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Review

Rostafuroxin: An ouabain-inhibitor counteracting specific forms of hypertension

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ABSTRACT

An innovative approach to the therapy of essential hypertension (EH) and the related complications has been pursued by our group with the aim of defining specific genetic-molecular mechanisms underlying the disease in sub-sets of patients. This approach is anticipated to have a major effect on the clinical practice, diagnostics and development of new drugs able to selectively target such mechanisms. The final achievement is the definition of biomarkers for identifying patients who more likely should benefit for a given therapy both in terms of efficacy and reduction of the adverse reactions. Among many, two mechanisms have been defined and addressed:

1) polymorphisms in the gene coding for the cytoskeletal protein, adducin;
2) increased levels of the salt and blood pressure-regulating hormone, endogenous ouabain (EO).

Both alterations lead to hypertension, organ hypertrophy, negative vascular remodeling and increased cardiovascular risk by affecting the renal Na^+ handling, through the up-regulation of the Na^+–K^+ pump and the activation of the Src-dependent signal transduction pathway. A novel antihypertensive agent, rostafuroxin (PST2238), has been selected and developed for its ability to correct the renal Na^+–K^+ pump abnormalities sustained by the mutant adducin and EO-dependent mechanisms. It is endowed with high potency and efficacy in reducing blood pressure (BP) and preventing organ hypertrophy in animal models representative of both adducin and EO mechanisms. At molecular level, in the kidney, rostafuroxin normalizes the enhanced activity of the Na^+–K^+ pump induced by mutant adducin and antagonizes the EO triggering of the Src-EGFr-dependent signaling pathway leading to renal Na^+–K^+ pump and ERK phosphorylation and activation. In the vasculature, it normalizes the increased myogenic tone caused by ouabain. A very high safety ratio and the absence of interaction with other mechanisms involved in BP regulation, together with 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. A recently concluded Phase II clinical trial (OASIS) has provided the proof of concept that such a compound is effective in the subset of patients where these two mechanisms are at work.

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1. Introduction

Primary hypertension is a complex polygenic disorder affecting 30–40% of the adult population in the industrialized countries and representing the major determinant for cardiovascular (CV), renal and cerebral organ complications [1]. The interaction between common genetic variants and environment unmasks the disease through the modulation of renal, vascular, nervous and hormonal functions [2]. This complexity accounts for the poor understanding of the specific mechanisms of hypertension and also for the marked variability in the individual response to the therapy [3,4]. It is calculated that only one third of patients who are aware on their hypertensive status have their blood pressure (BP) well controlled [5,6]. Among many reasons of this failure (poor efficacy, low compliance, adverse side effects), a prominent one concerns the lack of knowledge of the genetic-molecular basis of BP control. Defining the genetic-molecular mechanisms underlying different forms of hypertension and associated organ complications is needed to target treatments to the characteristics of the patient and optimize their response to the therapy [7–9].

Among many functional alterations, it is well established that the impairment of the pressure-natriuresis mechanism leading to salt retention and elevated peripheral resistance is a landmark of hypertension development [10–13]. This finding has been consistently confirmed both in animal models of genetic hypertension [10,14] and

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in patients [11,15]. The Na^+–K^+ pump, the enzyme located on the basolateral side of the renal tubuli, is the driving force for renal sodium reabsorption. Any mechanism altering the Na^+–K^+ pump function/expression may therefore be candidate for hypertension pathogenesis and represents a potential new therapeutic target [16].

During the last 20 years, studies spanning from the whole body to the gene level, let us to contribute at clarifying the clinical impact of two mechanisms that, by affecting the renal Na^+–K^+ pump function, are associated to hypertension in rats and humans:

a) the endogenous ouabain (EO), a salt modulating hormone, whose circulating levels are under a genetic control [17–19];
b) the polymorphism of the genes encoding for the cytoskeletal protein adducin [20–22].

There is considerable evidence that supports the clinical impact of these two genetic-molecular mechanisms on hypertension and related organ complications [23–27]. Two other chapters in this issue [28,29] are specifically dedicated to detail such evidence. Here, we report only a summary of the peculiarities of these mechanisms.

