The Novel Phosphate and Bile Acid Sequestrant Polymer SAR442357 Delays Disease Progression in a Rat Model of Diabetic Nephropathy[§]

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ABSTRACT

As a gut-restricted, nonabsorbed therapy, polymeric bile acid sequestrants (BAS) play an important role in managing hyperlipidemia and hyperglycemia. Similarly, nonabsorbable 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 Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) with SAR442357 resulted in enhanced sequestration of BAs and phosphate in the gut, improved glycemic control, lowering of serum cholesterol, and attenuation of diabetic kidney disease (DKD) progression. In comparison, colestevam, a BAS with poor phosphate binding properties, did not prevent DKD progression, whereas 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 of an 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 nonabsorbed 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 incidence 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, USRDS, 2015; 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 patients with DKD with

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ABBREVIATIONS: BA, bile acid; BAS, bile acid sequestrant; BES, N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; CKD, chronic kidney disease; CV, cardiovascular; DKD, diabetic kidney disease; ESRD, end-stage renal disease; FDR, false discovery rate; GC, glycocholic acid; GCDL, glycochenodeoxycholic acid; GI, gastrointestinal; HbA1c, Glycated Haemoglobin; KIM 1, Kidney Injury Molecule 1; PAA, poly(allylamine); PAS, periodic acid–Schiff; PDA, poly(diallylamine); T2D, type 2 diabetes; ZSF1, Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid.

ESRD compared with non-DKD ESRD (Giorda et al., 2018; Thomas, 2019). With nearly 160 million patients with DKD worldwide, it is projected there will be 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 and hypertension and use of renin-angiotensin-aldosterone system inhibitors and sodium-dependent glucose cotransporter-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 (Block et al., 1998, 2004; Goodman et al., 2000; Hruska et al., 2008). Therefore, pharmacological intervention to restrict phosphate absorption using phosphate binders is a mainstay of ESRD treatment. Sevelamer is a nonabsorbable polymeric phosphate binder used to lower serum phosphorus levels in patients with CKD on dialysis. Sevelamer also exhibits pleiotropic beneficial effects. For example, sevelamer improves lipid and glucose metabolism (Vlassara et al., 2012; Brønden et al., 2018), reduces inflammation and oxidative stress (Caglar et al., 2008; Sun et al., 2009; Navarro-González et al., 2011; Vlassara et al., 2012; Yilmaz et al., 2012; Rastogi, 2013; Brønden et al., 2020), clears uremic toxins (Garg et al., 2005; Vlassara et al., 2012), and prevents progression of aortic and coronary calcification (Chertow et al., 2002; Moe and Chen, 2004; Floege and Ketteler, 2004; Asmus et al., 2005; Rastogi, 2013). These benefits result in significant survival in sevelamer-treated patients compared with those receiving metal-based phosphate binders (Block et al., 2007; Di Iorio et al., 2012, 2013; Rodríguez-Ororio et al., 2015; Floege, 2016; Patel et al., 2016).

Colesevelam is a nonabsorbed 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 glycemia by eliciting changes in BAS-mediated modulation of nuclear farnesoid X bile acid receptor-dependent signaling pathways that regulate hepatic gluconeogenesis (Takebayashi et al., 2010; Handelsman, 2011; Kodera et al., 2011; Hansen et al., 2014; Inzucchi et al., 2015). Moreover, colesevelam enhances the conversion of intestinal BA toward secondary BAs, thereby stimulating secretion of the incretin, glucagon-like peptide 1 (GLP-1) from enteroendocrine L cells (Fuchs et al., 2018).

Colesevelam and sevelamer possess similar chemical attributes and thus bind similar substrates in the gut; however, with different binding affinities and capacities towards these substrates. 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 physicochemical properties as well as optimum binding affinity and capacity toward 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 of DKD using the ZSF1 rat model, which manifests disease progression and pathology of human DKD, including hypertension, obesity, hyperglycemia, and T2D mellitus (Bilan et al., 2011; van Dijk et al., 2016; Su et al., 2016; Dower et al., 2017).

Material and Methods

Synthesis of Hydrogels

Poly(allylamine hydrochloride) and poly(diallylamine hydrochloride) 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 made up of PAA and PDA components are described here using SAR442357 as the specific example. Detailed parameters for the syntheses of a series of such hydrogels are summarized in Supplemental Table 1. In a jacketed vessel with lid, a mixture of poly(allylamine hydrochloride) and poly(diallylamine hydrochloride) (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- μ m 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 minutes 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 <50 μ S/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 μ m).

