Antihypertensive and Laxative Effects by Pharmacological Inhibition of Sodium-Proton-Exchanger Subtype 3–Mediated Sodium Absorption in the Gut

Dominik Linz,* Klaus Wirth,* Wolfgang Linz, Hubert O. O. Heuer, Wendelin Frick, Armin Hofmeister, Uwe Heinelt, Petra Arndt, Uwe Schwahn, Michael Böhm, Hartmut Ruetten

Abstract—High intestinal sodium absorption is one mechanism of hypertension and constipation. The sodium-proton-exchanger subtype 3 (NHE3) is an important mediator of sodium absorption in the gut. SAR218034 (SAR) is an orally nonabsorbable specific NHE3 inhibitor. The effect of SAR (1 mg/kg per day in chow) on feces sodium excretion, systolic blood pressure via tail cuff, and gene expression of NHE3 in the gut were studied in senescent lean hypertensive rats (spontaneously hypertensive rats-lean, loaded with NaCl 0.7% in drinking water) and in hypertensive, obese, and hyperinsulinemic rats (spontaneously hypertensive rats-obese, not loaded with NaCl). In spontaneously hypertensive rats-lean, inhibition of intestinal NHE3 by SAR increased feces sodium excretion and reduced urinary sodium excretion, whereas absolute sodium balance and serum sodium concentration were not changed. This suggests reduced intestinal sodium absorption in SAR-treated animals and was associated with increased feces water content (58% versus 42% in placebo treated animals; P=0.0001) and reduction in systolic blood pressure from 222±7 to 198±2 mm Hg (P=0.0001). Angiotensin-converting enzyme inhibition by ramipril plus NHE3 inhibition resulted in an additive blood pressure–lowering effect. In spontaneously hypertensive rats-obese, SAR lowered systolic blood pressure but did not modify serum insulin or cholesterol levels. Gene expression of NHE3 was upregulated in the ileum and colon but not in the jejunum of SAR-treated rats. Reduction of intestinal sodium absorption by selective NHE3 inhibition in the gut reduces high blood pressure and increases feces water excretion. Intestinal NHE3 blockade could be a new treatment strategy for elderly patients suffering from high blood pressure and constipation. (Hypertension. 2012;60:1560-1567.) ● Online Data Supplement

Key Words: sodium-proton-exchanger subtype 3 inhibition ■ intestinal sodium absorption ■ constipation ■ blood pressure ■ spontaneously hypertensive rats ■ obese spontaneously hypertensive rats

A
terial hypertension is the leading cause of cardiovascular diseases, including stroke, heart failure, and coronary heart disease.1 Salt plays a major role in the regulation of blood pressure and is one of the most critical factors for hypertension and stroke.2,3 There is a considerable body of evidence linking higher salt intake with higher blood pressure and increased cardiovascular risk.4 The sodium-proton-exchanger subtype 3 (NHE3) is highly expressed at the apical membrane of the intestine and colon and mainly regulates intestinal salt and water absorption in the gut.5,6 Enhanced sodium absorption favors high blood pressure in the elderly patients.3 Other than hypertension, increased absorption of sodium in the gut reduces feces water content and causes constipation.4 Constipation can be complicated by fecal impaction and incontinence, particularly in elderly people with reduced mobility.2 Pharmacological inhibition of the intestinal NHE3 might lead to reduced sodium absorption and could be therapeutically equivalent to well-known benefits of dietary sodium restriction.4,8–10 Additionally, not-absorbed sodium might bind water in the gut resulting in a laxative effect. Consistently, NHE3-knockout mice show loose stools and a lower blood pressure compared with wild-type mice.11

Because reduced sodium absorption in the gut is an interesting therapeutic target for hypertension and constipation, we tested the use of intestinal NHE3 inhibition in 2 hypertensive rat models. We used senescent lean spontaneously hypertensive rats (SHR-lean) loaded with sodium chloride via drinking water and treated them with a nonabsorbable specific NHE3 inhibitor, SAR218034 (SAR) with and without the angiotensin-converting enzyme (ACE) inhibitor ramipril (RAM). Because insulin stimulates the NHE3 exchanger,12,13 we additionally tested the effect of long-term treatment with the NHE3 inhibitor in obese hyperinsulinemic SHR (SHR-ob).14

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Materials and Methods
All animal studies were performed in accordance to the German law for the protection of animals.

