The novel phosphate and bile acid sequestrant polymer SAR442357 delays disease progression in a rat model of diabetic nephropathy

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List of abbreviations:

BA and BAs: Bile acid and Bile acids

BAS: Bile acid sequestrants

CV: Cardiovascular

CKD: Chronic kidney disease

DKD: Diabetic kidney disease

ESRD: End-stage renal disease

FXR: nuclear farnesoid X bile acid receptor

GI: Gastrointestinal

PAA: Poly(allylamine)

PDA: Poly(diallylamine)

RAAS: Renin–angiotensin–aldosterone system

SGLT-2: Sodium-dependent glucose cotransporters-2

T2D: Type 2 diabetes

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Abstract

As a gut-restricted non-absorbed therapy, polymeric bile acid sequestrants (BAS) play an important role in managing hyperlipidemia and hyperglycemia. Similarly, non-absorbable sequestrants of dietary phosphate have been used for the management of hyperphosphatemia in end stage renal disease. To evaluate the potential utility of such polymer sequestrants to treat type 2 diabetes (T2D) and its associated renal and cardiovascular complications, we synthesized a novel polymeric sequestrant, SAR442357 possessing optimized bile acid (BA) and phosphate sequestration characteristics. Long-term treatment of T2D obese ZSF1 rats with SAR442357 resulted in enhanced sequestration of BAs and phosphate in the gut, improved glycemic control, lowering plasma cholesterol and triglycerides and attenuated diabetic kidney disease (DKD) progression. In comparison, colesevelam, a BAS with poor phosphate binding property did not prevent DKD progression, while losartan, an angiotensin II receptor blocker that is widely used to treat DKD, showed no effect on hyperglycemia. Analysis of hepatic gene expression levels of the animals treated with SAR442357 revealed upregulation of genes responsible for the biosynthesis of cholesterol and BAs, providing clear evidence of target engagement and mode of action of the new sequestrant. Additional hepatic gene expression pathway changes were indicative that there was interruption of the enterohepatic BA cycle. Histopathological analysis of ZSF1 rat kidneys treated with SAR442357 further supported its nephroprotective properties. Collectively, these findings reveal the pharmacological benefit of simultaneous sequestration of BAs and phosphate in treating T2D and its associated comorbidities and cardiovascular complications.
Significance Statement

- A new non-absorbed polymeric sequestrant with optimum phosphate and bile salt sequestration properties was developed as a treatment option for DKD.
- The new polymeric sequestrant offered combined pharmacological benefits including glucose regulation, lipid lowering and attenuation of DKD progression in a single therapeutic agent.
Introduction

Increased prevalence of diabetes has resulted in increased incidents of renal impairments leading to diabetic kidney disease (DKD). As a microvascular complication, DKD often progresses to end-stage renal disease (ESRD) requiring dialysis or kidney replacement therapy. DKD has become the leading cause of ESRD (Cameron, 2006; Alicic et al., 2017) with approximately 35% of all deaths from chronic kidney disease (CKD) being attributed to DKD (Thomas, 2019). Moreover, a higher rate of cardiovascular (CV) mortality is observed among DKD patients with ESRD compared to non-DKD ESRD (Giorda et al., 2018; Thomas, 2019). With nearly 160 million DKD patients worldwide, it is projected to reach 212 million by 2040 (Alicic et al., 2017).

Currently, there is no cure for CKD, including DKD. The available treatment options are limited to relieving the symptoms and slowing down disease progression. These include management of hyperglycemia, hypertension, use of renin–angiotensin–aldosterone system (RAAS) inhibitors, and sodium-dependent glucose cotransporters-2 (SGLT-2) inhibitors (Lytvyn et al., 2020). Losartan, an angiotensin II type 1 receptor blocker is one of the first line therapies employed to ameliorate hypertension associated CV disease and DKD. By inhibiting arteriolar contraction and sodium retention, losartan decreases proteinuria, albuminuria, and markers of tubular damage (Nielsen et al., 2011; Katsiki et al., 2018, Francischetti et al., 2020). Moreover, diuretics, cholesterol lowering drugs, and phosphate binders are used to treat CKD (Holman et al., 2008; Bilous, 2008; Floege, 2016; Alicic et al., 2017).

Hyperphosphatemia is a manifestation of renal failure and positively correlates with progression to ESRD. In ESRD, hyperphosphatemia is associated with secondary hyperparathyroidism, metabolic bone disease, and progressive vascular calcification, resulting in significant CV morbidity and mortality (Hruska et al., 2008; Block et al., 1998; Block et al., 2004; Goodman et al., 2000). Therefore, pharmacological intervention to restrict phosphate absorption using phosphate binders is a mainstay of ESRD treatment. Sevelamer
is a non-absorbable polymeric phosphate binder used to lower serum phosphorus levels in CKD patients on dialysis. Sevelamer also exhibits pleiotropic beneficial effects. For example, sevelamer improves lipid and glucose metabolism (Bronden et al. 2018; Vlassara et al., 2012), reduces inflammation and oxidative stress (Caglar et al., 2007; Navarro-Gonzalez et al., 2011; Sun et al., 2009; Rastogi, 2013; Yilmaz et al., 2012; Vlassara et al., 2012), clears uremic toxins (Garg et al., 2005; Vlassara et al., 2012), and prevents progression of aortic and coronary calcification (Moe and Chen, 2004; Floege and Ketteler, 2004; Asmus, 2005; Rastogi, 2013; Chertow et al., 2002). These benefits result in significant survival in sevelamer treated patients compared to those receiving metal based phosphate binders (Block et al., 2007; Di Iorio et al., 2012; Di Iorio et al., 2013; Patel et al., 2016; Rodriguez-Osorio et al., 2015; Floege, 2016).

Colestevelam is a non-absorbable polymeric cholesterol lowering drug, which belongs to the class of bile acid sequestrants (BAS). Colesevelam is approved for the treatment of hyperlipidemia and T2D. Its therapeutic action is derived from its ability to interrupt enterohepatic circulation of BAs by sequestering and removing BAs from the gastrointestinal (GI) tract (Rosenbaum et al. 1997; Stroeve et al., 2010; Herrema et al., 2010; Potthoff et al., 2013). Colesevelam improves glycaemia by eliciting changes in BAS-mediated modulation of nuclear farnesoid X bile acid receptor (FXR)-dependent signaling pathways that regulate hepatic gluconeogenesis (Hansen et al., 2014; Handelsmann, 2011; Inzucchi et al., 2015; Takebayashi et al., 2010; Kodera et al., 2011). Moreover, coleselvelam enhances the conversion of intestinal BA towards secondary BAs, thereby stimulating secretion of the incretin GLP-1 from enteroendocrine L-cells (Fuchs et al., 2018).