Characterization of Hydrogels

Elemental analyses were carried out at Quantitative Technologies Inc (QTI) laboratories (Whitehouse, NJ). 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 deuterium oxide (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 (Polomoscianik et al., 2012). Total titratable amine 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 1.0 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 toward bile salts such as sodium salts of glycocholic acid (GC) and glycochenodeoxycholic acid (GCDCA) were determined according to a published procedure (Braunlin et al., 2002). Equilibrium binding capacities of the hydrogels for phosphate ions were measured in N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (^bBES) 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-week-old male Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) rats (Charles River, Kingston, NY) were housed under a 12-hour light/dark cycle (lights on at 6 AM) with ad libitum access to a standard diet for this animal model (modified PMI 5008 diet; Ssniff, Soest, Germany) and tap water. Body weight, tail blood, urine, and fecal basal values were collected at 10 to 11 weeks of age. Subsequently, the obese ZSF1 rats were randomized to four groups based on body weight, random blood glucose, ^aHbA1c (%), and urinary albumin. From 12 weeks of age, the corresponding groups were treated daily with losartan (by mouth, 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 diets were cold-pelleted. Selection of the doses used for the polymers was based on published literature on polymeric sequestrants (such as 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, two nonprogressor 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 (ACCUCHEK Aviva, model: NC; Roche Diagnostics, Mannheim, Germany) and HbA1c (%) with a Cobas 6000 analyzer (Roche Diagnostics). 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 an 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 minutes at room temperature, and centrifuged (4°C, 10 minutes, 4000 rpm) for serum collection. Serum triglycerides, total cholesterol, inorganic phosphate, urea, and creatinine were measured (Cobas 6000 analyzer; Roche Diagnostics). Measurements of serum triglycerides and total cholesterol at the 2-month time point for the obese ZSF1 SAR442357-treated (2%) group and 3-month time points for the obese ZSF1 colesevelam-treated (2%) group were not recorded because of the insufficient sample volume. One measurement for serum inorganic phosphate was missed at the 3-month time point for the obese ZSF1 colesevelam (2%) group and at the 2-month time point for the lean ZSF1 and obese ZSF1 (2% and 4%) groups because of the lack of adequate sample volume.

Serum insulin was measured with an ELISA kit (Mercodia rat/mouse insulin Fluorescent Immunoassay (FIA) number 10-1248-10) following the 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) with subsequent RNA quality control with an Agilent 2100 bioanalyzer using an Agilent RNA 6000 Nano Kit (Agilent technologies, Santa Clara, CA). RNAs with a RNA integrity number (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 was done on an Illumina NextSeq 550 (Illumina, Inc., San Diego, CA).

Ribonucleic acid sequence (RNA-Seq) data analysis was performed using ArrayStudio (QIAGEN Inc.). Briefly, raw data quality control is performed, followed by a filtering step to remove reads corresponding to ribosomal ribonucleic acids (rRNAs) as well as reads that have a low quality score or are shorter than 25 nucleotides. Reads were further mapped to the rat genome (rn6) using Omicsoft Sequence Aligner version 4 (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 the 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. Because of the technical issues during monitoring, one measurement for food consumption was excluded from the obese ZSF1 colesevelam (2%) group at 2- and 3-month time points as well as five 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 caloric value of 26.47 kJ g⁻¹ (Sigma-Aldrich, Darmstadt, Germany). Energy uptake (Eup) was determined as the product of food consumed and the caloric 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 (E_{met}), the energy content of feces and urine was subtracted from energy intake, calculated as $E_{\text{met}} = E_{\text{up}} - (E_{\text{loss fec}} + E_{\text{loss urine}})$ (Drozdz et al., 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). Because of the low urinary volume of the lean rats, we developed a procedure using cotton coils as a combustion aid (Heiland Vet GmbH, Germany). Depending on sample volume available, two different sizes of coils were used: 8 mm diameter $\times$ 19 mm length (for low volumes) and 10 mm $\times$ 19 mm (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 hours from single-housed rats using metabolic cages (model 3701M001; Techniplast, 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 solution (pH 11.0) with an Ultra-Turrax disperser (model Ultra-Turrax T25 basic; IKA-Works Inc., Wilmington, NC) to obtain a uniform slurry with a target concentration of 50 mg feces per milliliter 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. In total, 2 ml of aqueous potassium chloride solution (3.7 g/l) was added, and samples were incubated further for 1 hour and then centrifuged at 1500g for 10 minutes. The aqueous supernatant phase was removed, and its volume was determined and then stored at -20°C. Finally, samples were thawed at 40°C, centrifuged at 1500g for 10 minutes, and then analyzed for total bile acid, inorganic phosphate, and total nonesterified fatty acid 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 (model 3701M001; Techniplast) after 8 and 16 hours to have in total 24 hours of urine collection. Urine inorganic phosphate, glucose, total protein, albumin, and creatinine were measured using commercially available enzymatic assay kits and normalized by 24-hour urine volume (Beckman Coulter AU680; Beckman Coulter Inc.). Basal urine measurements were missed for one rat from the obese ZSF1 SAR442357 (2%) group because of the accidental loss of the sample. Furthermore, one basal value was missing from the lean ZSF1 control group because albumin was under the lower limit of detection, and one outlier value for inorganic