IC₅₀ and Intestinal Absorption Determination
IC₅₀ values of SAR were determined by fluorometric imaging plate reader measurements in NHE-deficient mouse fibroblast cell line lipopolysaccharide-associated protein 1 with transfected NHE1, NHE3, and NHE5 cDNAs (please see the online-only Data Supplement). Pharmacokinetic studies were done in 6 Sprague Dawley rats to obtain a plasma drug concentration versus time plot for SAR (1 mg/kg) after both intravenous and oral administration. SAR plasma concentration was determined every hour up to the 24th hour. The absolute bioavailability was the dose-corrected area under curve non-intravenous divided by area under curve intravenous. To further characterize intestinal absorption, Caco-2/TC7 cell line experiments were performed as described elsewhere.

Vascularly Perfused In Situ Preparations of the Small Intestine
To investigate intestinal sodium and water transport, vascularly perfused in situ half-open colon/intestinal reperfusion preparations of the small intestine were used as described elsewhere (please see the online-only Data Supplement).

In Vivo Experiments
Dose-dependent increase of intestinal sodium excretion and water content of feces by SAR (0.07, 0.23, and 0.70 mg/kg) was investigated in 24 Sprague Dawley rats, 6 rats per group. Individual rats were loaded with NaCl 3% in drinking water. Twenty-four-hour sodium excretion after 7 days oral feeding with SAR was investigated.

Seventy-eight-week-old SHR-lean were randomized in 4 groups (n=14) and treated for 14 weeks: (1) placebo (SHR), (2) NHE3 inhibitor (1 mg/kg per day in chow; SAR), (3) ACE inhibitor (RAM; 1 mg/kg per day via drinking water), and (4) SAR+RAM. Metabolic investigations (plasma, feces, and urine samples) and blood pressure measurements were performed in the 92nd week of age (please see the online-only Data Supplement). SHR-lean were loaded with NaCl 0.7% in drinking water.

Male, 8-week-old SHR-ob (Charles River Laboratories) were randomized in 2 groups, placebo (n=7) and NHE3 inhibitor (1 mg/kg per day in chow; SAR, n=8) and treated for 6 weeks. Metabolic investigations (plasma, feces, and urine samples) and blood pressure measurements were performed (please see the online-only Data Supplement). SHR-ob were not loaded with NaCl in drinking water.

Statistical Analysis
Data are presented as mean±SEM. Depending on the homogeneity of variances (Levene test), significant differences were calculated by a 1-way ANOVA followed by a post hoc Dunn test. Kruskal-Wallis test was used, if variances were not homogeneous. When testing for differences between repeated measured parameters, a 2-way ANOVA (for repeated measures) followed by a post hoc Dunnett test was used. For repeated measured parameters, a rank transformation was performed if variances were not homogeneous. For all statistical calculations, software Everstat V5 based on SAS 8 was used. *P<0.05 was considered to be statistically significant.

Results
Expression Profile of NHE3
A screening of the rat NHE3 expression profile revealed highest expression in the proximal tubule of the kidney, whole small intestine, and colon (Figure 1A). Amino acid sequence comparison revealed high homologies between orthologous NHE proteins in rats and humans ranging from 90% to 98% identity (data not shown).

Characterization of NHE3 Inhibitor SAR218034
SAR218034 (SAR; Figure 1B) is a new NHE3 inhibitor. SAR has a molecular weight of 512.4 (free base). The inhibitory potency of SAR was tested on different recombinant NHE cell lines expressing NHE3 from human and rat, human NHE1, human NHE2, human NHE3, human NHE4, mouse NHE1, mouse NHE3, mouse NHE5, rat NHE1, rat NHE3, and rat NHE5.

Figure 1. A. Rat sodium-proton-exchanger subtype 3 (NHE3) gene expression profiling (n=6). B. Chemical structure of the selective nonabsorbable NHE3 inhibitor SAR218034 (SAR). C. Dose-dependent intestinal sodium excretion by SAR and D. dose-dependent intestinal water excretion by SAR in sodium-loaded normotensive Sprague Dawley rats (6 rats per dosage). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.
and human NHE5. Dose-dependent inhibition resulted in highly potent IC$_{50}$ values on human NHE3 with an IC$_{50}$ of 12 nmol/L and on rat NHE3 with an IC$_{50}$ of 22 nmol/L. Inhibition of human NHE5 resulted in an IC$_{50}$ of 0.97 µmol/L showing 80-fold less potency compared with NHE3. On human NHE1, there was a 1000-fold less potent IC$_{50}$ with 12.4 µmol/L found. Oral bioavailability of SAR is <1%. An oral dose of 1 mg/kg corresponded with a plasma concentration of 1 nmol/L. This was 13-fold below the rat IC$_{50}$ value for NHE3. In a permeability assay (Caco-2/TC7 cells), SAR showed a very low value of 0.27 x 10$^{-7}$ cm/s (Table 1). The compound was synthesized within Sanofi.