Ouabain/EO levels associate to BP and CV complications both in rats infused with ouabain [24] and in humans suffering from early and mild to long-lasting and more severe hypertension [19,25–27], thus indicating the primacy of EO in causing cardiac damage. The pathophysiological consequences of EO level alterations have been recently attributed to the ability of low ouabain concentrations to trigger the Na^+,K^+ATPase-Src-ERK1/2-dependent signaling in caveolae and to determine the up-regulation of the renal Na^+–K^+ pump activity and the expression of growth-related genes [24,30–32]. In addition, evidence has been reported indicating on how EO/ouabain, beside its renal effects, increases the myogenic tone of small resistance arteries, thus contributing to a sustained increase of BP through the increase of total peripheral resistance [33].

Adducin is a cytoskeletal protein, formed by three isoforms (α, β and γ), which participates in many cellular processes such as actin polymerization, cell volume and ion transport regulation, cell-to-cell contact, control of the residential time of integral membrane proteins (Na^+–K^+ATPase, Integrins), calcium handling and signal transduction [34,35,36, Ferrandi M., personal communication). The role of α-adducin polymorphisms on rodent (F316Y) and human (G460W) hypertension and associated CV risk has been widely documented and demonstrated to be dependent from the genetic and environmental context [23,28]. Studies aimed at clarifying the molecular mechanisms through which adducin polymorphisms affect BP and organ complications have indicated that mutant α-adducin variants increase renal Na^+–K^+ATPase activity and expression through a direct interaction between adducin and the Na^+–K^+ATPase [37]. More recently, a selective activation of the Na^+–K^+ATPase-Src-dependent signaling induced by the mutant α-adducin has been documented in renal caveolae of congenic rats (MHS.H-*Add*1), carrying the mutant α-adducin locus (*Add*1) introgressed from the Milan Hypertensive rat strain (MHS) into the Milan Normotensive rat strain (MNS) background (Ferrandi M., personal communication). This suggests that mutant adducin may alter the Na–K pump function and the expression of growth-related genes involved in CV complication, through molecular mechanisms having similar features as those described for the EO/ouabain.

These findings have then been used to address two objectives: 1) to develop a new therapeutic intervention able to target the altered molecular mechanisms triggered by EO and adducin mutations, and 2) to identify biomarkers for selecting essential hypertensive patients who are likely to successfully respond to the new therapeutic intervention.

In this respect, the enhanced activity of the renal Na^+–K^+ATPase triggered by mutant adducin and EO and the Src-dependent activation of signal transduction induced by EO represent new pharmacological targets for a tailored antihypertensive therapy. Moreover, the genetic profile underlying such altered mechanisms may represent the biomarker/s for identifying the patients prone to be cured with the new therapy.

Based on this, our group developed a new comprehensive line of research which led to the identification of a number of original compounds with antihypertensive properties, being able to antagonize the hypertensive effect and the molecular alterations induced by EO (or ouabain) or adducin polymorphism, without interfering with other known physiological mechanisms.

Among the compounds studied, a novel antihypertensive agent rostafuroxin (PST2238), has been selected in preclinical studies for its ability to selectively correct the molecular alterations sustained by adducin mutations and EO [38]. Its pharmacological profile and the mechanism of action will be summarized in the present review. Furthermore, the pharmacogenomic strategy adopted for testing the clinical efficacy of rostafuroxin in subset of patients carrying the adducin and EO-dependent hypertensive mechanisms will be also reported.

2. Rostafuroxin

Rostafuroxin (PST2238: 17beta-(3-furyl)-5beta-androstan-3beta, 14beta, 17alpha-triol-hydrate) is a digitoxigenin derivative that displaces ouabain binding from the dog kidney Na^+–K^+ATPase with an IC50 of 2 μM without interfering with other receptors or enzymatic activities known to be involved in the BP regulation or hormonal steroid control [39].

Its ability to counteract the molecular effects of EO/ouabain and mutant α-adducin on the Na^+–K^+ pump function has been proved in cultured renal cells either exposed to nanomolar ouabain for a long-term [39] or transfected with the mutant α-adducin variants [40]. In both conditions, the Na^+–K^+ pump activity is significantly increased as compared with the respective control cells and rostafuroxin is able to normalize it to the physiological levels, at concentrations ranging from 10^{-10} to 10^{-8} M, without altering the Na^+–K^+ pump activity in control cells [39,40] (Fig. 1).