phosphate at the 2-month time point was removed from the obese ZSF1 losartan group.

Urine KIM1^d and cystatin C were measured with an ELISA kit (Quantikine ELISA, catalog number RKM100 for KIM1, catalog number MSCCTC0 for cystatin C; R&D Systems), following the manufacturer's guidelines. Basal urine measurements were missed for one rat from the obese ZSF1 SAR442357 (2%) group because of the 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 (model $\times$ 204; Mettler Toledo).

Histology and Pathologic Analysis

Kidneys were sampled and fixed in 4% neutral buffered formaldehyde for 48 hours and embedded in paraffin. Cross sections were obtained from each sample. Afterward, the dehydration of the tissue was done automatically with a vacuum infiltration processor (VIP) tissue processor (Sakura Tissue-Tek VIP 5 Tissue Processor; Sakura, Torrance, CA) for approximately 20 hours followed by the blocking procedure. Slice sections 4 μ m thick 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, Great Britain).

Hematoxylin and Eosin Staining. Tissue deparaffination was carried with xylene three times for 3 minutes, followed by rehydration in decreasing ethanol solutions of 100%, 95%, and 70% for 1 minute each. Staining of the nuclei was done with Haemalum from Mayer (blue) for 5 minutes, followed by rinsing with distilled water for 10 minutes and ascending ethanol solutions of 70% and 99%. Final tissue staining was done with eosin (red) for 30 seconds, and ethanol and xylene were used three times for dehydration before permanent covering.

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

For pathology analysis, whole-slide scanned (20 $\times$) images were evaluated qualitatively and semiquantitatively by two board-certified veterinary pathologists (K.S. and D.S.B.). Criteria for semiquantitative evaluation included assessment based on the severity of a morphologic change and the extent of tissue involvement. For each rat, four parameters were assessed independently: 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 morphologic changes in more than 75% glomeruli, up to 40% tubules, and contained relatively medium-sized aggregates of inflammatory cells along with the interstitial fibrosis. Grade 5 was assigned when more than 40% tubules exhibited morphologic changes. Model-related unilateral or bilateral hydronephrosis was observed in multiple animals. Sections exhibiting hydronephrotic changes were excluded from

Fig. 1. Representative structure of SAR442357. X is the counter anion of the acid, in this case HCO₃⁻ (bicarbonate)

histopathological assessment, such as sections from one obese ZSF1 rat treated with colesevelam (2%).

Statistical Analysis

Results are represented as means $\pm$ S.D. or medians $\pm$ range for histologic 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) with two-way ANOVA followed by Dunnett's multiple comparisons test. $P \leq 0.05$ 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 correction (Benjamini and Hochberg, 1995). $FDR \leq 0.05$ was considered significant.

Kidney weight and histologic 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. $P \leq 0.05$ was considered significant.

Results

Synthesis and Characterization of Sequestrant Polymers

Sequestrants of phosphate and bile salts represent a class of amine-containing (cationic) water-swallowable 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 nonabsorbable 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 strategy in which the effects of variation of experimental parameters such as relative amounts of PAA, PDA, and epichlorohydrin in the polymer hydrogels were assessed. Toward that end, a library of 21 polymer hydrogels of different compositions were synthesized. The resulting hydrogels were characterized by using different physical and chemical techniques. The results of

characterization and in vitro substrate binding properties of these hydrogels are summarized in the supporting information (Supplemental Table 1). High-resolution two-dimensional solid-state NMR spectroscopy was used to systematically characterize the molecular structure of SAR442357. In particular, two-dimensional ¹H-¹³C correlation NMR spectroscopy (Supplemental Fig. 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. GC and GCDC were used as representative bile acids for in vitro binding experiments. Based on optimized physicochemical properties and in vitro substrate binding properties (Supplemental Table 1), SAR442357 was selected as the lead sequestrant for further pharmacological evaluation.