Effect of SAR on Feces Sodium and Water Excretion

In situ half-open colon/intestinal perfusion experiments, SAR increased sodium concentration (155±2 versus 148±1 mmol/L in controls; P<0.01) and volume (9.2±0.2 versus 8.3±0.1 mL in controls; P<0.01) of perfusate at the end of a 3-hour perfusion period. Oral treatment for 7 days with SAR (0.01–0.30 mg/kg) dose-dependently increased feces sodium concentration and water content in sodium-loaded normotensive Sprague Dawley rats (Figure 1C and 1D).

Effect of Long-Term Intestinal NHE3 Inhibition in SHR-Lean

In Figure 2, the effect of long-term intestinal NHE3 inhibition with or without RAM and of RAM alone on oral sodium intake (top) and urinary and feces sodium excretion (bottom) is shown. Food consumption did not differ between the groups, but water consumption (0.7% sodium content) was higher in SHR-treated SHR-lean. In SHR-treated animals, feces sodium excretion was increased and urinary sodium excretion was reduced. This suggests reduced intestinal sodium absorption in SAR-treated animals. Individual total oral sodium intake and total sodium excretion were similar in all groups. Changes in sodium excretion in SAR-treated SHR-lean were not modified by RAM cotreatment. Intestinal inhibition of NHE3 reduced systolic blood pressure and showed an additive effect in combination with RAM (Figure 3A). Heart rate was similar in all groups (Figure 3B). SAR increased feces water content in SHR-lean and in RAM-treated SHR-lean (Figure 3C). However, total water intake and total water excretion did not differ within groups. Sodium serum concentration was similar in all groups (Figure 3D). RAM alone had no effect on feces water content or heart rate but reduced systolic blood pressure. Long-term intestinal inhibition of NHE3 by SAR moderately increased aldosterone plasma concentration (Figure 4A) or renin (Figure 4B) and ACE plasma activity (Figure 4C). RAM alone and SAR in combination with RAM reduced ACE activity, increased renin activity, and reduced aldosterone concentration in SHR-lean to a similar extent. Low-sodium diet (0.01 g NaCl/100 g of rat chow) for 10 days reduced urinary sodium excretion and reduced blood pressure in SHR-lean (Table S1, please see the online-only Data Supplement).

Effect of Long-Term Intestinal NHE3 Inhibition in SHR-ob

Figure 5 summarizes the effect of intestinal NHE3 inhibition for 6 weeks on oral sodium intake (top) and urinary and feces sodium excretion (bottom) in SHR-ob. SAR treatment in SHR-ob did not modify oral sodium intake. Drinking water did not contain sodium in this study (0% sodium content). In SAR-treated SHR-ob, feces sodium excretion was increased and urinary sodium excretion was reduced, suggesting reduced intestinal sodium absorption in SAR-treated animals. Individual total oral sodium intake and total sodium excretion were similar within all groups. Inhibition of intestinal NHE3-mediated sodium absorption resulted in a significant reduction of systolic blood pressure (Figure 6A). Heart rate remained unchanged (Figure 6B). Feces water content was significantly

### Table 1. Characterization of NHE3 Inhibitor SAR218034

<table>
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<tr>
<th>Characterization</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Molecular weight</td>
<td>512.4 (free base)</td>
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<tr>
<td>Oral bioavailability</td>
<td>&lt;1%</td>
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<tr>
<td>Permeability assay (Caco-2/TC7)</td>
<td>0.27 x 10$^{-7}$ cm/s</td>
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<table>
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<tr>
<th>IC$_{50}$ values</th>
<th>Value</th>
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<tr>
<td>Rat NHE3</td>
<td>22 nmol/L</td>
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<tr>
<td>Human NHE3</td>
<td>12 nmol/L</td>
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<tr>
<td>Human NHE1</td>
<td>12.4 µmol/L</td>
</tr>
<tr>
<td>Human NHE5</td>
<td>0.97 µmol/L</td>
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</table>

NHE3 indicates sodium-proton-exchanger.

