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Rostafuroxin: an ouabain antagonist that corrects renal and vascular Na\(^+\)-K\(^+\)-ATPase alterations in ouabain and adducin-dependent hypertension

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Rostafuroxin: an ouabain antagonist that corrects renal and vascular Na\(^+\)-K\(^+\)-ATPase alterations in ouabain and adducin-dependent hypertension. Am J Physiol Regul Integr Comp Physiol 290: R529–R535, 2006. doi:10.1152/ajpregu.00518.2005.—The genetic and environmental heterogeneity of essential hypertension is responsible for the individual variability of antihypertensive therapy. An understanding of the molecular mechanisms underlying hypertension and related organ complications is a key aspect for developing new, effective, and safe antihypertensive agents able to cure the cause of the disease. Two mechanisms, among others, are involved in determining the abnormalities of tubular Na\(^+\) reabsorption observed in essential hypertension: the polymorphism of the cytoskeletal protein α-adducin and the increased circulating levels of endogenous ouabain (EO). Both lead to increased activity and expression of the renal Na\(^+\)-K\(^+\) pump, the driving force for tubular Na transport. Morphological and functional vascular alterations have also been associated with EO. Rostafuroxin (PST 2238) is a new oral antihypertensive agent able to selectively antagonize EO, adducin pressor, and molecular effects. It is endowed with high potency and efficacy in reducing blood pressure and preventing organ hypertrophy in animal models representative of both adducin and EO mechanisms. At molecular level, in the kidney, Rostafuroxin antagonizes EO triggering of the Src-epidermal growth factor receptor (EGFr)-dependent signaling pathway leading to renal Na\(^+\)-K\(^+\) pump, and ERK tyrosine phosphorylation and activation. In the vasculature, it normalizes the increased myogenic tone caused by nanomolar ouabain. A very high safety ratio and an absence of interaction with other mechanisms involved in blood pressure regulation, together with initial evidence of high tolerability and efficacy in hypertensive patients, indicate Rostafuroxin as the first example of a new class of antihypertensive agents designed to antagonize adducin and EO-hypertensive mechanisms.

adducin; endogenous ouabain; Na\(^+\)-K\(^+\) pump; antihypertensive therapy
ment, but also genetic markers to characterize essential hypertensive patients who are likely to respond successfully to treatment with such compounds. The strategy we adopted includes studies of renal function and cellular, biochemical, molecular, and genetic characterization of Milan hypertensive rats (MHS) (3, 4, 21, 22), an animal model that shares some pathophysiological abnormalities with a subset of hypertensive patients (22). Through these studies, we have been able to identify two genetic-molecular mechanisms underlying the disease in both species: 1) mutations of genes coding for the cytoskeletal protein adducin (4, 9, 10) and 2) increased circulating levels of endogenous ouabain (EO) (15, 17, 42), the mammalian counterpart of plant ouabain (33). These two mechanisms lead to the increased function of renal Na\(^{+}\)-K\(^{+}\)-ATPase, the transport system driving sodium from the luminal to the interstitial side of the renal tubular cell. This conclusion was reached by in vivo studies on MHS rats (16) and experimental rat models made hypertensive by chronic infusion of low doses of ouabain (OS rats) (23), as well as by studies on renal cultured cells expressing the adducin variants (25, 56) or exposed to ouabain (23). Moreover, recent data indicate that EO also acts at the vascular level and is responsible for an increase in myogenic tone of small resistance arteries, thus contributing to a sustained increase of blood pressure through the enhancement of total peripheral resistance (35, 59).

There is considerable evidence to support the clinical impact of these two molecular mechanisms on hypertension and related cardiovascular complications. The role of hypertension of the human α-adducin (ADD1) Trp allele is confirmed by several studies, as recently reviewed (6, 7): 1) six linkage studies performed with appropriate DNA markers; 2) 18 out 20 association studies in which, besides blood pressure, the variable involved in its regulation was also considered; 3) four out of five studies on cardiovascular complications in hypertensive patients (6, 7). Mixed results were obtained in case-control studies carried out in different ethnic populations (6, 7).

