Rostafuroxin protects from podocyte injury and proteinuria induced by adducin genetic variants and ouabain

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Abbreviations
BP, blood pressure; EO, endogenous ouabain; HR, heart rate; KO, β-adducin knockout mice; OHR, ouabain hypertensive rats; MHS, Milan hypertensive rats; MNS, Milan normotensive rats; NA, congenic rats for mutant α-adducin from MHS rats; NB, congenic rats for mutant β-adducin from MHS rats; SBP, systolic blood pressure; WT, wild-type mice.

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ABSTRACT

Glomerulopathies are important causes of morbidity and mortality. Selective therapies addressed to the underlying mechanisms are still lacking. Recently, two mechanisms, mutant β-adducin and ouabain, have been involved in glomerular podocytopathies and proteinuria through nephrin down-regulation. The main purpose of the present study was to investigate whether rostafuroxin, a novel antihypertensive agent developed as a selective inhibitor of Src-SH2 interaction with mutant adducin and ouabain-activated Na-K ATPase, may protect podocytes from adducin and ouabain-induced effects, thus representing a novel pharmacological approach for the therapy of podocytopathies and proteinuria caused by the above mentioned mechanisms. To study rostafuroxin effect on podocyte protein changes and proteinuria, mice carrying mutant β-adducin and ouabain hypertensive rats, OHR, were orally treated with 100 μg/kg/day rostafuroxin. Primary podocytes from congenic rats carrying mutant α- (NA) or β-adducin (NB) from Milan hypertensive rats, MHS, and normal rat podocytes incubated with 10^-9 M ouabain, were cultured with 10^-9 M rostafuroxin. The results indicated that mutant β-adducin and ouabain caused podocyte nephrin loss and proteinuria in animal models. These alterations were reproduced in primary podocytes from NB rats and normal rats incubated with ouabain. Treatment of animals, or incubation of cultured podocytes with rostafuroxin, reverted mutant β-adducin and ouabain-induced effects on nephrin protein expression and proteinuria. We conclude that rostafuroxin prevented podocyte lesions and proteinuria due to mutant β-adducin and ouabain in animal models. This suggests a potential therapeutic effect of rostafuroxin also in patients with glomerular disease progression associated with these two mechanisms.
INTRODUCTION

Glomerular diseases leading to progressive kidney failure represent a pivotal cause of morbidity and mortality. Proteinuria is the hallmark of a broad spectrum of podocytopathies characterized by podocyte foot process effacement and alterations of the slit diaphragm, a dynamic signaling hub able to initiate complex signaling networks and integrate multiple intercellular signals via actin cytoskeleton (Grahammer et al., 2013).

The understanding of the genetic molecular mechanisms leading to glomerular podocyte injury and proteinuria may help identifying potential target molecules and developing new and specific therapies for the prevention and treatment of glomerular diseases.

Mutations in several podocyte genes have been associated with monogenic and polygenic syndromes that feature proteinuria (Grahammer et al., 2013; Liu et al., 2001; Mathieson, 2008; Piscione et al., 2011; Wiggins, 2007). In this context, we have contributed to unravel the role of a polymorphism of the cytoskeletal protein adducin and high levels of endogenous ouabain (EO) in hypertension and glomerular podocyte damage, both in animal models and in patients (Bianchi et al., 1994; Bianchi, 2005; Manunta et al., 2009; Ferrandi et al., 2010a; Ferrandi et al., 2010b). These studies have shown that mutant α-adducin and ouabain activate Src tyrosine kinase and the Src-dependent Na-K ATPase phosphorylation and its signaling network, associated with an overall increase of renal tubular sodium reabsorption and blood pressure (BP) (Ferrandi et al., 2010a). Rostafuroxin, a novel anti-hypertensive compound (Quadri et al., 1997), disrupts mutant α-adducin and ouabain-mediated interactions, favoring the normalization of renal Src-Na-K ATPase signaling and BP in hypertensive rats (Ferrandi et al., 2010a; Ferrari et al., 1998; Ferrari et al., 1999a; Ferrari et al., 1999b) and in patients, carrying a specific genetic profile that includes genes encoding for adducin variants and enzymes controlling ouabain synthesis and transport (Lanzani et al., 2010).
Recently, we produced data indicating that mutant β-adducin and EO/ouabain affect the expression of nephrin (Bignami et al., 2013; Ferrandi et al., 2010b), a selective podocyte marker protein (Verma et al., 2006), both in animal models and in patients.