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Figure 2. Effect of long-term treatment for 14 weeks with SAR218034 (SAR) with or without the angiotensin-converting enzyme (ACE)-inhibitor ramipril (RAM) and of RAM alone in spontaneously hypertensive rats (SHR)-lean (n=14/group) on oral water (grey) and chow (black) sodium intake (top), total bars represent cumulative sodium intake) and urinary (grey) and feces (black) sodium excretion (bottom), total bars represent cumulative sodium excretion; 0.7% sodium content in drinking water). n.s. indicates not significant.

Figure 5 summarizes the effect of intestinal NHE3 inhibition for 6 weeks on oral sodium intake (top) and urinary and feces sodium excretion (bottom) in SHR-ob. SAR treatment in SHR-ob did not modify oral sodium intake. Drinking water did not contain sodium in this study (0% sodium content). In SAR-treated SHR-ob, feces sodium excretion was increased and urinary sodium excretion was reduced, suggesting reduced intestinal sodium absorption in SAR-treated animals. Individual total oral sodium intake and total sodium excretion were similar within all groups. Inhibition of intestinal NHE3-mediated sodium absorption resulted in a significant reduction of systolic blood pressure (Figure 6A). Heart rate remained unchanged (Figure 6B). Feces water content was significantly
increased in SAR-treated rats (Figure 6C). Serum sodium concentration did not differ between groups (Figure 6D). Body weight was not changed by SAR. Water consumption was increased in NHE3 inhibitor–treated SHR-ob when compared with the placebo-treated SHR-ob. Serum insulin, glucose, or hemoglobin A1c, and lipids were not changed by NHE3 inhibitor treatment. SAR did not change potassium serum concentration (Table 2). Gene expression of NHE3 was upregulated in the ileum (Figure 7A) and jejunum (Figure 7B) of SAR-treated SHR-ob rats. Changes in gene expression of NHE3 in colon (Figure 7C) of SAR-treated SHR-ob rats just failed significance.

**Discussion**

This is the first study showing that inhibition of intestinal sodium absorption by selective pharmacological inhibition of the NHE3 exchanger in the gut reduces systolic blood pressure and increases feces water content in 2 hypertensive rat models. Long-term treatment with the nonabsorbable NHE3 inhibitor SAR does not cause hyperkalemia or metabolic disarranges.

In rats, NHE3 is expressed predominantly in the kidney and intestinal segments. NHE3 plays a central role in absorptive functions that profoundly influence systemic electrolyte and blood pressure homeostasis. The new NHE3 inhibitor SAR demonstrated high potency and selectivity and has approximately equivalent inhibitor action on rat and human NHE3. SAR did not interact with the physiological functions of human NHE5 or NHE1 isoforms. Amino acid sequence comparison revealed high homologies between orthologous NHE proteins in rats and humans ranging from 90% to 98% identity. Therefore, IC50 values for different NHEs should not differ significantly between species.

The bioavailability of SAR is very low as demonstrated in a permeability assay (Caco-2 in TC7 cells) and in oral bioavailability studies. An oral dose of 1 mg/kg as used in our study corresponds to a maximal plasma concentration of ≈1 nmol/L. This is 13-fold below the IC50 value for rat NHE3. Derivatives of SAR with lower

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**Figure 3.** Effect of long-term treatment for 14 weeks with SAR218034 (SAR) with or without ramipril (RAM) and of RAM alone on (A) blood pressure, (B) heart rate, (C) feces water content, and (D) serum sodium concentration in spontaneously hypertensive rats (SHR)-lean (n=14/group; 0.7% sodium content in drinking water).