Colestevelam and sevelamer possess similar chemical attributes and thus bind similar substrates in the gut, however, with different binding affinities and capacities. Therefore, we hypothesized that the discovery of a novel polymeric sequestrant that combines the efficacy and pleiotropic benefits of phosphate and BA binding with optimum capacity and affinity may offer significant clinical benefit in delaying progression to ESRD and reducing CV risk. We have synthesized a novel polymeric sequestrant (SAR442357) with optimum physico-
chemical properties as well as optimum binding affinity and capacity towards both phosphate and BA anions. Such a compound could serve as an innovative new therapy for the treatment of DKD with potentially fewer side effects. Here, we report the preclinical results and mechanistic insights into the pharmacological action of SAR442357 as a treatment for DKD using the ZSF1 rat model, which manifest disease progression and pathology of human DKD including hypertension, obesity, hyperglycemia and T2D mellitus (Bilan et al., 2011; Dower et al., 2017; van Dijk et al., 2016; Su et al. 2016).
Material and Methods

Synthesis of hydrogels

Poly(allylamine hydrochloride) (PAA.HCl) and poly(diallylamine hydrochloride) (PDA.HCl) were synthesized by free radical polymerization of allylamine and diallylamine respectively, in the forms of their hydrochloride salts by following reported procedures (Harada and Hasegawa, 1984; Jang and Rasmussen, 1998). The crosslinked polymer networks comprised of PAA and PDA components is described here using SAR442357 as the specific example. Detailed parameters for the syntheses of a series of such hydrogels are summarized in Supplementary table 1. In a jacketed vessel with lid, a mixture of PAA·HCl and PDA·HCl (14.3:1 molar ratio) was diluted with distilled water to achieve the desired polymer concentration of 40% (w/v). The reaction mixture was subsequently warmed to 30°C and the pH of the solution was adjusted to 11.0 by adding sodium hydroxide (NaOH) pellets (for smaller batches 50% (w/v) aqueous NaOH solution can be used), while maintaining the temperature of the reaction mixture at 25°C. The temperature of the stirred reaction mixture was slowly brought to room temperature followed by the addition of an appropriate amount of epichlorohydrin (14.3 mmol) to the reaction mixture via a syringe. The temperature of the reaction mixture was subsequently brought to 40°C and stirred until the reaction mixture became a gel. At this point the stirring was stopped and reaction mixture was cooled down to room temperature. The gel was placed in a glass drying dish and allowed to cure overnight at 20°C in a vacuum oven. The gel was subsequently broken into smaller pieces and pushed through a 200 μm sieve to produce smaller particles. The polymer particles were suspended in deionized water and the pH of the suspension was adjusted to 13.0 by the addition of 50% (w/v) aqueous NaOH solution. The resulting suspension was stirred for 20 min and filtered over filter paper (113 grade). The gel particles were subjected to a series of washing (using deionized water) and filtration steps until the conductivity of the suspension reached a value of <50μS/cm. Subsequently, the suspension was bubbled with appropriate amounts of CO₂ gas to reach the carbonation level of ~13%. The gel particles were filtered and dried at 60°C under reduced pressure to constant weight.
The dried particles were ground using a mill grinder or cryomilling and were fractioned into particles of different sizes using sieves of appropriate mesh size (50-100 µm).

**Characterization of hydrogels**

Elemental analyses were carried out at QTI laboratories (Whitehouse, New Jersey, USA). The percentage of carbonate counter ions was determined by thermogravimetric analysis. Particle size measurement was carried out using Malvern Mastersizer 3000 instrument (Malvern Panalytical Ltd, Malvern, UK). Solid state magic angle spinning NMR spectra of the swollen gels (in D2O) were measured using a Bruker AVANCE 400 MHz NMR spectrometer (Bruker BioSpin GmbH, Ettlingen, Germany). Equilibrium swelling indices of these hydrogels in PBS medium were determined by following a published method (Polomoscanik et al., 2012). Total titratable amine (TTA) contents in the hydrogels were determined by potentiometric titration. The titration process involves conversion of the polymer gel into its HCl salt by treatment with excess HCl. The resulting HCl salt of the gel is titrated with 1 N NaOH. Two inflection points were obtained on the titration curve; the first one corresponding to HCl (strong acid) neutralization and the second one corresponding to amine hydrochloride (weak acid) neutralization. The amount of amine groups in the polymer gel is estimated as a difference between the second and the first inflection points.

**Equilibrium binding of phosphate and bile acid salts**

Equilibrium binding capacities of the hydrogels towards bile salts such as sodium salts of glycocholic acid (GC) and glycochenodeoxy cholic acid (GCDC) were determined according to a published procedure (Braunlin et al., 2002). Equilibrium binding capacities of the hydrogels for phosphate ions were measured in BES buffer using potassium phosphate, monobasic (KH₂PO₄) as the substrate followed by estimation of unbound phosphate ions by ion chromatography. The experimental details for these measurements are described elsewhere (Swearingen et al., 2004).

**Animals**
Eight weeks old male ZSF1 (Lepr<sup>by</sup>/Lepr<sup>e</sup>) rats (Charles River, Kingston, NY, USA) were housed under a 12:12 h light–dark cycle (lights on at 6 a.m.) with ad libitum access to a standard diet for this animal model (PMI 5008 mod., Ssniff, Soest, Germany) and tap water. Body weight, tail blood, urine and fecal basal values were collected at 10-11 weeks of age. Subsequently, the obese ZSF1 rats were randomized to 4 groups based on body weight, random blood glucose, HbA1c (%), and urinary albumin. From 12 weeks of age, the corresponding groups were treated daily with losartan (p.o., 10 mg/kg in the diet) (Hennig, Germany), colesevelam (2% in the diet) or SAR442357 (2% or 4% in the diet) for 3 months. The drugs in the form of dry powder were dry mixed with the powdered standard Sniff diet at appropriate ratios. After homogenization in dry form, the drug containing diets as well as drug free diet were cold pelleted. Selection of the doses used for the polymers was based on published literature on polymeric sequestrants like colesevelam, colestilan and also sevelamer, that for example described the 2% colesevelam dose as an efficacious dose in rodent models when added to the diet of the animals (Herrema et al., 2010; Meissner et al., 2011). Initially each group had 10 animals. Based on urine albumin/creatinine values, 2 non-progressor rats were removed from the obese ZSF1 control group. Measurements were taken at baseline (age: 11 weeks) and at the end of second and third month of treatment (age: 20 and 24 weeks, respectively). All experiments were approved by the Ethics Committee of the State Ministry of Agriculture, Nutrition and Forestry (State of Hesse, Germany).

**Body weight, blood and serum parameters**

Body weight was monitored weekly with a balance (Mettler Toledo, Modell New Classic MF, Greifensee, Switzerland). Blood glucose was measured with a glucometer (ACCU-CHEK Aviva, Roche Diagnostics, Mannheim Germany, Model:NC) and HbA1c (%) with a Cobas 6000 analyzer (Roche Diagnostics, Mannheim, Germany). Final measurements were performed before the animals were anesthetized.
Tail blood collection was performed for basal and 20-week time-points. For final measurements, rats were euthanized in ad libitum state under deep isoflurane anesthesia by aortic exsanguination. Blood was collected (S-Monovette Z-Gel, clot activator, Sarstedt, Nümbrecht, Germany), coagulated for 20 min at room temperature and centrifuged (4°C, 10 min, 4000 rpm) for serum collection. Serum triglycerides, total cholesterol, inorganic phosphate, urea and creatinine were measured (Cobas 6000 analyzer, Roche Diagnostics, Mannheim, Germany). Measurements of serum triglycerides and total cholesterol at the 2-month time-point for the obese ZSF1 SAR442357 (2%) treated group and 3-month time-points for the obese ZSF1 colesevelam (2%) treated group were not recorded due to insufficient sample volume. One measurement for serum inorganic phosphate was missed at 3-month time-point for the obese ZSF1 colesevelam (2%) group and at 2-month time-point for the lean ZSF1 and obese ZSF1 (2% and 4%) groups due to lack of adequate sample volume.