Regarding EO, in addition to its direct influence on blood pressure (30, 32, 40, 45, 53), recent data demonstrate its critical role in favoring cardiac and renal complications associated with hypertension, both in rat models (19) and in hypertensive patients (6, 7). Mixed results were obtained in case-control studies carried out in different ethnic populations (6, 7).

It seems reasonable to assume, therefore, that a new molecule able to antagonize the pathological molecular effects of EO and adducin mutation.

ROSTAFUROXIN, A NOVEL ANTIHYPERTENSIVE COMPOUND

Rostafuroxin (PST 2238) (17β-(3-furyl)-5β-androstan-3β, 14β,17α-triol) (Fig. 1) is a digitoxigenin derivative (51) that displaces in vitro specific \(^3\)H-ouabain binding from dog kidney Na\(^{+}\)-K\(^{+}\)-ATPase with an IC\(_{50}\) of 2 \(\times\) \(10^{-6}\) M and affects enzymatic activity at 10\(^{-5}\) M (23). Its ability to specifically interact with the Na\(^{+}\)-K\(^{+}\)-ATPase is supported by two observations: 1) the absence of any significant in vitro binding of the compound up to 10\(^{-4}\) M to a panel of general and hormonal receptors, known to be involved in blood pressure regulation or hormonal steroid control (23), and 2) the capacity to display its pharmacological and therapeutic activity by correcting the functional alterations of the Na\(^{+}\)-K\(^{+}\)-ATPase associated with hypertension at concentrations ranging from 10\(^{-11}\) to 10\(^{-9}\) M (23, 25).

ROSTAFUROXIN NORMALIZES THE ALTERED RENAL Na\(^{+}\)-K\(^{+}\) PUMP FUNCTION IN ADDUCIN- AND OUABAIN-DEPENDENT FORMS OF HYPERTENSION

Adducin-dependent Na\(^{+}\)-K\(^{+}\) pump alterations: effect of rostafuroxin. Functional increase of the activity and expression of the basolateral renal Na\(^{+}\)-K\(^{+}\)-ATPase is a feature of animal models with adducin-dependent forms of hypertension (16). Similarly, congenic Milan normotensive rats in which the DNA segment containing the locus for the MHS-mutated adducin variant has been introgressed into the MNS genetic background (NUA) show increased blood pressure and renal Na\(^{+}\)-K\(^{+}\)-ATPase activity with respect to the MNS controls (57). In humans, erythrocyte Na\(^{+}\) content is lower and the Na\(^{+}\)-K\(^{+}\) pump activity faster in carriers of the mutated Trp ADD1 allele than in homozygotes for the Gly allele (26). Moreover, renal cells transfected with the mutated adducin variants display an increased Na\(^{+}\)-K\(^{+}\)-ATPase activity compared with cells transfected with wild-type adducin (56). The increased cellular expression and activity of the Na\(^{+}\)-K\(^{+}\)-ATPase caused by the expression of the mutated adducin variants were recently investigated by studying the dynamics of the Na\(^{+}\)-K\(^{+}\) pump endocytosis process. Indeed, cells transfected with both rat and human mutated adducins show an impairment of pump endocytosis in basal condition, as well as after hormonal natriuretic stimuli, such as dopamine (13), due to an adducin-dependent alteration of the phospho-dephosphorylation cycle of the pump endocytosis process.

![Fig. 1. Structural formula of rostafuroxin (PST 2238) (17β-(3-furyl)-5β-androstan-3β,14β,17α-triol).](http://ajpregu.physiology.org/Downloadedfrom)
ROSTAFUROXIN, A NEW ANTIHYPERTENSIVE OUABAIN ANTAGONIST