The key binding parameters for colesevelam, the two homopolymers of PAA and PDA, and SAR442357 are shown in Table 1. The chemical structure of colesevelam comprises an epichlorohydrin crosslinked PAA backbone with pendant hydrophobic alkyl chains tethered to the amine groups of PAA (Steinmetz and Schonder, 2005). SAR442357 maintains the good substrate binding properties of the PAA homopolymer hydrogel, yet with a lower swell index. The favorable swelling index of SAR442357 compared with 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-month period of the study (from 10 to 24 weeks of age), lower body weight compared with the obese ZSF1 control group.

Three months of treatment of the obese ZSF1 rats with SAR442357 (2% or 4%) did not change body weight compared with the obese ZSF1 control group (Fig. 2A), although food intake was higher with SAR442357 4% from 2 months onward 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 onward for 4% SAR442357 treatment group and in the 2% SAR442357 (Fig. 2E) at 3 months compared with 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 onward compared with the obese ZSF1 control

TABLE 1
Synthesis and characterization of epichlorohydrin crosslinked PAA-PDA copolymer hydrogel networks

Polymer ID	Monomer or Copolymer	PAA/PDA Molar Ratio	Swelling Index	In Vitro Binding		
				GC Bile Acid Binding	GCDC Bile Acid Binding	Phosphate Binding
PAA	Poly(allylamine) homopolymer	1/0	11.9	g/g 1.89	g/g 2.43	mmol/g 6.07
PDA	Poly(diallylamine) homopolymer	0/1	15.1	1.41	2.28	2.62
SAR442357	PAA-PDA copolymer	14.3/1	8.1	1.96	2.46	5.65
Colesevelam	Hydrophobically modified PAA	1/0	6.4	0.77	1.64	1.29

group. Energy loss via feces and urine was consistently low at all measured time points (Fig. 2, E and F).

No changes in body weight, food intake, food efficiency, metabolized energy, or 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. 2, A–D, F). Regarding energy loss via feces, only the obese ZSF1 colesevelam-treated (2%) group showed higher energy loss from 2 months onward, similar to the SAR442357-treated groups, compared with 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 with the obese ZSF1 control group (Fig. 3).

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 with the obese ZSF1 control group. Furthermore, SAR442357 (2% or 4%) reduced HbA1c (%) starting at 2 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 with the ZSF1 obese control group.

The losartan (10 mg/kg) group did not show differences in these metabolic parameters compared with 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 acid levels along the treatment period. Fecal bile acids were lower in the lean ZSF1 control group from 2 months onward, and urine bile acids were lower at all measured time points compared with the obese ZSF1 control group (Fig. 4, A and B). Fecal free fatty acids were also lower at all measured time points (Fig. 4C).

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

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

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. 5).

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% colesevelam and 4% SAR442357 compared with the obese ZSF1 control group. However, this effect on cholesterol waned after 3 months with 2% colesevelam. 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 onward (Fig. 5B).

Hepatic Gene Expression for Cholesterol, Bile Acids, and Fatty Acid Synthesis Pathways

To test for hepatic effects of the different treatments, ribonucleic acid sequence analysis was performed on liver samples taken at study termination. The global number of significantly regulated genes for the different treatment groups compared with the obese ZSF1 control group is illustrated in volcano plots in Supplemental Fig. 2. Genes that were downregulated in the obese ZSF1 control group and recovered upon treatment with either colesevelam or SAR442357 are summarized in a heat map (Supplemental Fig. 3). 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 (*Acl*, *Acaca*, *Acacb*, *Fasn*) synthesis pathways were higher in those animals treated with colesevelam (2%) and SAR442357 (2% or 4%) compared with the obese ZSF1 control group (Fig. 6). In all cases, there was a trend toward higher levels with SAR442357 (4%) compared with 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 was lower in the lean ZSF1 control group at all measured time points and serum inorganic phosphate from 2 months on compared with the obese ZSF1 control group (Fig. 7).

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 with 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 onward with SAR442357 (2% or 4%) in a dose-dependent manner compared with 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 decrease in serum inorganic phosphate at 2 months compared with the obese ZSF1 control group (Fig. 7C).