Serum insulin was measured with an ELISA kit (Mercodia rat/mouse insulin FIA No. 10-1248-10) following manufacturer’s guidelines. For insulin measurements, one outlier value was removed from the obese ZSF1 SAR442357 (2%) group from the 2-month time-point.

**Gene expression analysis**

**Sequencing of mRNA.** Liver samples were collected in RNAlater™ solution (Invitrogen, Thermo Fisher Scientific, Darmstadt, Germany) and stored at -80°C until further processing. Total RNA was extracted from the samples using RNeasy Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer’s instructions. Total RNA purity and concentration was determined on a Nanodrop8000™ spectrophotometer (Thermo Fisher Scientific, Darmstadt, Germany) with subsequent RNA quality control with an Agilent 2100 bioanalyzer using an Agilent RNA 6000 Nano Kit (Agilent technologies, Santa Clara, CA, USA). RNAs with a RIN score of 8 or higher were used for RNA sequencing. Library
preparation was done with the Illumina TruSeq RNA Sample Prep Kit v2 and subsequent sequencing on an Illumina NextSeq 550 (Illumina, Inc., San Diego, CA, USA).

RNA-seq data analysis was performed using ArrayStudio (QIAGEN Inc.). Briefly, raw data QC is performed followed by a filtering step to remove reads corresponding to rRNAs as well as reads having low quality score or shorter than 25 nt. Reads were further mapped to the rat genome (rn6) using OSA4 (Hu et al., 2012) (Omicsoft Sequence Aligner, version 4) and quantified using ENSEMBL gene model of transcriptome, with parameters enabling identification of new splice variants. Differential analysis of gene expression was performed at gene level using Voom module integrated to Array Studio (Law et al., 2014). This module uses Voom in the Limma R/Bioconductor package.

**Bomb calorimetry**

Combustion of food and feces. Food consumption was measured and corrected by separating the unconsumed food from feces. Due to technical issues during monitoring, one measurement for food consumption was excluded from the obese ZSF1 colesevelam (2%) group at 2- and at 3-month time-points, as well as 5 measurements from the obese ZSF1 losartan (10 mg/kg) group. Samples of chow and collected feces (~1 g) were dried at 60°C for up to 7 days, homogenized in a coffee grinder and squeezed to pills. Energy contents in these samples were determined in an oxygen bomb calorimeter (model 6300, Parr Instruments Deutschland GmbH, Frankfurt a.M., Germany). For the analysis of solid feces samples, the bomb calorimeter was calibrated using benzoic acid for calorimetric determination with a guaranteed calorific value of 26.47 kJ g⁻¹ (Sigma-Aldrich, Darmstadt, Germany). Energy uptake (Eup) was determined as the product of food consumed and the calorific value of the food. The energy loss was defined as the sum of fecal (Eloss fec) and urinary caloric loss (Eloss urine, for details see below) and calculated from the feces/urine produced per 24 hours multiplied by its respective energy content. To obtain energy metabolized (Emet) the energy content of feces and urine were subtracted from energy intake calculated as Emet = Eup - (Eloss fec + Eloss urine) (Drozdz A, and Weiner J, 1975).
The utilization of food energy is expressed as food efficiency corresponding to the percentage of metabolized energy from energy intake.

Combustion of urine. Urinary samples were collected using metabolic chambers to separate urine from food and feces. For determination of urinary caloric value, samples were usually freeze-dried, pulverized and combusted in bomb calorimeters to obtain an estimate of excretory energy losses (Raman et al., 2007; Singh et al., 2009). Due to the low urinary volume of the lean rats, we developed a procedure using cotton coils as combustion aid (Heiland Vet GmbH, Germany). Depending on sample volume available, two different sizes of coils were used: 8mm diameter x 19mm length (for low volumes) and 10mm x 19mm (for high volumes). The caloric value of the combustion aid was 16.49 kJ g⁻¹. Results of urinary samples were corrected automatically for the value of the aid. Calibration and testing for validity were performed as previously reported (Elvert et al., 2013). The analysis of urine followed the same procedure used for the feces samples. One outlier was excluded from the obese ZSF1 colesevelam (2%) group at the 2-month time-point.

Analysis of fecal bile acids, fecal phosphate and fecal fatty acids

Fecal samples were collected over 24 h from single housed rats using metabolic cages (Techniplast, model 3701M001, Buguggiate VA, Italy). The feces were dried, weighed and frozen at -20°C for later analysis. Extraction and measurement of fecal total bile acids were performed according to the method described by Dvir et al. (2000) with minor modifications. Fecal inorganic phosphate and fecal volatile fatty acids were measured from the identical extraction solutions.

Briefly, dried feces were homogenized in a potassium hydroxide (KOH) solution (pH 11.0) with an ultra-turrax disperser (model Ultra-Turrax T25 basic, IKA®-Works Inc., Wilmington, North Carolina, USA) to obtain a uniform slurry with a target concentration of 50 mg feces per mL of homogenate. Bile acid, phosphate and free fatty acid extraction was performed by agitating the slurry on a shaker using the solvents dichloromethane and methanol. An aliquot of the feces homogenate containing 500 mg feces was extracted over 4
hours at room temperature with 5 mL of a dichloromethane:methanol (2:1, v/v) mixture. Two mL of aqueous potassium chloride (KCl) solution (3.7 g/L) were added and samples were incubated further 1 h and then centrifuged at 1500×g for 10 min. The aqueous supernatant phase was removed, its volume was determined and then stored at -20°C. Finally, samples were thawed at 40°C, centrifuged at 1500×g for 10 min and then analyzed for total bile acid, inorganic phosphate and total non-esterified fatty acid (NEFA) concentrations using commercially available enzymatic and colorimetric assay kits on a chemistry analyzer (Beckman Coulter AU680, Beckman Coulter Inc., Clare, Ireland).

**Urine parameters**

Urine was collected in metabolic cages (Techniplast, Modell 3701M001, Buguggiate VA, Italy) after 8 and 16 h to have in total 24 h of urine collection. Urine inorganic phosphate, glucose, total protein, albumin and creatinine were measured using commercially available enzymatic assay kits and normalized by 24 h urine volume (Beckman Coulter AU680, Beckman Coulter Inc., Ireland). Basal urine measurements were missed for one rat of the obese ZSF1 SAR442357 (2%) group due to accidental loss of the sample. Furthermore, one basal value was missing from the lean ZSF1 control group due to albumin being under the lower limit of detection and one outlier value for inorganic phosphate at 2-month time-point was removed from the obese ZSF1 losartan group.

Urine KIM1 and cystatin C were measured with an ELISA kit (R&D Systems, Quantikine ELISA, Cat.No RKM100 for KIM1, Cat.No. MSCTC0 for Cystatin C) following manufacturer’s guidelines. Basal urine measurements were missed for one rat from the obese ZSF1 SAR442357 (2%) group due to accidental loss of the sample and one KIM1 value of the obese ZSF1 losartan group at 3-month time-point for being an outlier.