The possible protective effect of rostafuroxin from glomerular injury has not been yet documented. Therefore, the main purpose of the present study was to investigate whether rostafuroxin can counteract mutant β-adducin and ouabain mediated effects, being able to prevent podocyte protein loss and the onset of proteinuria. To this aim, we investigated the effect of rostafuroxin on BP, urinary protein excretion and podocyte protein expression in mice carrying mutant β-adducin, compared to mice with the knockout of β-adducin, and in ouabain hypertensive rats, OHR, obtained by a chronic subcutaneous infusion of a low dose of ouabain. The effect of rostafuroxin on podocyte protein expression has been also investigated in cultured podocytes from congenic rat substrains for α- and β-adducin, obtained by introgressing the mutant α-adducin (NA congenic substrain) or β-adducin (NB congenic substrain) from the Milan hypertensive rats, MHS, into the Milan normotensive, MNS, genetic background, and in normal rat podocytes incubated with ouabain.

The results indicated that rostafuroxin exerts a protective effect on podocyte damage induced by mutant β-adducin and ouabain in animal models. In view of the possible translatability of the data from animals to humans, it is reasonable to speculate that the compound may represent a drug of choice also for patients.
MATERIALS AND METHODS

Chemicals

Rostafuroxin (PST2238) (17β-(3-furyl)-5β-androstan-3β, 14β, 17α-triol) is a digitoxigenin derivate synthesized in Prassis sigma-tau Research Institute, Settimo Milanese, Milan, Italy and developed in sigma-tau, Pomezia, Rome, Italy and CVie Therapeutics, Hong Kong, China. The pharmacological characteristics of the compound are described elsewhere (Quadri et al., 1997; Ferrari et al., 1998; Ferrari et al., 1999a; Ferrari et al., 1999b).

Animal models

Ethical approval

Care and husbandry of animals complied with the guidelines of the Prassis sigma-tau Research Institute for Animal Care, approved by the Italian Ministry of Health, with the European Directive 86/609 and with the Italian Law (DLI 16, January 27, 1992). Animals were monitored by a veterinarian.

Mouse model: β-Adducin null mouse model (official strain designation 129-Add2tm1Llp/Llp) has been previously described (Gilligan et al., 1999). Wild-type 129/Sv mice, carrying the same β-adducin polymorphism (Q529R) described in the Milan hypertensive (MHS) rats (Tripodi et al., 1996), were used as controls. Starting after weaning, male wild-type mice (n=26) were divided into two groups receiving rostafuroxin (100 μg/kg/day in 0.5% Methocel, n=13) or vehicle (0.5% Methocel, n=13) by gavage for 25 weeks. Male β-Adducin null mice (n=10) were used as controls and two β-Adducin null mice received rostafuroxin (100 μg/kg/day) for 25 weeks.
Mice were used to test the effect of rostafuroxin on the following parameters: 1) systolic blood pressure (SBP) and heart rate (HR), that were recorded weekly in conscious mice by an indirect tail-cuff plethysmographic method (BP recorder, U. Basile, Varese, Italy); 2) urinary parameters, that were measured monthly in conscious mice; 3) podocyte protein expression. After 25 weeks of rostafuroxin treatment, all mice were sacrificed to prepare renal microsomes for immunoblotting quantifications of podocyte proteins.

**Rat models:** Two rat models were studied: (i) the congenic rat substrains from MHS and MNS rats and (ii) the ouabain hypertensive rats.