EO-dependent Na\(^{+}\)-K\(^{+}\) pump alterations: effect of rostafuroxin. The molecular mechanism(s) through which EO, at subnanomolar concentrations, favors the increase of tubular reabsorption at the renal level, as well as an increase of myogenic tone and resistance at the vascular level, is (are) the subject of extensive studies and debate. This is because the renal effect implies the ability of EO to stimulate Na transport across the tubular cell by activating the basolateral α1 Na\(^{+}\)-K\(^{+}\) pump (16); the vascular effect involves inhibition of the α2 Na\(^{+}\)-K\(^{+}\) pump that, by reducing the Na\(^{+}\) concentration gradient across the plasma membrane, promotes both Na\(^{+}\)/Ca\(^{2+}\) exchanger-mediated Ca\(^{2+}\) entry into the myocytes and contraction (35, 59). A unifying view of these two apparently contradictory hypotheses is reported in Fig. 3 which indicates, as supported by recent data, that endogenous ouabain (EO) not only acts as a classical Na\(^{+}\)-K\(^{+}\) pump inhibitor but, at very low concentrations (nanomolar or lower), may act as a signal transducer inducing, via a Src-EGFr-dependent pathway, tyrosine phosphorylation of the renal α1 Na\(^{+}\)-K\(^{+}\) pump with consequent activation of its function (19, 31, 39). We, therefore, propose that EO may increase peripheral resistances by inhibiting the ouabain high-affinity α2-Na\(^{+}\)-K\(^{+}\) pump isoform in the vasculature and, in parallel, it may enhance tubular Na reabsorption in the kidney by acting as a signal transducer, to phosphorylate the renal α1-Na\(^{+}\)-K\(^{+}\) pump and activate its function, with consequent volume expansion and hypertension. It is physiologically relevant that both ouabain stimulatory effect on renal α1-Na\(^{+}\)-K\(^{+}\) pump mediated by Tyr phosphorylation and the inhibitory one on the vascular α2-Na\(^{+}\)-K\(^{+}\) pump leading to increased arterial myogenic tone happen, at least in rodents, at similar concentrations (ouabain effective concentrations: 10\(^{-10}\)–10\(^{-9}\) M on α1 isoform in rat renal

adaptor protein subunit (or AP\(_{2}\mu\)) protein responsible for the clathrin-dependent pump endocytosis. Deficient Na\(^{+}\)-K\(^{+}\)-ATPase endocytosis may therefore be a common factor contributing to increased tubular sodium reabsorption both in rats and humans carrying the mutated adducin variants.

Rostafuroxin reduces blood pressure without affecting heart rate, and restores the normal activity of the renal Na\(^{+}\)-K\(^{+}\)-ATPase in MHS rats when orally treated at doses from 1 to 100 μg·kg\(^{-1}\)·day\(^{-1}\) [half-maximal effective dose (ED\(_{50}\)): 4 μg/kg] for 4–6 wk (25) (Fig. 2A). Similarly, NUA rats have their blood pressure normalized by rostafuroxin (Ferrandi M and Ferrari P unpublished results) (Fig. 2A). In normotensive MNS or Sprague-Dawley rats, the compound does not affect either blood pressure (Fig. 2A) or renal Na\(^{+}\)-K\(^{+}\)-ATPase activity, even at doses of 30 mg/kg po (23, 25). The ability of rostafuroxin to correct the adducin-dependent Na\(^{+}\)-K\(^{+}\)-ATPase alteration is shown in studies in adducin-transfected normal rat kidney (NRK) cells. Incubation of NRK cells, expressing the MHS adducin variant, with rostafuroxin at 10\(^{-9}\)–10\(^{-10}\) M for 5 days, normalizes Na\(^{+}\)-K\(^{+}\) pump activity to the level of cells expressing the wild-type variant, whose pump activity is not affected by the compound (25).

Fig. 2. Antihypertensive activity of rostafuroxin in different genetic and experimental rat models of hypertension. A: adducin-dependent genetic models: Milan hypertensive strain (MHS) and the congenic Milan normotensive congenic strain (NUA) strain, in which the DNA segment containing the alpha-adducin locus from MHS has been introgressed into the Milan normotensive strain (MNS). Rats (n = 8) from each strain were treated with vehicle or rostafuroxin 100 μg/kg po from weaning for 6 wk. Tail systolic blood pressure at the 6th wk is reported (25); B: volume-dependent experimental models: rats made hypertensive by chronic infusion of low doses of ouabain and Sprague-Dawley rats made hypertensive by an 8-wk infusion of 15 μg·kg\(^{-1}\)·day\(^{-1}\) ouabain. Rats (n = 8) were treated with rostafuroxin 10 μg/kg po for 6 wk (19); deoxycorticosterone acetate (DOCA)+salt rats made hypertensive by weekly subcutaneous treatments with 30 mg/kg DOCA and 1% NaCl in drinking water. Rats (n = 8) were treated with vehicle or rostafuroxin 10 mg/kg po for 3 wk (24); reduced renal mass (RRM) rats made hypertensive by 70% surgical reduction of renal mass and drinking 1% NaCl. Rats (n = 8) were treated with vehicle or rostafuroxin 1 mg/kg po for 2 wk (24, 27, 28). Values are expressed as means ± SE; *P < 0.05, **P < 0.001 vs. vehicle; §§P < 0.01 vs. MHS and MNS. Student’s t-test was done for comparison between rostafuroxin and vehicle-treated groups of rats; two-way ANOVA was performed followed by Dunnett’s test for multiple comparisons.