Both kidneys were removed after euthanasia and weighed (Mettler Toledo, Model X204, Greifensee, Switzerland).

**Histology and pathological analysis**
Kidneys were sampled and fixed in 4% neutral buffered formaldehyde for 48 h and embedded in paraffin. Cross sections were obtained from each sample. Afterwards the dehydration of the tissue was done automatically with a VIP 5 tissue processor (Sakura® Tissue-Tek VIP 5 Tissue Processor, Torrance, CA, USA) for approximately 20 h, followed by the blocking procedure. Slice sections of 4 µm thickness were taken from each sample and dried overnight on a stretching table at 45°C.

Staining was performed automatically using a Gemini AS automatic slide stainer (Thermo Fisher Scientific, Runcorn, WA7 1TA, Great Britain).

**Hematoxylin and eosin (H&E) staining.** Tissue deparaffination was carried with xylene 3 times for 3 min followed by rehydration in decreasing ethanol solutions 100 %, 95 % and 70 % for 1 min each. Staining of the nuclei was done with Haemalum from Mayer (blue) for 5 min, followed by rinsing with distilled water for 10 min and ascending ethanol solutions of 70 and 99%. Final tissue staining was done with Eosin (red) for 30 sec; 3 times ethanol and xylene for dehydration and permanent covering.

**Periodic Acid-Schiff (PAS) staining.** Tissue deparaffination was done with xylene 3 times for 3 min followed by rehydration in decreasing ethanol solutions 100 %, 95 % and 70 % for 1 min each. For preservation the slices were exposed to periodic acid for 10 min and subsequently rinsed with tap water for 1 min. Staining of glycoproteins was done with Schiff’s reagent (pink) for 10 min followed by rinsing with distilled water for 1 min. Staining of the nuclei was done with Haemalum (blue) for 2 min followed by rinsing with distilled water for 1 min 3 times ethanol and xylene for dehydration and permanent covering.

For pathology analysis, whole slide scanned (20x) images were evaluated qualitatively and semiquantitatively by 2 board-certified veterinary pathologists (KS and DSB). Criteria for semiquantitative evaluation included assessment based on the severity of a morphological change and the extent of tissue involvement. For each rat, 4 parameters were assessed independently that included glomerular pathology, tubular pathology, tubular protein casts, and interstitial inflammation. Briefly, a score of 0 was assigned to kidneys that
exhibited minimal changes within a normal range for 6-month-old rats; score 1 was assigned to kidneys that exhibited morphologic changes in approximately 25% glomeruli, up to 10% tubules and contained focal small interstitial infiltrate composed of mononuclear inflammatory cells; score 2 was assigned to kidneys that exhibited involvement of approximately 50% glomeruli, up to 20% tubules and contained multiple small foci of inflammatory cells within the interstitium; grade 3 was assigned to kidneys that showed involvement of approximately 75% glomeruli, up to 30% tubules and contained medium sized aggregate of inflammatory cells within the interstitium; grade 4 was assigned to kidneys that exhibited morphological changes in more than 75% glomeruli, up to 40% tubules and contained relatively medium sized aggregates of inflammatory cells along with the interstitial fibrosis; and grade 5 was assigned when more than 40% tubules exhibited morphological changes. Model-related unilateral or bilateral hydronephrosis was observed in multiple animals. Sections exhibiting hydronephrotic changes were excluded from histopathological assessment like one obese ZSF1 rat treated with colesevelam (2%).

**Statistical analysis**

Results are represented as mean ± standard deviation (S.D.) or median ± range for histological parameters. Statistical analysis (GraphPad Prism software) for treatment effects versus the obese ZSF1 control group was performed using GraphPad Prism (GraphPad Prism Software Inc., San Diego, CA, USA) with two-way ANOVA followed by Dunnet’s multiple comparisons test. P ≤ 0.05 was considered significant.

Gene expression values are represented as fold change for treatment groups versus the obese ZSF1 control group. The variable multiplicity was considered and false discovery rate (FDR) adjusted p-values were calculated using the Benjamini-Hochberg (BH) correction (Benjamin and Hochberg, 1995). FDR ≤ 0.05 was considered significant.

Kidneys weight and histological values were analyzed for normality with Kolmogorov-Smirnov test and subsequently with one-way ANOVA for treatment effects versus the obese ZSF1 control group with Kruskal-Wallis test. P ≤ 0.05 was considered significant.
Results

Synthesis and characterization of sequestrant polymers

Sequestrants of phosphate and bile salts represent a class of amine containing (cationic) water-swellable polymer networks. These polymer networks, commonly addressed as hydrogels, are obtained by covalent crosslinking of soluble polymer chains. The crosslinking process renders the polymers to be non-absorbable from the GI tract. By remaining confined to GI tract without systemic exposure, these hydrogels bind target anions in the gut, primarily via electrostatic interactions. SAR442357 is a structurally optimized amine functionalized copolymer hydrogel, which is obtained by crosslinking of PAA and PDA using epichlorohydrin as the crosslinker. Figure 1 represents the schematic structure of a representative portion of SAR442357. This polymer was identified through a parallel synthesis process. Systematic optimization of the physicochemical properties was pursued using a design of experiment (DOE) strategy, where the effects of variation of experimental parameters such as relative amounts of PAA, PDA, and epichlorohydrin in the polymer hydrogels were assessed. Towards that end, a library of 21 polymer hydrogels of different compositions was synthesized. The resulting hydrogels were characterized by using different physical and chemical techniques. The results of characterization and \textit{in vitro} substrate binding properties of these hydrogels are summarized in the supporting information (Supplementary Table 1). High resolution 2-D solid state NMR spectroscopy was used to systematically characterize the molecular structure of SAR442357. In particular, 2-D $^1\text{H}$-$^{13}\text{C}$ correlation NMR spectroscopy (Supplementary Figure 1) enabled us to unequivocally assign the structure of composition of this polymer.

Relative ratios of PAA and PDA contents in the hydrogel can lead to distinct spatial distribution of the amine functionalities across the polymer network that may influence the electrostatic interaction with the anionic substrates. On the other hand, the amount of epichlorohydrin modulates the physical properties of the hydrogels including ease of particle formation and swelling properties. With the appropriate degree of crosslinking, hydrogels will
exhibit adequate availability of binding sites for the electrostatic interactions with the substrates in the gut without significant increase in swelling. Furthermore, the particle size of the resulting hydrogel has significant influence on its binding behavior, which can be explained by differences in diffusion rates of the substrates across the hydrogel matrix as a function of particle size.

The in vitro phosphate and bile acid binding properties of these hydrogels were assessed under equilibrium conditions in a physiologically relevant buffer. Glycocholic acid (GC) and glycochenodeoxycholic acid (GCDC) were used as representative bile acids for in vitro binding experiments. Based on optimized physicochemical properties and in vitro substrate binding properties (Supplementary Table 1), SAR4422357 was selected as the lead sequestrant for further pharmacological evaluation.

