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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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IN RECENT YEARS, PHARMACOLOGICAL treatment of essential hypertension has evolved from an empirical approach based on “group therapy” to a more individual approach, which addresses specific hypertensive mechanisms affecting patient subgroups (38, 44). A large selection of different classes of antihypertensive drugs is available today, and their efficacy is widely recognized. However, during the past 15 years, this strategy has not brought improvement in blood pressure control in the general population of hypertensive patients (2), leading to a very high incidence of organ complications with related in conveniences and costs. Several factors may account for this failure, such as the side effects of the available drugs that cause their discontinuation and the variability of the blood pressure response (a given drug produces a clinically relevant fall in blood pressure in only 30–40% of patients, reducing the patient’s faith in the physician) (58). Primary hypertension, triggered and sustained by a variety of environmental and genetic factors (34), affects up to 40% of the adult population in industrialized countries. This heterogeneity is certainly responsible for the above-mentioned failure, as only the removal or correction of specific factors at work in a subset of patients can ensure blood pressure regulation and prevention of organ complications. Unfortunately, very little is known about the ability of available drugs to intervene in these factors. Therefore, the success of future hypertension treatment will depend upon the understanding of the genetic molecular mechanisms operating in subsets of patients and the ability of new drugs to specifically correct such alterations.

The difficulty of the kidney to excrete sodium is one of the main mechanisms responsible for blood pressure rise in both experimental (8, 48) and genetic rat models (22, 52), and, at least, in some forms of monogenic human hypertension (55). For many years now, our group has tried to determine whether molecular mechanisms leading to this renal defect are involved in primary forms of hypertension and to use the results we obtained to identify not only new targets for innovative treat-
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 \(\alpha\)-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).

Fig. 1. Structural formula of rostafuroxin (PST 2238). (17\(^\beta\)-(3-furyl)-5\(^\beta\)-androstan-3\(^\beta\),14\(^\beta\),17\(\alpha\)-triol).
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, 39). 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-EGF-receptor-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\(_{3}\mu\)) 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 (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).
caveolae (19); from 1 nM on α2 isoform in mouse resistance arteries (59)).

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_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 α1, β1, and γ\(\_\)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 μg/kg po, being ineffective at 0.01 μg/kg (23). Similarly, the increased V_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\(^{-9}\)–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 ± 110 mg vs. saline control rats (n = 6): 527 ± 66.8 mg, P < 0.01] is completely normalized by a 4-wk treatment of OS rats with 100 μg/kg po rostafuroxin (580.8 ± 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.

Fig. 4. Molecular mechanism of action of the ouabain antagonist rostafuroxin in renal caveolae: subnanomolar ouabain/EO concentrations activate an Src-dependent signaling pathway that induces enrichment of the tyrosine-phosphorylated forms of Src, epidermal growth factor receptor (EGFr), and Na\(^+\)-K\(^+\)-ATPase in renal caveolae, increases Na\(^+\)-K\(^+\)-ATPase activity on the cell membrane and activates the p42/44 MAPK in the cytosol, thus leading to hypertension and organ hypertrophy. Rostafuroxin, at nanomolar concentrations, antagonizes the ouabain/EO-Src-Na\(^+\)-K\(^+\)-ATPase interaction, normalizes the Na\(^+\)-K\(^+\)-ATPase and p42/44 MAPK activities, thus reducing blood pressure and preventing organ hypertrophy.
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 LD50 > 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 ED50 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>
<thead>
<tr>
<th>Subjects</th>
<th>Observations</th>
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<tbody>
<tr>
<td><strong>Tolerability</strong></td>
<td></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)</td>
</tr>
<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)</td>
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<tr>
<td><strong>Efficacy</strong></td>
<td></td>
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<tr>
<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)</td>
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<tr>
<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)</td>
</tr>
<tr>
<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)</td>
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Rosta, Rostafuroxin group; Los, losartan group; EH, essential hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure.
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 pathogenic 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 pathogenic mechanisms.

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REFERENCES