The efficacy and potency of rostafuroxin in antagonizing the EO and mutant α-adducin hypertensive effects have been also proved in "in vivo" rat models carrying specific forms of hypertension. Ouabain increases BP in Sprague Dawley rats when infused at very low doses (15 μg/kg/day) for 4–8 weeks (OS rats) [39]. This hypertensive effect is paralleled by an increased function and expression of the renal Na^+–K^+ pump [24,39] and, in the long run, by left ventricle and renal hypertrophy [24]. All of these ouabain-dependent effects are prevented by chronic administration of rostafuroxin at oral doses ranging from 0.1 to 10 μg/kg [24,39] Fig. 1. The ability of rostafuroxin to antagonize the hypertensive 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 is completely normalized by a 4-week treatment of OS rats with 100 μg/kg os rostafuroxin [41]. Moreover, it has been demonstrated that the increase of mouse mesenteric artery myogenic tone caused by "in vitro" exposure to ouabain, is completely antagonized by rostafuroxin [33]. The involvement of EO in volume-dependent forms of hypertension is supported by data obtained in different experimental models [18,38,42] and also in humans [19,26,27]. Rostafuroxin displays a clear antihypertensive effect in these forms of volume-dependent hypertension such as DOCA-salt and Reduced-Renal-Mass hypertensive rats [38,42], while no activity is observed in SHR and normotensive animals [42].

The Milan strain of spontaneously hypertensive rats (MHS) is an animal model in which mutations on the α-adducin [20] and increased EO levels [17] are associated to hypertension, cardiac hypertrophy and increased expression and function of the renal Na^+–K^+ pump [24]. Rostafuroxin reduces BP and cardiac hypertrophy in MHS rats [40] and in parallel normalizes their Na^+–K^+ pump activity [40]

when orally given at doses ranging from 1 to 100 μg/kg once a day, for several weeks (ED_{50} = 4 μg/kg os) (Fig. 1).

The antihypertensive activity of rostafuroxin is long-lasting since it is still present 24 h after oral treatment [40].

Despite its digitoxigenin-like structure, rostafuroxin is devoid of any digitalis-like cardiac effect in intravenously treated guinea pigs up to 6.7 mg/kg i.v. [42].

2.1. Molecular mechanisms

Studies aimed at characterizing the molecular mechanism of action of this novel agent are in progress. The available data show that rostafuroxin is able to normalize the alterations of the Na^+–K^+ATPase trafficking affected by both ouabain and mutant adducin [35].

Furthermore, the effect of rostafuroxin on the ouabain-dependent hypertensive and pro-hypertrophic cardiac and renal effects has been recently elucidated [24]. In specialized membrane districts, the caveolae, where signal transduction proteins are clustered, sub-nanomolar ouabain concentrations induce the enrichment of Na^+–K^+ATPase isoforms, Src and EGFr signaling molecules [24]. This process leads to the activation of the membrane Na^+–K^+ATPase and ERK1/2 in the cytosol thus favoring the increase of trans-epithelial Na^+ transport and the transcription of growth-related genes in the nucleus [45]. Rostafuroxin, by specifically displacing ouabain binding from the high affinity Na^+–K^+ATPase isoform present in the caveolae, antagonizes all the ouabain functional effects [24] (Fig. 1).

Similarly to what was observed for the ouabain-dependent molecular mechanism, it has been recently demonstrated that adducins (both from the rat model and humans) interact with the Na^+–K^+ATPase-Src-dependent signaling pathway, triggering its activation in renal caveolae (Ferrandi M., personal communication). This interaction is however different for the mutant adducin variants as compared with the normal ones causing a further activation of the Src-dependent signaling which induces an increase of the Na^+–K^+ATPase tyrosine phosphorylation and activity. In several experimental settings (MHS.H-*Add*1 congenic rats, cultured renal cells transfected with the human adducin variants, cell-free system with recombinant adducins and Src), rostafuroxin completely antagonizes the molecular effects of the mutant but not of the normal adducin variants, normalizing the triggering of the Na^+–K^+ATPase-Src-dependent signal transduction pathway at concentrations ranging from 10^{-10} to 10^{-11} M (Ferrandi M., personal communication) (Fig. 1). The functional consequence of this molecular mechanism is a selective normalization of the Src-dependent signaling and reduction of the up-regulated renal Na^+–K^+ATPase activity (Fig. 1). Consequently, a reduction of the tubular Na^+ reabsorption is expected in those subjects carrying the mutant adducin variants.