The key binding parameters for colesevelam, the two homopolymers of PAA and PDA, and SAR4422357 are shown in Table 1. The chemical structure of colesevelam comprises of an epichlorohydrin crosslinked PAA backbone with pendant hydrophobic alkyl chains tethered to the amine groups of PAA (Steinmetz and Schonder, 2005). SAR4422357 maintains the good substrate binding properties of the PAA homopolymer hydrogel, yet with a lower swell index. The favorable swelling index of SAR4422357 compared to the corresponding homopolymer hydrogels PAA and PDA is expected to reduce potential swelling mediated undesired GI side effects.

**Body weight and food efficiency**

The lean ZSF1 control group had over the full 3 months period of the study (from 10 to 24 weeks old age) lower body weight compared to the obese ZSF1 control group.

Three months treatment of the obese ZSF1 rats with SAR442357 (2% or 4%) did not change body weight compared to the obese ZSF1 control group (Fig. 2A), although food intake was higher with SAR442357 4% from 2 months onwards and with SAR442357 2% also at 3 months (Fig. 2B). No differences were observed in food efficiency (Fig. 2C). However, energy metabolized (Fig. 2D) and energy loss via feces were higher from 2 months
onwards for 4% SAR442357 treatment group and in the 2% SAR442357 (Fig. 2E) at 3 months compared to the obese ZSF1 control group. No differences were observed in energy loss via urine (Fig. 2F) between the SAR442357 treatment groups and the obese ZSF1 control group.

The lean ZSF1 control group exhibited higher food intake at 3 months and higher food efficiency and metabolized energy from 2 months onwards compared to the obese ZSF1 control group. Energy loss via feces and urine was consistently low at all measured time points (Fig. 2E-F).

No changes in body weight, food intake, food efficiency, metabolized energy and energy loss via urine were observed for the obese ZSF1 rats treated with losartan (10 mg/kg) or 2% colesevelam mixed in the diet (Fig. 2A-D, F). Regarding energy loss via feces, only the obese ZSF1 colesevelam (2%)-treated group showed higher energy loss from 2 months onwards, similar to the SAR442357-treated groups, compared to the obese ZSF1 control group (Fig. 2E).

**Diabetes progression**

Throughout the study period, the lean ZSF1 control group exhibited lower circulating insulin, random glucose and HbA1c (%) levels compared to the obese ZSF1 control group (Fig. 3A-C).

Treatment of obese ZSF1 rats with SAR442357 (2% or 4%) for 3 months delayed diabetes progression as indicated by higher circulating insulin (Fig. 3A) and lower glucose (Fig. 3B) levels compared to the obese ZSF1 control group. Furthermore, SAR442357 (2% or 4%) reduced HbA1c (%) starting at two months of treatment (Fig. 3C). These effects were observed at an earlier time-point with the highest dose of SAR442357 (4%), as insulin levels were higher and glucose values lower after 2 months of treatment compared to the ZSF1 obese control group.

The losartan (10 mg/kg) group did not show differences in these metabolic parameters compared to the obese control group. In the colesevelam (2%) group a
statistically significant lowering of HbA1c (%) values was noted at 2 months (Fig. 3C). The HbA1c (%) value at 3 months was lower than obese ZSF1 controls but did not reach statistical significance.

**Bile acids and free fatty acids**

Sequestration of bile acids by SAR442357 was assessed by measuring fecal and urinary bile acids levels along the treatment period. Fecal bile acids were lower in the lean ZSF1 control group from 2 months onwards and urine bile acids lower at all measured time points compared to the obese ZSF1 control group (Fig. 4A-B). Fecal free fatty acids were also lower at all measured time points (Fig. 4C).

In the obese ZSF1 rats treated with 2% coleselam, or SAR442357 (2% or 4%), fecal bile acids levels were consistently higher in a dose independent manner from 2 months onwards compared to the obese ZSF1 control group (Fig. 4A). However, regarding urine bile acids, only 2% coleselam showed a transient decrease urinary in bile acids at 2 months compared to the obese ZSF1 control group (Fig. 4B).

Fecal free fatty acids showed a profile parallel to fecal bile acids, with higher levels in the obese ZSF1 rats treated with 2% coleselam and SAR442357 (2% or 4%) in a dose independent manner from 2 months onwards compared to the obese ZSF1 control group (Fig. 4C).

Losartan (10 mg/kg) had no effect on bile acids or free fatty acid levels (Fig.4A-C).

**Serum triglycerides and total cholesterol**

Over the course of the study the lean ZSF1 control group showed lower serum triglycerides and total cholesterol levels than the obese ZSF1 control group (Fig. 5A-B).

None of the therapeutic agents had any impact on serum triglyceride levels (Fig. 5A). On the other hand, serum cholesterol levels were lower after 2 months of treatment with 2% coleselam and 4% SAR442357 compared to the obese ZSF1 control group. However, this effect on cholesterol waned after 3 months with 2% coleselam. In comparison, both
losartan (10 mg/kg) and SAR442357 (2% or 4%) decreased serum cholesterol at 3 months with 4% SAR442357 having an effect from 2 months onwards (Fig. 5B).

**Hepatic gene expression for cholesterol, bile acids and fatty acid synthesis pathways**

To test for hepatic effects of the different treatments, RNASeq analysis was performed on liver samples taken at study termination. The global number of significantly regulated genes for the different treatment groups compared to the obese ZSF1 control group is illustrated in volcano plots in supplementary Figure S2. Genes that were down-regulated in the obese ZSF1 control group and recovered upon treatment with either colesevelam or SAR442357 are summarized in a heat map (supplementary Fig. S3). In line with the expected mechanism of action, hepatic mRNA levels for marker genes belonging to cholesterol (Hmgcr, Mvd, Sqle), bile acids (Cyp7a1) and fatty acid (Acly, Acaca, Acacb, Fasn) synthesis pathways were higher in those animals treated with colesevelam (2%) and SAR442357 (2% or 4%) compared to the obese ZSF1 control group (Fig. 6). In all cases, there was a trend towards higher levels with SAR442357 (4%) compared to SAR442357 (2%) that was, however, not statistically significant. Losartan treatment had no effect on expression of these genes.

**Inorganic phosphate**

Sequestration of dietary phosphate by SAR442357 was assessed by measuring fecal, urinary and serum phosphate levels during the treatment period. Fecal and urine inorganic phosphate were lower in the lean ZSF1 control group at all measured time points and serum inorganic phosphate from 2 months on compared to the obese ZSF1 control group (Fig. 7A-C).

Although fecal inorganic phosphate levels were higher in the obese ZSF1 rats treated with colesevelam (2%) or SAR442357 (2% or 4%) after 2 months compared to the obese ZSF1 control group, these differences only persisted with colesevelam (2%) treatment at 3 months (Fig. 7A). However, urine inorganic phosphate levels were low from 2 months onwards with SAR442357 (2% or 4%) in a dose-dependent manner compared to the obese.
ZSF1 control group (p<0.05). After 3 months, treatment with colesevelam (2%) led to lower urinary inorganic phosphate levels (Fig. 7B).

Only treatment with SAR442357 (2%) led to a transitory decreased in serum inorganic phosphate at 2 months compared to the obese ZSF1 control group (Fig. 7C).

Treatment with losartan (10 mg/kg) had no effect on inorganic phosphate levels in any of the biological mattresses analyzed.