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**Figure 4.** Effect of long-term treatment for 14 weeks with SAR218034 (SAR) with (n=14) or without (n=14) ramipril (RAM) and of RAM alone (n=14) on (A) plasma aldosterone concentration and (B) renin and (C) angiotensin-converting enzyme (ACE) activity in spontaneously hypertensive rats (SHR)-lean.
bioavailability demonstrated less potency and selectivity for NHE3. Highly absorbable NHE3 inhibitors with comparable IC50 values for NHE3 resulted in a less pronounced intestinal sodium excretion compared with SAR in pilot experiments (unpublished data). NHE3 is located on the apical plasma membrane and may have an extracellular domain exposed intraluminally with which SAR can interact. This explains the increased efficacy of the nonabsorbable NHE3 inhibitor SAR compared with a highly absorbable NHE3 inhibitor in the gut. Additionally, in vascularly perfused in situ preparations of the small intestine, intraluminal SAR application increased sodium concentration and volume of the perfusate. This underlines the local effect of SAR at the luminal intestinal membrane. The observed changes in sodium excretion in our study suggest an isolated effect of NHE3 inhibition by SAR in the gut. In this study, we measured very low SAR plasma concentrations. However, SAR levels may be higher in the renal tubule because of water extraction. NHE3 inhibition in the kidney by biological relevant SAR levels would have theoretically resulted in increased urinary sodium excretion and in a diuretic effect. Additionally, NHE3 inhibition in the kidney should have resulted in an increase in urine pH. By contrast, we observed reduced urinary sodium excretion and lower pH in SAR-treated animals possibly representing a compensatory mechanism mediated by the kidney to correct for acidosis and hyponatremia in NHE3 inhibitor–treated animals. A comparable reduction in renal sodium excretion was also observed in SHR-lean fed with low-sodium diet. In addition, with very potent and orally highly bioavailable NHE3 inhibitors, we could not see a significant influence on renal function, for example, natriuresis (unpublished data). This is the reason that no renal-specific NHE3 inhibitor was developed for clinical use. Altogether, we suggest that SAR218034 can be considered to be a functionally nonabsorbable NHE3 inhibitor suitable to block NHE3 locally in the gut.

Enhanced activity of intestinal NHE3 increases salt and water absorption in the gut, possibly resulting in hypertension.

**Figure 5.** Effect of long-term treatment for 6 weeks with SAR in spontaneously hypertensive rats (SHR)-ob (n=7/group) on oral sodium intake (top, total bars represent cumulative sodium intake) and urinary (grey) and feces (black) sodium excretion (bottom, total bars represent cumulative sodium excretion; 0% sodium content in drinking water). n.s. indicates not significant.

**Figure 6.** Effect of long-term treatment for 6 weeks with SAR218034 (SAR) on (A) blood pressure, (B) heart rate, (C) feces water content, and (D) sodium serum concentration in spontaneously hypertensive rats (SHR)-ob (n=7/group; 0% sodium content in drinking water).
Reduced intestinal sodium absorption in SAR-treated animals was associated with lowered blood pressure in old SHR-lean, a rat model for genetic hypertension. Interestingly, a similar reduction in blood pressure was observed in SHR-lean fed with a low-salt diet resulting in a comparable change in urinary sodium excretion, suggesting that reduced sodium intake or absorption might partly explain blood pressure reduction by SAR. Because insulin stimulates the NHE3 exchanger,12,13 we additionally tested the effect of long-term treatment with the NHE3 inhibitor in SHR-ob.14 SAR resulted in a comparable effect on blood pressure in SHR-lean and SHR-ob, 2 hypertensive rat models, and in both, sodium-loaded and -nonloaded animals. Increased sodium concentration in the feces in SAR-treated animals binds water in the gut. A stool water content of 60% to 70% in SAR-treated animals results in a softer but still solid stool consistency and does not cause diarrhea. Our findings are in line with recent studies in NHE3 null mice, which showed increased feces water content and hypotension20 and confirm the role of intestinal NHE3 in the development of hypertension and constipation.

NHE3 regulation is modulated by a number of mechanisms.6 Most of the NHE3 regulations in the literature describe acute regulation that occurs within the time span of minutes of cellular activation. Chronic regulation of NHE3 mainly involves transcriptional modification of NHE3. In NHE3 inhibitor–treated SHR-ob, we found an upregulation of NHE3 gene expression in the ileum and colon but not in the jejunum. However, our functional measurements after long-term SAR treatment suggest that the observed NHE3 upregulation does not limit the blood pressure–lowering and laxative effects of SAR. Whether SAR treatment results in changes in renal NHE3 expression needs to be investigated in future studies. These findings have several therapeutic implications. Up to now, saluretics are effectively used to treat high blood pressure and to improve congestion in congestive heart failure, but their use is often limited by constipation.21 We provide a novel alternative treatment strategy to reduce sodium and water load by selective NHE3 inhibition in the gut. In SHR-ob and SHR-lean, long-term intestinal NHE3 inhibition did not result in the development of hypokalemia, diabetic condition, or impaired lipid