Fig. 3. Proposed dual mechanisms for the hypertensogenic role of endogenous ouabain (EO) as therapeutic targets for new antihypertensive ouabain antagonists: EO may act by increasing peripheral vascular resistances (TPRs) and blood pressure by, on the left, reducing vascular α2-Na\(^{+}\)-K\(^{+}\)-ATPase activity and promoting cytosolic Ca\(^{2+}\) increase via Na\(^{+}\)/Ca\(^{2+}\) exchange activation and, on the right, by enhancing tubular Na\(^{+}\) reabsorption through activation of the renal α1 Na\(^{+}\)-K\(^{+}\)-ATPase induced by a Src-EGFr-dependent tyrosine phosphorylation pathway.
Indeed, experimental data demonstrate that chronic infusion of ouabain at doses leading to subnanomolar plasma concentrations in normotensive rats and mice elevates blood pressure (12, 40), increases myogenic tone (35), and stimulates renal Na\(^{+}\)-K\(^{+}\)-ATPase activity (19). Moreover, incubation of NRK cells with nanomolar ouabain for 5 days increases the maximal activity rate (\(V_{\text{max}}\)) of the Na\(^{+}\)-K\(^{+}\) pump (23). Evidence of the molecular mechanism through which EO exerts its signal transduction function has been recently provided by studying the effect on renal caveolae of chronic infusion of subnanomolar ouabain concentrations in OS rats (19). As shown in the diagram in Fig. 4, exposure to ouabain of this specialized membrane subdomain, at doses producing plasma concentrations similar to those observed for EO in human and rat hypertension (0.5–0.7 nM), enhances the expression of the \(\alpha_{1}\), \(\beta_{2}\), and \(\gamma_{2}-\text{Na}\(^{+}\)-K\(^{+}\)-ATPase in its total and tyrosine-phosphorylated forms, together with that of total and phosphorylated Src and EGFr (19). This molecular effect is paralleled by an increase of the Na\(^{+}\)-K\(^{+}\)-ATPase activity in membrane caveolae and activation of the p42/44 MAPK in the cytosol (19), mechanisms that produce an increase of tubular Na transport (14) and nuclear gene transcription (31). Consequently, this may explain both the hypertension and the cardiac and renal hypertrophy observed in OS rats (19). At the vascular level, resistance arteries from OS rats display an increased contraction in response to high extracellular K\(^{+}\) when assayed in vitro and compared with vessels from saline-treated controls (36). Moreover, in intact pressurized small mouse mesenteric arteries, nanomolar ouabain concentrations increase cytosolic Ca\(^{2+}\) and myogenic tone with predictable physiological consequences on an increase of total peripheral resistance (TPRs) in vivo (35, 59).