**Diabetic nephropathy progression**

*Urine and serum parameters*

Throughout the course of the study, the lean ZSF1 control group had lower urine volume, glucose, total protein, albumin/creatinine ratio, urinary KIM1 and cystatin C compared to the obese ZSF1 control group (Fig. 8A-F).

Urinary volume and glucose were decreased only with SAR442357 (4%) after 3 months of treatment compared to the obese ZSF1 control group (Fig. 8A-B). SAR442357 (4%) decreased urinary total protein from 2 months onwards. Similarly, after 3 months both losartan (10 mg/kg) and SAR442357 (2%) decreased urine total protein compared to the obese ZSF1 control group (Fig. 8C). Colesevelam (2%) had no effects on urinary total protein.

Urinary albumin/creatinine ratio was decreased from 2 months onwards with losartan (10 mg/kg) and SAR442357 (4%) and at 3 months with SAR442357 (2%) compared with the obese ZSF1 control group. Colesevelam (2%) decreased the albumin/creatinine ratio transiently at 2 months (Fig. 8D).

Losartan (10 mg/kg) and SAR442357 (4%) decreased urine KIM1 from 2 months onwards compared to the obese ZSF1 control group (Fig. 8E).

SAR442357 (4%) decreased urine cystatin C from 2 months onwards and at 3 months also losartan (10 mg/kg) and SAR442357 2% decreased cystatin C compared to the obese ZSF1 control group (Fig. 8F).
In addition to the urinary kidney disease progression markers measured throughout the course of the study, we measured kidney weights (Fig. 9) and final concentrations of the biomarkers attributed to chronic tubular damage serum urea and serum creatinine (Supplemental Figure S7) at the end of the study.

Kidneys weights were decreased only with SAR442357 (2% or 4%) following 3 months of treatment compared to the obese ZSF1 control group (Fig. 9).

Serum urea concentration was significantly lower in lean ZSF1 rats compared to the obese ZSF1 control animals. Furthermore, it significantly decreased in obese ZSF1 rats treated with losartan (10 mg/kg) and SAR442357 (4%) (Supplemental Figure S7A). On the other hand, no differences in serum creatinine values were observed between the treated groups and the control group in obese ZSF1 rats (Supplemental Figure S7B).

Histopathological findings

Rats treated with 4% SAR442357 showed a significant (p<0.05) decrease in the progression of renal injury including glomerular pathology, tubular intraluminal protein casts and tubular pathology (Figs. 10, 11 and Supplemental Figure S6). Though statistically insignificant, improvement in interstitial inflammation was also observed in rats treated with 4% SAR442357. In comparison, obese ZSF1 control rats showed most severe changes in the glomerular and tubular compartments along with the segmental and multifocal tubulointerstitial areas. Glomerular changes included mesangial thickening by periodic acid Schiff (PAS) stained material, synechae and crescents formation, splitting and thickening of Bowman's capsule, thickened capillary walls and lower luminal diameter, lobulation of glomerular tufts, obliteration of glomerular spaces with eosinophilic material, and regional to global obsolescence. Tubulo-interstitial changes included dilation of cortical and medullary tubules, presence of intraluminal protein casts, thickened tubular basement membrane, tubular epithelial cell degeneration, cortical perivascular aggregates of mononuclear inflammatory cells, and regional interstitial collapse with tubular atrophy and fibrosis (Fig. 11). Compared to 4% SAR442357, rats treated with losartan (10 mg/kg/day) showed progression
of glomerular pathology whereas the treatment effects on assessed parameters such as tubular protein casts, tubular pathology, and interstitial inflammation were comparable (Figs. 10, 11 and Supplemental Figure S6). Similarly, rats treated with 2% colesvelam exhibited no improvement in glomerular pathology, tubular protein casts, and tubular pathology parameters when compared to the treatment with 4% SAR442357 (Figs. 10, 11 and Supplemental Figure S6).
Discussion

The goal of this investigation was to evaluate pharmacological benefits of a non-absorbed polymeric sequestrant (SAR442357), possessing optimum phosphate and bile acid binding characteristics, in attenuating the progression of DKD and preventing associated cardiovascular complications in T2D using a relevant preclinical animal model. Our findings suggest that treatment of obese ZSF1 rats with SAR442357 for 3 months may attenuate the progression of DKD in a manner similar to losartan, an angiotensin II type 1 receptor blocker approved for this purpose. On the other hand, use of colesvelam, a clinically approved BAS (with poor phosphate binding characteristics), did not offer any therapeutic benefits for DKD. However, unlike losartan, both colesvelam and SAR442357 improved glycemia in these animals. This was evident from an increase in plasma insulin and decrease in plasma glucose and HbA1c in the colesvelam or SAR442357 treated animals.

The pharmacological actions of lumen restricted BASs stem from their abilities to sequester and remove bile acids from the intestinal lumen, thereby decreasing their enterohepatic circulation. This process leads to increased hepatic synthesis of new bile acids from cholesterol, which in turn increases the requirement for cholesterol in the liver. This results in increased uptake of cholesterol via the LDL receptor pathway, and an overall decrease in blood cholesterol. Moreover, BASs such as colesvelam have been shown to improve hyperglycemia in preclinical animal models of diabetes (Herrema et al., 2010; Sedgeman et al., 2018) as well as in T2D patients (Takebayashi et al. 2010; Handelsmann, 2011; Holst Nerild et al. 2018). Various mechanisms have been proposed for the anti-diabetic properties of BASs including increased secretion of GLP-1 through activation of TGR5 and decreased activation of intestinal FXR (Herrema et al. 2010; Takebayashi et al. 2010; Potthoff et al. 2013). Although colesvelam has been reported to improve glycemia in diabetes models such as ZDF (fa/fa) rats and db/db mice (Shang et al., 2012; Sedgeman et al., 2018), it has not been evaluated in ZSF1 rats, a model of diabetic kidney disease (Griffin et al., 2007; Homer and Dower, 2018). In the present study, colesvelam exhibited a modest effect on improving glycemic control and progression of renal dysfunction in comparison to
untreated obese ZSF1 rats. At similar dose levels, SAR442357 improved glycemia and
delayed progression of kidney impairment in the ZSF1 rat model of DKD. In contrast,
losartan failed to improve glycemic control as expected (Castoldi et al., 2019), but improved
renal decline in ZSF1 rats, as reported previously (Su et al., 2018), and its renal effects were
comparable to SAR442357 treatment. Improved therapeutic benefits offered by SAR442357
may be attributed to its higher potency at reducing enterohepatic uptake of bile acids
compared to colesevelam (Fig. 4A, p<0.05). These results are supported by lower serum
total cholesterol and higher hepatic gene expression of markers for cholesterol and bile acid
biosynthesis in the SAR442357 treated group compared with the obese ZSF1 control group.
In addition, SAR442357 is a significantly stronger binder of phosphate than colesevelam
(Fig. 7). Elevated serum phosphate has emerged as an important cardiovascular risk factor
in diabetes patients with kidney disease. Numerous preclinical and clinical studies have
shown that there is linear correlation between serum phosphate T2D, DKD, and coronary
artery disease (Hutchison et.al. 2011; Streja et.al. 2013; Vervloet et. al. 2017). Removal of
dietary phosphate from GI tract using phosphate sequestrants has been shown to reduce
systemic absorption of phosphate and can lead to improvements in progression of CKD and
associated cardiovascular complications. Thus, optimized phosphate and bile acid binding
characteristics of SAR442357 may be attributed to its markedly improved therapeutic benefit
as compared to colesevelam and losartan.