Table 2. Effect of Intestinal NHE3 Inhibition on Metabolic Data in SHR-ob

<table>
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<th>Characteristics</th>
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<th>P Value</th>
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<td>Water consumption, mL/kg per day</td>
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<td>Glucose metabolism</td>
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<tr>
<td>Serum potassium, mmol/L</td>
<td>5.8±0.1</td>
<td>6.0±0.2</td>
<td>0.793</td>
</tr>
</tbody>
</table>

NHE indicates sodium-proton-exchanger; SHR, spontaneously hypertensive rats; LDL, low-density lipoprotein; HbA1c, hemoglobin A1c.

Figure 7. Effect of long-term treatment for 6 weeks with SAR218034 (SAR) on sodium-proton-exchanger subtype 3 (NHE3) gene expression in (A) ileum, (B) jejunum, and (C) colon in spontaneously hypertensive rats (SHR)-ob (n=7/group). Expression levels of NHE3 mRNA in every individual RNA sample were normalized to the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the same sample.
status. This is important because adverse effects such as worsening of the metabolic situation favoring a prodiabetic metabolism, hypokalemia favoring arrhythmogenic death, and worsening of kidney function occur during salidiuretic treatment.

We tested whether a combination of systemic ACE inhibition and intestinal NHE3 inhibition would have particular use. NHE3 inhibitor treatment can be safely combined with ACE inhibitors and the combination results in additive blood pressure-lowering effect. NHE3 inhibitor treatment reduced blood pressure by an increased fecal sodium and water content. A modification of fecal sodium and water content was not observed in ACE inhibitor–treated animals, suggesting a different independent mode of action.

Low-salt intake results in an activation of the renin-angiotensin-aldosterone system, which serves to conserve sodium at the level of the renal tubule via increased reabsorption of filtered sodium and possibly explaining reduced renal sodium excretion in SAR-treated animals. Although the statistical analysis data give evidence for no significant changes of the renin-angiotensin-aldosterone system, there is a clear trend toward increased renin activity and ACE activity as well as aldosterone in NHE3 inhibitor–treated SHR. However, most of the elderly patients are treated with ACE inhibitors or angiotensin receptor blockers reducing the adverse effect of renin-angiotensin-aldosterone system activation. The combination of ACE/angiotensin receptor blockers with NHE3 inhibitor treatment should be beneficial.

Our study might have some limitations. For blood pressure measurement, we used the tail-cuff method rather than telemetry. Telemetry would have added further information about blood pressure pattern. Terminal clearance experiments examining renal filtration and tubular function were not investigated in our study, but should be addressed in further experiments.

Conclusion and Perspectives
Selective inhibition of NHE3-mediated sodium absorption in the gut has the potential to reduce high blood pressure and can be safely combined with ACE inhibitor treatment. In addition, NHE3 inhibition in the gut may display a laxative effect in elderly patients suffering from constipation. Contrary to diuretics, NHE3 inhibitor treatment does not impair glucose metabolism and does not result in hypokalemia. These results show that SAR218034 inhibiting intestinal NHE3-mediated sodium absorption has potential as a representative of a new class of antihypertensive drugs. Intestinal NHE3 inhibitors may be particularly useful as an adjunct to other therapies. The real potential of the NHE3 inhibitor is to help accomplish a truly low-salt intake from the gut, because it is notoriously difficult to sufficiently reduce salt in the human diet.

Acknowledgments
We thank Gerald Fischer, Silke Loy, Simone Stengelin, and Melanie Medem for helpful support. We thank Katarina Mertsch for the pharmacokinetic data and Jörg Peters (University of Greifswald) for the plasma renin activity and aldosterone data. W. Linz and H.O.O. Heuer designed and performed the study. D. Linz performed cardiovascular measurements and wrote the manuscript. P. Arndt generated and determined IC50 values on different NHE isoforms recombinant cell lines, and U. Schwahn performed the mRNA investigations. K. Wirth developed the theoretical concept and M. Böm gave his clinical input regarding the new treatment strategy. W. Frick, A. Hofmeister, and U. Heinelt synthesized and optimized the NHE3 inhibitor. H. Rutten designed the research.