2.2. Safety and tolerability

Rostafuroxin is characterized by 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} > 2000 mg/kg [42]. Chronic toxicological studies, performed in rats and monkeys, indicate a NOAEL (no observed adverse effect level) of 250 μmol/kg/day os for rats and 450 μmol/kg/day os for monkeys [42]. Therefore, at least in rats, the ratio between the effective antihypertensive and the toxic dose appears to be higher than 1 to 10,000, considering an oral effective doses ranging from 0.25 to 25 nmol/kg/day [42].

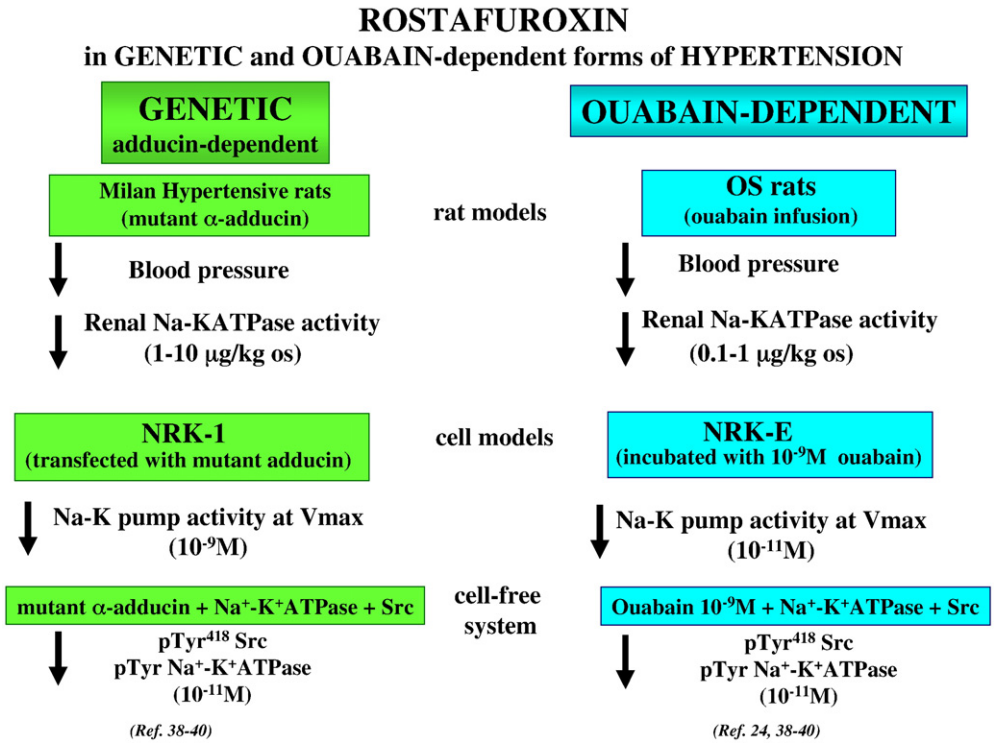

Fig. 1. Summary of the activities of rostafuroxin in different preclinical experimental settings: a) "in vivo" animal models representative of forms of hypertension sustained either by adducin mutation (Milan hypertensive strain, MHS) or altered circulating levels of ouabain/endogenous ouabain (EO) (ouabain infused rats, OS): rostafuroxin significantly reduces blood pressure and normalizes the up-regulated renal Na^+–K^+ATPase function both in MHS and OS rats at oral doses of 1–10 and 0.1–1 μg/kg/day, respectively [38–40]; b) cultured rat renal cells (NRK) either stably transfected with the rat mutant α-adducin variant (NRK-1) or incubated with 10^{-9} M ouabain for 5 days (NRK-E) display a significant increase of the Na^+–K^+ATPase activity at Vmax as compared with the respective controls cells (NRK expressing the wild type α-adducin variant or NRK incubated only with medium): rostafuroxin completely normalizes the Na^+–K^+ATPase activity at Vmax, without affecting the pump activity in control cells, at concentrations ranging from 10^{-9} to 10^{-11} M, respectively [38–40]. c) in "cell-free system" either mutant α-adducin variant or 10^{-9} M ouabain significantly increase the Src-dependent Tyr phosphorylation of the renal Na^+–K^+ATPase and ERK, as compared with the respective control conditions: rostafuroxin nullifies both the difference between the mutant and wild type α-adducin and the ouabain-dependent triggering mechanism of the Src-dependent signaling pathway, at concentrations ranging from 10^{-9} to 10^{-11} M [24, Ferrandi M., personal communication].