In terms of chemical structure, SAR442357 is different from colesevelam. The former
contains pendant primary and secondary amine groups, the latter being part of a rigid six-
membered ring structure (Fig. 1). On the other hand, in addition to limited number of primary
amine groups, colesevelam contains pendant hydrophobic alkyl chains and quaternary
ammonium groups (Steinmetz and Schonder 2005). Presence of higher amounts (mmol/g) of
primary amine groups in SAR442357 manifests its substantially higher in vitro binding
capacity for phosphate ions (5.65 mmol/g polymer) compared to colesevelam (1.29 mmol/g
polymer) (Table 1). In addition, SAR442357 also exhibits higher binding capacity for bile
salts. The differences in phosphate binding capacities in vitro between SAR442357 and
Colesvelam mirror the observed in vivo potencies. Thus, after two months of treatment, while SAR442357 treatment resulted in decreased urinary phosphate excretion in a dose-dependent manner (Fig. 7B, p<0.05), urinary phosphate lowering in colesvelam treated animals was very modest. Improved phosphate lowering effect of SAR442357 can be attributed to its ability to sequester dietary phosphate in the gut with higher binding strength and capacity compared to colesvelam. It may be noted that a clinically approved non-absorbed polymeric phosphate sequestrant, sevelamer, contains only primary amine groups along its backbone (Burke et al., 2001).

In our study, losartan treatment led to reduced urinary albumin/creatinine ratio, total protein, KIM1, cystatin C and serum total cholesterol in obese ZSF1 rats. These findings are similar to those reported in uninephrectomized ZSF1 rats treated with losartan at the same dose (Su et al., 2018). Increase in urinary KIM1 and cystatin C concentrations are markers for tubular damage and have been used to predict progression of diabetic nephropathy (Pais et al., 2019; Fernando et al., 2019; Conti et al., 2006; Herget-Rosenthal et al., 2007; Kim et al., 2013). Reduction of urinary KIM1 with losartan has also been observed in humans (Nielsen et al., 2011). However, to our knowledge, the effect of losartan on urinary cystatin C has only been studied in children with CKD and the results were inconsistent over three years of study (Webb et al., 2012). Similarly, in uninephrectomized ZSF1 rats, after 12 weeks of treatment with losartan, no change in urinary cystatin C was observed (Su et al., 2018). Although decrease in total cholesterol with losartan has been reported in Sprague Dawley rats fed with a high fat diet (Mourad et al., 2013), the results have not been translated in humans (Xiao et al., 2016). Furthermore, while losartan has been reported to improve insulin sensitivity in rodents and humans (Mourad et al., 2013; Xiao et al., 2016), in our studies using overtly diabetic ZSF1 rats, losartan, as noted previously, did not show any measurable impact on glycemia. In addition to lowering serum cholesterol, SAR442357 treatment resulted in decrease in urine volume, urine total protein, albumin/creatinine ratio, KIM1, cystatin C as well as slowing the progression of diabetes. The pharmacological benefits elicited by SAR442357 in improving both DKD and glycemia in obese ZSF1 rats combines...
those produced by losartan and colesevelam and reported in humans (Brenner et al., 2001; Rosenstock et al., 2010; Xiao et al., 2016).

In summary, our studies showed that developing a novel non-absorbed polymeric sequestrant with optimum phosphate and bile salt sequestration properties such as SAR442357 may offer combined pharmacological benefits including glucose regulation, lipid lowering and attenuation of DKD progression in a single therapeutic agent. These effects can result in overall improvements in kidney disease and pathology, which is evident from lower hypertrophy and the histopathological analysis. Furthermore, it should be noted that polypharmacological characteristics of SAR442357 are similar to those of SGLT2 inhibitor empagliflozin reported in ZSF1 rats (Park et al., 2020). The latter has been approved as a treatment option for CKD/DKD patients. Taken together, the results presented here suggest that SAR442357 exerts a number of pharmacological benefits in improving kidney functions and reducing cardiovascular complications in a rodent model of DKD. Finally, being systemically non-absorbed this compound could be a safe and effective drug candidate to treat DKD.
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Disclosures

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References


Figure Legends

Figure 1. Representative structure of SAR442357.

Figure 2. Body weight and food efficiency. Values represented as mean ± S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnet’s multiple comparisons test for statistical differences; *p<0.05, **p<0.01, ***p<0.001 (lean ZSF1 n=10, obese ZSF1 n=8, obese ZSF1 Losartan n=5-10, obese ZSF1 Colesevelam n=8-10, obese ZSF1 SAR442357 2% n=10, obese ZSF1 SAR442357 4% n=10). NM: Not measured.

Figure 3. Diabetes progression. Values represented as mean ± S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnet’s multiple comparisons test for statistical differences; *p<0.05, **p<0.01, ***p<0.001 (lean ZSF1 n=10, obese ZSF1 n=8, obese ZSF1 Losartan n=10, obese ZSF1 Colesevelam n=10, obese ZSF1 SAR442357 2% n=9-10, obese ZSF1 SAR442357 4% n=10).

Figure 4. Bile acids. Values represented as mean ± S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnet’s multiple comparisons test for statistical differences; *p<0.05, **p<0.01, ***p<0.001 (lean ZSF1 n=10, obese ZSF1 n=8, obese ZSF1 Losartan n=10, obese ZSF1 Colesevelam n=9-10, obese ZSF1 SAR442357 2% n=9-10, obese ZSF1 SAR442357 4% n=10).

Figure 5. Serum triglycerides and total cholesterol. Values represented as mean ± S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnet’s multiple comparisons test for statistical differences; *p<0.05, **p<0.01, ***p<0.001 (lean ZSF1 n=10, obese ZSF1 n=8, obese ZSF1 Losartan n=10, obese ZSF1 Colesevelam n=9-10, obese ZSF1 SAR442357 2% n=9-10, obese ZSF1 SAR442357 4% n=10).

Figure 6. Hepatic gene expression for cholesterol, bile acids and fatty acid synthesis pathways. Values represented as fold change versus the obese ZSF1 control group. Analysis with false-discovery rate with Benjamini-Hochberg correction for multiplicity for statistical differences; *FDR<0.05, **FDR<0.01, ***FDR<0.001 (lean ZSF1 n=10, obese
ZSF1 n=8, obese ZSF1 Losartan n=10, obese ZSF1 Colesevelam n=10, obese ZSF1 SAR442357 2% n=10, obese ZSF1 SAR442357 4% n=10).

**Figure 7. Inorganic phosphate.** Values represented as mean ± S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnet's multiple comparisons test for statistical differences; *p<0.05, **p<0.01, ***p<0.001 (lean ZSF1 n=9-10, obese ZSF1 n=8, obese ZSF1 Losartan n=9-10, obese ZSF1 Colesevelam n=9-10, obese ZSF1 SAR442357 2% n=9-10, obese ZSF1 SAR442357 4% n=9-10).