Sources of Funding
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Disclosures
K. Wirth, W. Linz, H.O.O. Heuer, W. Frick, A. Hofmeister, U. Heinelt, P. Arndt, U. Schwahn, and H. Rutten are employees of Sanofi’s R&D Diabetes group and are involved in preclinical research and identification of new approaches in diabetes mellitus. The other authors have no conflicts to report.

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**Novelty and Significance**

**What Is New?**

- Selective inhibition of sodium-proton-exchanger subtype 3–mediated sodium absorption in the gut has the potential to reduce high blood pressure and to improve intestinal fluid homeostasis in elderly patients suffering from hypertension and constipation.

**What Is Relevant?**

- Contrary to diuretics, sodium-proton-exchanger subtype 3 inhibitor treatment does not impair glucose metabolism and does not result in hypokalemia or activation of the renin-angiotensin-aldosterone system.

**Summary**

- SAR218034 inhibiting intestinal sodium-proton-exchanger subtype 3 has potential as a representative of a new class of antihypertensive drugs. Intestinal sodium-proton-exchanger subtype 3 inhibitors may be particularly useful as an adjunct to other therapies.
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Antihypertensive and Laxative Effects by Inhibition of NHE3-Mediated Sodium Absorption in the Gut.

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Methods:

IC$_{50}$ determination:

Cell lines: The cDNAs of human NHE3 (hNHE3, Acc. No. NM_004174), NHE1 (hNHE1, Acc No. NM_003047), NHE5 (hNHE5, Acc No. NM_004594) and rat NHE3 (rNHE3, Acc. No. NM_012654) were cloned by reverse transcription-polymerase chain reaction (RT-PCR) from human and rat kidney mRNA. These cDNAs were cloned into the mammalian expression vector pMAMneo and transfected into the NHE-deficient mouse fibroblast cell line LAP1. Cells expressing the different NHE subtypes were selected by the acid load survival method. Clonal cell lines for each subtype were then used for studies of pH$_i$-recovery after acid load. Studies of pH$_i$ recovery after acid load were essentially performed as described by Faber et al., 1996. Briefly, the cells were scraped of the culture dishes, washed and incubated with 5 µmol/L BCECF-AM (2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein-acetoxy-methyl ester) for 20 min at 37°C in a buffer containing 20 mM NH$_4$Cl. The cells were then washed to remove extracellular dye and resuspended in the loading buffer without BCECF-AM. Intracellular acidification was induced by addition of 975 µl NH$_4$Cl- and HCO$_3$-free solution (so called "recovery medium": bicarbonate-free to inhibit the sodium-dependent chloride-bicarbonate exchanger of LAP1 cells). Single clones showing survival and recovery potency mediated by functional NHE expression were used for IC$_{50}$ determination in FLIPR (Fluorometric Imaging Plate Reader) measurements: LAP1 (mouse LTK- cell line) cells stably expressing hNHE3, rNHE3, hNHE1, and hNHE5 were seeded with a density of 25,000 cells/well/100 µl medium (ISCove-medium, 10 % FCS, 2 mM L-glutamine, 100 u/ml penicillin/streptomycine, 50 g/ml gentamicine, 400 g/ml G418) on black, clear bottom 96-well microplates (Costar, Corning Inc., Corning, NY). Cells were incubated overnight at 37°C, 5 % CO$_2$ and 90% humidity. The medium was discarded prior to measurement and 100 µl/well dye buffer (20 mM HEPES, pH 7.4 adjusted with KOH, 20 mM NH$_4$Cl, 115 mM choline chloride, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM KCl, 5 mM glucose, 5 M BCECF) were added. After incubation for 20 min at 37°C the cells were washed 3-times with Na$^+$-free washing buffer (5 mM HEPES, pH 7.4 adjusted with KOH, 133.8 mM choline chloride, 4.7 mM KCl, 1.25 mM CaCl$_2$, 1.25 mM MgCl$_2$, 0.97 mM KH$_2$PO$_4$, 0.23 mM KH$_2$PO$_4$, 5 mM glucose), leaving 90 µl of washing buffer per well. pH-recovery was measured with a FLIPR (Fluorometric Imaging Plate Reader, Molecular Devices, Sunnyvale, CA; laser power 0.3 W, aperture 4, measuring interval 2 s, time of measurement 120 s). For calculation of NHE activity, the increase in fluorescence between the 12th and 32nd s was calculated. For IC$_{50}$ determination different concentrations of SAR218034 were diluted in the range of 10 µM down to 0.1 nM.
Vascularly perfused in-situ preparations of the small intestine:
To investigate intestinal sodium and water transport, vascularly perfused in situ half-open colon/intestinal reperfusion preparations of the small intestine was used as described elsewhere. Briefly, 48 h fasted, 16 h water deprived male Wistar rats (n=8) were anesthetized with ketamin/midazolam. 15min before the beginning of small intestine perfusion, the abdominal cavity of an anaesthetised animal was exposed by laparotomy. The peritoneum is cut and the small intestine is exposed. The pylorus of the stomach is ligated. The gut is notched and a Luer-adapter is tied in. At 30cm distal to the first adapter a second Luer-adapter is tied in. Isotone NaCl-solution is used to clean the gut. Gut perfusion is performed by a perfusion pump at 37°C with a speed of 1ml/min. 3 h reperfusion with starting volume of 10 mL aqua bdest/or 0.9% NaCl with or without 1mg/ml SAR was performed. Volume of perfusate at the end of 3 h reperfusion period as indicator for water absorption rate and electrolytes in perfusate were determined (see figure S1).