Treatment with losartan (10 mg/kg) had no effect on inorganic phosphate levels in any of the biologic samples 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 with the obese ZSF1 control group (Fig. 8).

Urinary volume and glucose were decreased only with SAR442357 (4%) after 3 months of treatment compared with the obese ZSF1 control group (Fig. 8, A and B). SAR442357 (4%) decreased urinary total protein from 2 months onward. Similarly, after 3 months, both losartan (10 mg/kg) and SAR442357 (2%) decreased urine total protein compared with

Fig. 2. Body weight (A) and food efficiency (C). Further daily parameters analyzed were food intake (B), metabolized energy (D) and energy loss either via feces (E) or via urine (F). Values are represented as means $\pm$ S.D. Treatment effect versus the obese ZSF1 control group analyzed with two-way ANOVA with Dunnett'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.

Fig. 3. Diabetes progression as observed on the blood parameters insulin (A), random fed blood glucose (B) and HbA1c (C). Values are represented as means $\pm$ S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnett'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% $n = 9$ to 10, obese ZSF1 SAR442357 4% $n = 10$).

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 onward 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 onward compared with the obese ZSF1 control group (Fig. 8E).

SAR442357 (4%) decreased urine cystatin C from 2 months onwards, and at 3 months, both losartan (10 mg/kg) and SAR442357 (2%) decreased cystatin C compared with 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 Fig. 7) at the end of the study.

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

Serum urea concentration was significantly lower in lean ZSF1 rats compared with the obese ZSF1 control animals. Furthermore, it significantly decreased in obese ZSF1 rats treated with losartan (10 mg/kg) and SAR442357 (4%) (Supplemental Fig. 7A). 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 Fig. 7B).

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 and 11; Supplemental Fig. 6). Although 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, synechiae and crescent formation, splitting and thickening of Bowman's capsule, thickened capillary walls and lower luminal diameter, lobulation of glomerular tufts, obliteration of

Fig. 4. Fecal (A) and urine (B) bile acids and fecal free fatty acids (C). Values are represented as means $\pm$ S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnett'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 $n = 9$ to 10, obese ZSF1 SAR442357 2% $n = 9$ to 10, obese ZSF1 SAR442357 4% $n = 10$).

Fig. 5. Serum triglycerides (A) and total cholesterol (B). Values are represented as means $\pm$ S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnett'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 $n = 9$ to 10, obese ZSF1 SAR442357 2% $n = 9$ to 10, obese ZSF1 SAR442357 4% $n = 10$).

glomerular spaces with eosinophilic material, and regional-to-global obsolescence. Tubulointerstitial changes included dilation of cortical and medullary tubules, presence of intra-luminal 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 with 4% SAR442357, rats treated with losartan (10 mg/kg per day) showed progression of glomerular pathology, whereas the treatment effects on assessed

Fig. 6. Hepatic gene expression for cholesterol (A, B, C), bile acids (D), fatty acid synthesis (E, F, G, H) and cholesterol endocytosis (I) pathways. Values represented as fold change vs. 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% $n = 10$). 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Hmgcr); Mevalonate diphosphate decarboxylase (Mvd); Squalene monooxygenase (Sqle); ATP citrate lyase (Acly); Acetyl-CoA carboxylase alpha (Acaca); Acetyl-CoA carboxylase beta (Acacb); Fatty acid synthase (Fasn)

Fig. 7. Fecal (A), urine (B) and serum (C) inorganic phosphate. Values are represented as means $\pm$ S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnett's multiple comparisons test for statistical differences; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (lean ZSF1 $n = 9$ to 10, obese ZSF1 $n = 8$, obese ZSF1 losartan $n = 9$ to 10, obese ZSF1 colesevelam $n = 9$ to 10, obese ZSF1 SAR442357 2% $n = 9$ to 10, obese ZSF1 SAR442357 4% $n = 9$ to 10).

parameters such as tubular protein casts, tubular pathology, and interstitial inflammation were comparable (Figs. 10 and 11; Supplemental Fig. 6). Similarly, rats treated with 2% colesevelam exhibited no improvement in glomerular pathology, tubular protein casts, and tubular pathology parameters when compared with the treatment with 4% SAR442357 (Figs. 10 and 11; Supplemental Fig. 6).