**Figure 8. Diabetic nephropathy progression.** Values represented as mean ± S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnet's multiple comparisons test for statistical differences; *p<0.05, **p<0.01, ***p<0.001 (lean ZSF1 n=10, obese ZSF1 n=8, obese ZSF1 Losartan n=9-10, obese ZSF1 Colesevelam n=9-10, obese ZSF1 SAR442357 2% n=9-10, obese ZSF1 SAR442357 4% n=10).

**Figure 9. Kidneys weight.** Values represented as mean ± S.D. Treatment effect vs. the obese ZSF1 control group analyzed with one-way ANOVA on ranks (Kruskal-Wallis test) for statistical differences; *p<0.05, ***p<0.001 (lean ZSF1 n=10, obese ZSF1 n=8, obese ZSF1 Losartan n=10, obese ZSF1 Colesevelam n=10, obese ZSF1 SAR442357 2% n=10, obese ZSF1 SAR442357 4% n=10).

**Figure 10. Kidney histopathology scores.** Values represented as median with range. Treatment effect vs. the obese ZSF1 control group analyzed with one-way ANOVA on ranks (Kruskal-Wallis test) for statistical differences; *p<0.05, **p<0.01, ***p<0.0001 (lean ZSF1 n=10, obese ZSF1 n=8, obese ZSF1 Losartan n=9, obese ZSF1 Colesevelam n=10, obese ZSF1 SAR442357 2% n=10, obese ZSF1 SAR442357 4% n=10).

**Figure 11. Kidney histopathology.** Compared to the lean ZSF1 rat, kidney from control obese ZSF1 rat shows glomeruli surrounded by a thick PAS (Periodic acid Schiff) positive Bowman's capsule (blue arrow) with regional splitting (yellow arrow), thickened glomerular mesangium, narrow capillary lumina and synechae formation. Tubules are surrounded by a thick PAS positive basement membrane (green arrow). Mononuclear inflammatory cells are
present in the surrounding interstitium (yellow circle). Losartan, Colesevelam and SAR442357 treated rats show reduced glomerular and tubulo-interstitial pathology. Note relative lack of interstitial inflammatory cells in Colesevelam (2%) or SAR442357 treated rats. PAS Stain. Magnification 200x.
Table 1. Synthesis and characterization of epichlorohydrin crosslinked PAA-PDA copolymer hydrogel networks.

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In vitro binding
Fig. 1

[Chemical structure diagram with labels a, b, c, d, e, and n]
Fig. 2

(A) Body weight (g) over basal, 2 months, and 3 months for Lean ZSF1, Obese ZSF1, Obese ZSF1 Colesevelam (2%), Obese ZSF1 SAR442357 (2%), and Obese ZSF1 Losartan (10 mg/kg/d). (B) Food intake (g 24h) for the same groups. (C) Food efficiency (%) for the same groups. (D) Energy metabolized (kJ 24h) for the same groups. (E) Energy loss via feces (kJ 24h) for the same groups. (F) Energy loss via urine (kJ 24h) for the same groups.
Fig. 3

- **Lean ZSF1**
- **Obese ZSF1**
- **Obese ZSF1 Colesevelam (2%)**
- **Obese ZSF1 SAR442357 (2%)**
- **Obese ZSF1 Losartan (10 mg/kg/d)**
- **Obese ZSF1 SAR442357 (4%)**

**A**

- Insulin (µg/L)
- Basal (11 w age)
- 2 months (20 w age)
- 3 months (24 w age)

**B**

- Random glucose (mmol/L)
- Basal (11 w age)
- 2 months (20 w age)
- 3 months (24 w age)

**C**

- HbA1c (%)
- Basal (11 w age)
- 2 months (20 w age)
- 3 months (24 w age)
Fig. 4

A

Fecal bile acids (μmol 24 h)

Basal 2 months 3 months
(@ 11 w age) (@ 20 w age) (@ 24 w age)

B

Urine bile acids (μmol 24 h)

Basal 2 months 3 months
(@ 11 w age) (@ 20 w age) (@ 24 w age)

C

Fecal free fatty acids (μmol 24 h)

Basal 2 months 3 months
(@ 11 w age) (@ 20 w age) (@ 24 w age)
**Fig. 5**

- **Lean ZSF1**
- **Obese ZSF1**
- **Obese ZSF1 Losartan (10 mg/kg/d)**
- **Obese ZSF1 Colesevelam (2%)**
- **Obese ZSF1 SAR442357 (2%)**
- **Obese ZSF1 SAR442357 (4%)**

**A**

**Triglycerides (mmol/L)**

- Basal (@ 10 w age)
- 2 months (@ 20 w age)
- 3 months (@ 24 w age)

**B**

**Cholesterol (mmol/L)**

- Basal (@ 10 w age)
- 2 months (@ 20 w age)
- 3 months (@ 24 w age)
Fig. 8

(A) Urine volume (ml 24h) at Basal, 2 months, and 3 months for Lean ZSF1 and Obese ZSF1 with treatments: Obese ZSF1 Colesevelam (2%), Obese ZSF1 SAR442357 (2%), Obese ZSF1 Losartan (10 mg/kg/d), and Obese ZSF1 SAR442357 (4%).

(B) Urine glucose (mmol 24h) at Basal, 2 months, and 3 months for Lean ZSF1 and Obese ZSF1 with treatments: Obese ZSF1 Colesevelam (2%), Obese ZSF1 SAR442357 (2%), Obese ZSF1 Losartan (10 mg/kg/d), and Obese ZSF1 SAR442357 (4%).

(C) Urine total protein (mg 24h) at Basal, 2 months, and 3 months for Lean ZSF1 and Obese ZSF1 with treatments: Obese ZSF1 Colesevelam (2%), Obese ZSF1 SAR442357 (2%), Obese ZSF1 Losartan (10 mg/kg/d), and Obese ZSF1 SAR442357 (4%).

(D) Albumin/Creatinine at Basal, 2 months, and 3 months for Lean ZSF1 and Obese ZSF1 with treatments: Obese ZSF1 Colesevelam (2%), Obese ZSF1 SAR442357 (2%), Obese ZSF1 Losartan (10 mg/kg/d), and Obese ZSF1 SAR442357 (4%).

(E) Urine KIM1 (pg 24h) at Basal, 2 months, and 3 months for Lean ZSF1 and Obese ZSF1 with treatments: Obese ZSF1 Colesevelam (2%), Obese ZSF1 SAR442357 (2%), Obese ZSF1 Losartan (10 mg/kg/d), and Obese ZSF1 SAR442357 (4%).

(F) Urine Cystatin C (μg 24h) at Basal, 2 months, and 3 months for Lean ZSF1 and Obese ZSF1 with treatments: Obese ZSF1 Colesevelam (2%), Obese ZSF1 SAR442357 (2%), Obese ZSF1 Losartan (10 mg/kg/d), and Obese ZSF1 SAR442357 (4%).
Fig. 11

Lean ZSF1

Obese ZSF1

Obese ZSF1 Losartan (10 mg/Kg/d)

Obese ZSF1 Colesevelam (2%)

Obese ZSF1 SAR442357 (2%)

Obese ZSF1 SAR442357 (4%)