(i) Congenic rat substrains from MHS hypertensive and MNS normotensive parental strains. MHS and MNS rats were derived from the internal stock colony (Prassis sigma-tau Research Institute, Settimo Milanese, Milan, Italy). Congenic rat strains were obtained from MHS and MNS rats, as described (Tripodi et al., 2004). The donor strain was the MHS and the receiving genetic background was the MNS. Two congenic substrains were studied: NA (carrying the mutant 316Y α-adducin from MHS) and NB (carrying the mutant 529R β-adducin from MHS). NA congenic rats, but not NB, significantly raise systolic blood pressure (SBP) compared to MNS (Tripodi et al., 2004).

The congenic rat substrains were used to test the effect of rostafuroxin on the following parameters: 1) SBP and HR, that were recorded weekly in MNS, MHS and NA congenic rats by an indirect tail-cuff plethysmographic method (BP recorder, U. Basile, Varese, Italy); 2) podocyte protein expression: glomerular podocytes were isolated from neonatal NA and NB rats and cultured for 5 days without or with $10^{-9}$ M rostafuroxin for immunoblotting quantifications and immunofluorescence analysis.

(ii) Ouabain hypertensive rats (OHR). Hypertension was induced in male Sprague-Dawley rats, 5 week-old and weighing 110-120 g, by subcutaneous infusion of ouabain (15 μg/kg/day,
Sigma-Aldrich, n=30) with osmotic mini-pumps (Alzet, Charles River, Calco, Italy), for 12 weeks, as described (Ferrari et al., 1998). Normotensive control rats (n=15) received sterile saline solution through osmotic mini-pumps. After 4 weeks, OHR rats were divided into two groups receiving rostafuroxin (100 μg/kg/day in 0.5% Methocel, n=15) or vehicle (0.5% Methocel, n=15) by gavage for 8 weeks.

The effect of rostafuroxin in OHR rats was tested on the following parameters: 1) SBP and HR, that were recorded weekly by an indirect tail-cuff plethysmographic method (BP recorder, U. Basile, Varese, Italy). The initial SBP of controls and OHR rats was comparable (average 125-130 mmHg), then it increased in OHR rats as compared to controls. HR was not affected by ouabain treatment. After 12 weeks of ouabain infusion, plasma ouabain levels increased in OHR compared to controls (from 0.2±0.03 to 0.7±0.07 nM, P<0.001)(Ferrandi et al., 2004); 2) urinary parameters: at the end of rostafuroxin treatment, rats were acclimatized to metabolic cages and urines were collected for urinary parameter measurements; 3) podocyte protein expression: nephrin and synaptopodin were quantified by immunofluorescence and immunoblotting in renal microsomes. The effect of ouabain on nephrin and synaptopodin expression was replicated ex vivo by incubating podocytes primary cell cultures from Sprague Dawley neonatal rats with 10⁻⁹ M ouabain, without or with a simultaneous incubation with 10⁻⁹ M rostafuroxin for 5 days.

Biochemical assays for urinary parameters in mice and rats

Urinary parameters were measured in conscious wild-type and knockout mice monthly, starting from 5 months up to 25 months of age. Mice received a 1 ml bolus of physiological solution by gavage and urine was collected over a period of 6h.

Urinary parameters were also measured in conscious congenic male rats at 3 and 6 months of age and in OHR rats. Rats were housed in individual metabolic cages with free access to water and food. Urine samples were collected over a period of 24h and analyzed for the following
parameters: volume, pH, osmolality, Na⁺ and K⁺ concentrations (IL 943 photometer), total protein excretion (total protein Kit, Sentinel Diagnostics, Milan, Italy), and creatinine (colorimetric Kit, Sentinel Diagnostics).

**Immunostaining**

Immunofluorescence was performed on unfixed renal tissue embedded in OCT compound (Miles Scientific, Naperville, IL, USA), snap-frozen in a mixture of isopentane and dry-ice, and stored at −80°C. Five-micrometer sections were placed on slides and fixed in cold acetone. Cells were seeded on glass coverslips and fixed in buffered paraformaldehyde. In indirect immunofluorescence, the bound primary antibody (anti-nephrin, Progen; anti-synaptopodin, Sigma) was detected by Alexa Fluor 546 anti-guinea pig or anti-mouse IgG (Invitrogen). F-actin was stained by rhodamine-labelled phalloidin (Sigma). Slides were mounted with Vecta-shield aqueous mounting medium (Vector Laboratories, DBA Italia SRL).