Discussion

The goal of this investigation was to evaluate pharmacological benefits of a nonabsorbed polymeric sequestrant

(SAR442357) that possesses 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 colesevelam, a clinically approved BAS (with poor phosphate binding characteristics) did not offer any therapeutic benefits for DKD. However, unlike losartan, both colesevelam and SAR442357 improved glycemia in these animals. This was evident from an

Fig. 8. Diabetic nephropathy progression as observed on the urinary parameters volume (A), glucose (B), total protein (C), albumin/creatinine ratio (D) as well as the biomarkers KIM1 (E) and cystatin (F). Values are represented as means $\pm$ S.D. Treatment effect vs. the obese ZSF1 control group analyzed with two-way ANOVA with Dunnett'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 $n = 9$ to 10, obese ZSF1 colesevelam $n = 9$ to 10, obese ZSF1 SAR442357 2% $n = 9$ to 10, obese ZSF1 SAR442357 4% $n = 10$).

Fig. 9. Kidney weight. Values are represented as means $\pm$ 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% $n = 10$).

increase in plasma insulin and decrease in plasma glucose and HbA1c in the colesevelam- 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 low density lipoprotein receptor pathway and an overall decrease in blood cholesterol. Moreover, BASs such as colesevelam have been shown to improve hyperglycemia in preclinical animal models of diabetes (Herrema et al., 2010; Sedgeman et al., 2018) as well as in patients with T2D (Takebayashi et al., 2010; Handelsman, 2011; Nerild et al., 2018). Various mechanisms have been proposed for the antidiabetic properties of BASs, including increased secretion of glucagon-like peptide 1 through activation of Takeda G protein-coupled Receptor 5 (TGR5) and decreased activation of intestinal nuclear farnesoid X bile acid receptor (Herrema et al., 2010; Takebayashi et al., 2010; Potthoff et al., 2013). Although colesevelam has been reported

to improve glycemia in diabetes models such as Zucker Diabetic Fatty (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, colesevelam exhibited a modest effect on improving glycemic control and progression of renal dysfunction in comparison with 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 with 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 patients with diabetes and kidney disease. Numerous preclinical and clinical studies have shown that there is a linear correlation between serum phosphate and T2D, DKD, and coronary artery disease (Hutchison et al., 2011; Streja et al., 2013; Vervloet et al., 2017). Removal of dietary phosphate from the 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 with colesevelam and losartan.

In terms of chemical structure, SAR442357 is different from colesevelam. The former contains pendant primary and

Fig. 10. Kidney histopathology scores for tubular protein casts (A), tubular pathology (B), glomerular pathology (C) and interstitial inflammation (D). Values are represented as medians 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% $n = 10$).

Fig. 11. Kidney histopathology. Compared with the lean ZSF1 rat, kidney from control obese ZSF1 rat shows glomeruli surrounded by a thick PAS-positive Bowman's capsule (blue arrow) with regional splitting (yellow arrow), thickened glomerular mesangium, narrow capillary lumina, and synechiae 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 tubulointerstitial pathology. Note the relative lack of interstitial inflammatory cells in rats treated with colesevelam (2%) or SAR442357. PAS stain. Magnification, 200 $\times$.

secondary amine groups, with the latter being part of a rigid six-membered ring structure (Fig. 1). On the other hand, in addition to a limited number of primary amine groups, colesevelam contains pendant hydrophobic alkyl chains and quaternary ammonium groups (Steinmetz and Schonder 2005). Presence of higher amounts (millimoles per gram) of primary amine groups in SAR442357 manifests its substantially higher in vitro binding capacity for phosphate ions (5.65 mmol/g polymer) compared with 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 colesevelam mirror the observed in vivo potencies. Thus, after 2 months of treatment, although SAR442357 treatment resulted in decreased urinary phosphate excretion in a dose-dependent manner (Fig. 7B, $P < 0.05$), urinary phosphate lowering in colesevelam-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 with colesevelam. It may be noted that a clinically approved nonabsorbed 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 (Conti et al., 2006; Herget-Rosenthal et al., 2007; Kim et al., 2013; Pais et al., 2019; Fernando et al., 2019). 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 3 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, although 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, it did not show any measurable impact on glycemia. In addition to lowering serum cholesterol, SAR442357 treatment resulted in a decrease in urine volume, urine total protein, albumin/creatinine ratio, KIM1, and cystatin C and slowed 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 nonabsorbed 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 sodium-dependent glucose cotransporter-2 inhibitor empagliflozin reported in ZSF1 rats (Park et al., 2020). The latter has been approved as a treatment option for patients with CKD/DKD. 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 nonabsorbed, this compound could be a safe and effective drug candidate to treat DKD.

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