Hence, these findings support an integrated view of the renal and vascular molecular effects of EO that converge in causing certain forms of salt- and volume-dependent hypertension and related organ complications. In these forms of hypertension, rostafuroxin seems to exert potent and specific antihypertensive and antihypertrophic effects by acting at both renal and vascular levels. In OS rats, rostafuroxin normalizes high blood pressure (Fig. 2B), and the increased renal Na\(^{+}\)-K\(^{+}\)-ATPase activity in a range of doses from 0.1 to 100 \(\mu\)g/kg po, being ineffective at 0.01 \(\mu\)g/kg (23). Similarly, the increased \(V_{\text{max}}\) Na\(^{+}\)-K\(^{+}\) pump activity observed in NRK cells incubated with \(10^{-9}\) M ouabain for 5 days is completely normalized by \(10^{-11}\)–\(10^{-10}\) M rostafuroxin (23). At molecular level, the in vivo and in vitro effects of nanomolar ouabain on the signal transduction pathway mediated by the Src-dependent phosphorylation of the Na\(^{+}\)-K\(^{+}\)-ATPase in caveolae, are completely antagonized by rostafuroxin at \(10^{-9}\)

\(\times\) \(10^{-10}\) M (19) which, in vivo, not only prevents hypertension, but also ouabain-dependent cardiac and renal hypertrophy (19), as opposed to other antihypertensive agents such as Ca antagonists (19). The ability of rostafuroxin to antagonize the pressor effect of ouabain was also observed on the vascular bed. The increased contractile response of mesenteric arteries isolated from OS rats to 75 mM extracellular KCl [developed tension, OS \((n = 7)\): 958.1 \(\pm\) 110 mg vs. saline control rats \((n = 6)\): 527 \(\pm\) 66.8 mg, \(P < 0.01\)] is completely normalized by a 4-wk treatment of OS rats with 100 \(\mu\)g/kg po rostafuroxin (580.8 \(\pm\) 104.5 mg, \(n = 6\)) (Micheletti R, personal communication). Moreover, Zhang et al. (59) recently demonstrated that the increase of mouse mesenteric artery myogenic tone caused by in vitro exposure to ouabain, is completely antagonized by rostafuroxin. The involvement of EO in volume-dependent forms of hypertension is supported by data obtained in different experimental models (37, 54) and also in humans (47). As reported in Fig. 2B, rostafuroxin seems especially effective in these forms as indicated by its antihypertensive activity in DOCA+salt (24) and reduced renal mass rat models of hypertension (27, 28).

Overall, these findings indicate that rostafuroxin represents the first example of a new class of antihypertensive agents that reduce blood pressure and prevent hypertension-related organ complications by selectively correcting the molecular and functional alterations of the Na\(^{+}\)-K\(^{+}\) pump induced by genetic (i.e., Adducin) and/or hormonal (EO) mechanisms, without affecting the normal physiological mechanisms of blood pressure control.
Safety and tolerability of rostafuroxin. One of the principal causes of the low compliance of hypertensive patients in assuming lifelong antihypertensive therapy is a series of side effects that almost all of the available drugs induce, compromising the quality of life. For this reason, any new agent able to compete with the wide spectrum of available antihypertensive drugs must have very high tolerability and be devoid of side effects. This goal may be achieved by developing a compound that targets a specific molecular alteration without interfering with physiological homeostasis. Indeed, rostafuroxin displays such qualities, having a highly safe profile, as indicated by acute and chronic toxicological and pharmacological safety studies.

Acute oral toxicity of rostafuroxin in rats yields LD$_{50}$ > 2,000 mg/kg (24). One-and three-month chronic toxicological studies, performed in rats and monkeys, indicate that the compound does not induce mortality or any toxicological alterations at doses up to 100 mg/kg po for rats and 180 mg/kg po for monkeys, which appear to be the maximum tolerated doses in these two species (24). Therefore, at least in rats, the ratio between the effective antihypertensive and the toxic dose appears to be higher than 1 to 25,000, considering an ED$_{50}$ of 4 µg/kg po (24). Rostafuroxin has no mutagenic activity and displays a very clean pharmacological safety profile as shown by studies carried out in different species on hemodynamics, gastrointestinal system, central nervous system, respiratory functions, and steroidogenesis (24).

According to available data, a component of the antihypertensive activity of rostafuroxin is linked to its ability to normalize the increased tubular Na$^+$ transport; however, rostafuroxin is devoid of any natriuretic and diuretic effects and does not cause the typical diuretic’s side effects such as activation of the renin-angiotensin-aldosterone system, hypokalemia, alterations of lipidic and glucidic profiles (18). Indeed, rostafuroxin does not act as a diuretic, because it is able to reestablish a correct body hydrosaline set point by correcting the altered renal Na$^+$-K$^+$-ATPase defect without inhibiting physiological sodium transporters, as the diuretics do.

