectic polysaccharides with higher arabinose and galactose content (arabinogalactan) were stronger inhibitors of galectin-3 [30]. MCP originates from high molecular weight citrus pectin, which has been treated at elevated temperatures and a high pH resulting into chemical cleavage of the citrus pectin galacturonan backbone into smaller fragments. Next to MCP, other pectins have also shown their ability to inhibit galectin-3 induced processes [29]. With the use of pectolytic enzymes, we have isolated pectins with a reduced level of homogalacturonan segments by degrading the large pectin molecules into smaller fragments, in order to achieve pectin structures with a higher affinity for galectin-3. The enzymatic treatment of rhubarb pectin resulted in EMRP that indeed demonstrated higher affinity for galectin-3 in our in vitro assays compared to MCP. Further modification with a second enzyme did not result in a further increase of inhibitory properties.

Recent studies using pectic polysaccharides from different food sources showed that the galectin inhibitory capacity is enhanced in pectins with higher galactose and arabinose contents, which indicated that these sub-groups are important in galectin-3 inhibition [30]. Analysis of our tested pectin EMRP showed that this pectin was still rich in galacturonic acid, with rhamnogalacturonan I segments, and relatively rich in galactose, which may explain why this particular pectin shows strong galectin-3 inhibitory capacity (Supplemental Table 2). However, we observe that other pectins with high galactose content perform less well. Presumably, not only does the galactose content play a role, but also the position, length and the precise structure of the galactose side chains within the pectin molecule are important to have the required interaction with galectin-3.

In our in vivo experiments, the pectin with the highest affinity for galectin-3 from our in vitro assays, EMRP, was used in a murine model of heart failure. A model of Ang II infusion was used to resemble hypertensive heart disease, which is quite common, and is accompanied with fibrosis formation. Administration of our pectin prevented cardiac fibrosis and improved fractional shortening. This is in line with recent reports that MCP reduced cardiovascular levels of collagen and transforming and connective growth factors in the heart of obese animals [53] and prevented cardiac inflammation and fibrosis in aldosterone-salt-treated rats [42]. We demonstrate that several pectins, after enzymatic modification into smaller fragments, can prevent cardiac fibrosis and inflammation.

Our combined in vitro and in vivo studies suggest that pectins produce this effect through the direct binding of galectin-3. However, direct binding studies in vivo have not been performed, so an indirect effect cannot be ruled out. Fibers in general have also shown to affect oxidative stress and might also be protective by quenching or deleting free radicals, interchanging ions or counteract the deleterious action of free radicals with antioxidant compounds associated with their polysaccharide matrix [54]. Enzymatic treatment of pectins

creates multiple pectic fragments of different sizes, therefore it is difficult to decide which fragment binds to galectin-3. Consequently, it is challenging to develop an assay that can determine whether pectic fragment have entered the systemic bloodstream and can modify the activity of circulating galectin-3 herein.

Some limitations must be acknowledged. First, we were limited by the pectin library that was to our disposal. Our screen was limited in the number of available pectins, and like any other screen, could have been more comprehensive. Our primary aim was to show the feasibility of this approach. Future experiments should screen larger libraries. Most importantly, we demonstrated that more active pectins and pectin fragments can be identified by this screening method. Secondly, we used a murine model in which fibrosis was induced by Ang II infusion over a relative short time span and therefore, the model does not fully resemble the human situation. However, the process of Ang II induced hypertensive heart disease in mice resembles the pathophysiology of hypertensive heart disease development in humans.

In summary, the present study demonstrates that pectins can be used as galectin-3 inhibitors from natural origin. Altogether, pectins from natural food sources might be regarded as dietary sources with specific therapeutic effects with respect to the prevention of galectin-3 mediated (cardiac) fibrosis, and subsequent organ (heart) dysfunction and failure. We are currently conducting a longitudinal population study (LifeLines, 165,000 subjects, http://www.lifelines.net) with extensive food questionnaires, to gather evidence from the human setting for this hypothesis.

Contributors

CG, RV, HS and RB designed the experiment, HS created several pectins.

RV, MD and WM conducted the experiments and verified the data.

CG and RV have written the manuscript.

PM, MD, WG; HS, RB, and WM critically reviewed the manuscript.

Data sharing

The data generated and analyzed during this study are available from the corresponding author without any restrictions.

Declaration of Competing Interests

The UMCG, which employs several of the authors, has received research grants and/or fees from AstraZeneca, Abbott, Boehringer Ingelheim, Cardior Pharmaceuticals Gmbh, Ionis Pharmaceuticals, Inc., Novo Nordisk, and Roche. Dr. de Boer received speaker fees from Abbott, AstraZeneca, Bayer, Novartis, and Roche.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.retram.2021.103321.

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