In-vivo experiments:
All animals were maintained under standard laboratory housing conditions with free access to chow (Sniff, Soest/Germany, salt content 0.9% by weight) and drinking water ad libitum. Systolic blood pressure (BP) and heart rate (HR) were determined by the tail-cuff method (TSE Systems GmbH, Bad Homburg, Germany) at 0, 7, 10 and 14 weeks in SHR and at 0, 2, 4 and 6 weeks in SHR-ob. Serum insulin concentrations were determined using a commercial rat ELISA kit (MERCODIA, Upsala, Sweden). For the determination of urinary and feces sodium-excretion, the rats were placed over 24h in metabolic cages (Tecniplast S.p.a., Buguggiate, Italien) and the urine and feces was sampled. Ion concentrations in urine were measured (Hitachi 912 analyzer). The water content in the feces was measured by the Karl Fischer method. Feces sodium concentration was determined via flame photometry. In SHR-lean (14 SHR, 14 SAR, 14 RAM, SAR + RAM), plasma renin activity was measured according to Peters et al. Plasma aldosterone was determined with a commercial kit: .Aldosterone Coat-A-Count RIA 100T, Siemens Healthcare Diagnostics GmbH 65780 Eschborn, (125Jod-Basieter Radioimmunoassay). Plasma ACE activity was determined according to Santos et al. In SHR-ob, the expression levels of rat NHE3 mRNA in jejunum, ileum and colon were determined from frozen tissue samples homogenized by a Qiagen TissueLyser II bead mill. Total RNA from the lysates was extracted with the Agencourt RNAAdvance Tissue kit according to the manufacturers instructions. From these total RNA preparations, mRNA levels of NHE3 were determined with primer and probe sets supplied by Applied Biosystems in a two-step RT-PCR reaction with random hexamer priming in the RT step. Expression levels of NHE3 mRNA in every individual RNA sample were normalized to the mRNA levels of Gapdh (glyceraldehyde-3-phosphate dehydrogenase) from the same sample. NHE3-gene expression was additionally investigated in different tissues of 6 Sprague Dawley rats for expression profiling.

Effect of low sodium diet (0.01g NaCl/100g rat chow) for 10 days on urinary sodium excretion and blood pressure was investigated in 7 SHR-lean. Results see Table S1.
References:


Table S1: Effect of low salt diet in SHR-rats

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal Salt Diet (0.7 g NaCl/100g chow)</th>
<th>Low Sodium Diet (0.01g NaCl/100g chow)</th>
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<tbody>
<tr>
<td>SHR (n=7)</td>
<td></td>
<td></td>
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<tr>
<td>Bodyweight</td>
<td>372±10 g</td>
<td>391±12 g</td>
</tr>
<tr>
<td>Urine Flow</td>
<td>2.4±0.3 ml/kg/h</td>
<td>1.6±0.4 ml/kg/h</td>
</tr>
<tr>
<td>Sodium Excretion</td>
<td>1.1±0.1 g/kg/d</td>
<td>0.3±0.0 µmol/kg/h</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>219±4 mmHg</td>
<td>207±7 mmHg</td>
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</tbody>
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Figure S1: Vascularly perfused in-situ preparations of the small intestine.