Rostafuroxin clinical studies. Approximately one-third of essential hypertensive patients show increased circulating levels of EO (32, 40, 45, 53), and a similar percentage of hypertensive patients are heterozygous for the Trp ADD1 allele (6, 7). These subsets of patients may conceivably constitute the target population for rostafuroxin antihypertensive treatment.

Rostafuroxin’s specific preclinical pharmacological and toxicological profiles make it promising for clinical development. As reported in Table 1, rostafuroxin has already satisfied safety requirements in phase I studies on healthy volunteers showing complete tolerability either after single or repeated oral administrations up to a dose of 10 mg/day, without showing differences in side effect patterns compared with placebo (20). Rostafuroxin is currently under phase II clinical investigation (Table 1). In two small exploratory studies in patients with mild uncomplicated hypertension, rostafuroxin has been demonstrated to be effective in lowering blood pressure in a statistically significant way at oral doses from 0.1 to 1 mg/day (20).

On the basis of this finding, a multicenter European phase II study aimed at defining the dose-response curve of rostafuroxin in 440 hypertensive patients is currently ongoing (53a). The protocol also includes a definition of the genetic profile of the responder patients for gene coding for adducins, enzymes involved in EO synthesis and found to be overexpressed in

Table 1. Summary of rostafuroxin phase I-II clinical studies

<table>
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<tr>
<th>Subjects</th>
<th>Observations</th>
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<td><strong>Tolerability</strong></td>
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<tr>
<td>Single dose (placebo, 1, 2.5, 5, and 10 mg/day)</td>
<td>Healthy volunteers ($n = 9$ each group) No significant modification of clinical, instrumental, laboratory parameters as compared to placebo. Very few and mild adverse events, both in the placebo and drug-treated groups.</td>
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<tr>
<td>Multiple doses (placebo, 0.5, 2.5, and 5 mg twice a day for 7 days)</td>
<td>Healthy volunteers ($n = 9$ each group) No significant modification of clinical, instrumental, laboratory parameters as compared to placebo. Very few and mild adverse events, both in the placebo and drug-treated groups.</td>
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<td>Multicenter dose-titration study—three sequential 4-wk schedule (0.1, 1, and 5 mg/day)</td>
<td>Never-treated EH patients ($n = 25$) Statistically significant mean blood pressure fall at any dose as compared to baseline.</td>
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<td>Multicenter, double-blind single-dose controlled study comparing rostafuroxin (0.5 mg/day for 3 mo) with losartan (50 mg/day for 3 mo)</td>
<td>Mild, untreated EH patients (Rosta: $n = 42$; Los: $n = 21$) Similar percentage of normalized (N) (38%) and responder (R) (Rosta: 28.6% vs. Los: 23.8%) patients in the two groups. Statistically significant SBP and DBP fall in both groups. Rosta produced a smaller BP fall than Los because of a sharper division between Rosta and not-Rosta patients in the former than in the latter group. N: DBP$&lt; = 90$ mmHg; R: reduction in mean DBP$&gt; = 10$% with respect to the baseline assessment</td>
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<td>Multicenter, double-blind, dose-range controlled study of Rostafuroxin (0.05, 0.15, 0.5, 1, and 1.5 mg/day) within cross-over design vs. placebo (OASIS Study) (53a)</td>
<td>Stable, uncomplicated EH patients ($n = 440$) Currently ongoing (September 2005).</td>
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Rosta, Rostafuroxin group; Los, losartan group; EH, essential hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure.
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Invited Review

MHS rats (46), ouabain transporters, and other proteins regulating tubular Na⁺ reabsorption.

In conclusion, several experimental and clinical lines of evidence support the notion that adducin polymorphisms and EO play a pathogenetic role in hypertension and related organ complications. These effects occur through a complex interaction between genetic-molecular mechanisms regulating renal sodium reabsorption, vascular reactivity, and environmental variables such as salt intake. The new antihypertensive agent rostafuroxin, described here, may represent a novel therapeutic approach tailored to the individual patients carrying these specific pathogenetic mechanisms.

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