Table 1
Comparison between therapeutic "traditional approach" (one drug in the general patient population) with the "present approach" (one specific drug in a population selected by genotyping).

	Traditional	Present
SBP fall by a single drug (placebo-corrected effect)	6–12 mm Hg	14–23 mm Hg[a]
Difference in SBP fall among single drugs	None or −3 mm Hg	5–12 mm Hg[a]
Reduction of CV complications (vs. placebo)	~20%	Expected >20%[b]

[a] Depending upon the composition of the genetic profile.
[b] Due to the larger BP fall and rostafuroxin effect on the specific mechanisms underlying CV complications.

Rostafuroxin has no genotoxic activity and displays a very clean pharmacological safety profile [42]. Although a component of the antihypertensive activity of rostafuroxin is linked to its ability to normalize the increased tubular Na^+ transport, as stated above, it is completely devoid of any diuretic activity either after acute or chronic treatments [43]. As consequence, rostafuroxin does not cause the typical diuretic's side effects such as activation of the Renin–Angiotensin–Aldosterone (RAA) system, hypokalemia, alterations of lipidic and glucidic profiles.

2.3. Clinical studies

Phase I clinical studies confirmed the safety of rostafuroxin also in healthy volunteers after both single and repeated administrations [38].

Two explorative small Phase II clinical trials aimed at testing the antihypertensive efficacy and tolerability of rostafuroxin in mild essential hypertensive patients, have confirmed the safety of the drug and its ability to lower BP at the low oral doses ranging from 0.1 to 1 mg/day [38].

A Phase II European multicentric, dose-range finding, clinical study on 440 patients with moderate, uncomplicated essential hypertension was recently concluded [44]. The primary end-points were aimed at identifying the effective doses on systolic and diastolic BP fall, both as office and 24-h BP measurements. The influence of the variations within the genes coding for adducin isoforms (ADD1, ADD2 and ADD3) and some enzymes involved in EO synthesis and metabolism (LSS, MDR2, HSD) on the magnitude of BP fall after rostafuroxin was also included as secondary end point [44]. A wide genome scanning (WGS), using the Illumina technology on 1 million SNPs for each patient, has been performed. The analysis of these data allowed us to identify a defined subset of patients carrying specific combinations of these genetic variants whose BP was normalized by rostafuroxin at nanomolar oral doses and unaffected by placebo. Based on the available data, we may predict that the clear separation would occur in at least 20–25% of hypertensive patients. This finding is consistent with the preclinical evidence of the adducin and/or EO-mediated activation of signaling pathways associated with hypertension and related organ complications. Moreover, it supports the pharmacogenomic strategy by which rostafuroxin has been developed as the first example of a novel antihypertensive agent targeted to patients carrying specific genetic fingerprints.

When this strategy is compared with the traditional therapeutic approach where one antihypertensive drug should be effective in the whole hypertensive population, the advantages in terms of improvement of efficacy, safety and reduction of the healthy costs are evident. From an overview of the available literature (see Table 1), it emerges that the systolic BP fall induced by a single antihypertensive drug (either diuretics or Angiotensin Converting Enzyme (ACE) inhibitors or Angiotensin Receptor Blockers (ARBs) or renin inhibitors, etc.) ranges from 6 to 12 mm Hg, when corrected for the placebo effect [46–50]; moreover, the difference in terms of efficacy among the different classes of drugs is no more higher than 3 mm Hg and the organ complication reduction does not exceed the 20% [51]. The data obtained in the OASIS study on the efficacy of rostafuroxin in a selected, genotyped population indicate an improved efficacy both with respect to placebo and a traditional antihypertensive agent. Moreover, the targeting and correction of the altered mechanisms responsible for the disease are in keeping with an improved control of the organ complications and a consequent reduction of the health burden.

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