**Renal microsome preparations and caveolae isolation**

Renal microsomes were prepared from mice and rats. Kidneys were homogenized in 250 mM sucrose, 30 mM histidine, 1 mM EDTA pH 7.2, centrifuged at 6000g for 15 minutes. The supernatant was centrifuged at 48000g for 30 min. The pellet, containing renal microsomes, was resuspended in sucrose-histidine buffer and analyzed by Western blotting.

Caveolae enriched microdomains were purified according to a detergent-free procedure, as described (Ferrandi et al., 2004). Briefly, renal tissues were homogenized in 200 mM sodium carbonate, pH 11, 2 mM sodium orthovanadate, 100 mg/liter Pefabloc and centrifuged at 6000g for 10 min. The supernatant was sonicated and fractionated on a 5-45% sucrose gradient. The gradients were centrifuged at 150000g for 18h and thirteen fractions were automatically collected and protein content measured. The expression of the specific proteins in the single fractions was determined by
Western blotting. Low-density fractions (fraction 2-5), containing the specific caveolin marker, were referred to as caveolae.

**Glomeruli and podocyte isolation**

Kidneys were removed from 7- to 10-day-old male and female rats. Glomeruli were isolated by sieving then seeded in culture flasks pre-coated with collagen type IV (Sigma-Aldrich) at 37°C in 5% CO2 atmosphere. On days 4 to 5, podocyte growth started and, by day 8, glomeruli were detached using trypsin–EDTA and filtered through the 36-μm mesh to discard glomeruli. Second passage podocytes, >90% pure as judged by light microscopy, were seeded in flasks and chamber slides. Cell characterization was performed by immunofluorescence, using markers for podocytes (nephrin, podocin, synaptopodin), epithelial (cytokeratin), smooth muscle (α-SMA), and endothelial cells (CD31).

Second passage podocytes from neonatal NA and NB rats were seeded in flasks and incubated for 5 days with 10^{-9} M rostafuroxin and analyzed by Western blotting. Podocytes were also obtained from neonatal Sprague Dawley rats and incubated with 10^{-9} M ouabain alone or in the presence of 10^{-9} M rostafuroxin and analyzed by Western blotting.

Measurement of actin stress fibers in podocytes was performed on digitized images using the ImageJ software (www.imagej.nih.gov). After manual selection of the cell area as a region of interest (ROI), a color threshold procedure allowed selective highlighting of stress fibers in gray mode. The software was programmed to calculate automatically the area occupied by staining. An operator blind to the experiments evaluated fifty cell images per experimental procedure. Data were exported in an Excel file and mean, standard deviation and standard error calculated. The statistical differences were then analyzed by ANOVA.
Adducin purification and tyrosine kinase phosphorylation assay in a cell-free system

Recombinant wild-type and mutant rat β-adducin were prepared according to previously published procedures (Ferrandi et al., 1999).

Tyrosine kinase phosphorylation was measured in a cell-free system by Western blotting. Human recombinant full-length kinases belonging to the Src family (such as Src, Yes, Fyn, Lyn) and ABL1 and VEGFR/Flt-1 (50 ng) (Upstate Millipore) were incubated without or with adducin (0.75 μg) in a medium containing 10 mM Tris, 10 mM MgCl2, 5 mM MnCl2, 0.25 mM EGTA, 0.025 mM orthovanadate, 80 mM NaCl, and 2 mM ATP, pH 7.4 for 10 min at 30°C. Rostafuroxin (concentration range from 10^{-13} to 10^{-9} M) was simultaneously added to the kinases. The reaction was stopped by adding Laemmli sample buffer and the samples were then analyzed by Western blotting. Adducin and tyrosine kinase phosphorylation were investigated by using an anti-phospho-tyrosine antibody (clone 4G10, Upstate Millipore) or specific antibodies as indicated.

Western blot analysis

Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Criterion XT, Bio-Rad), blotted on nitrocellulose membrane (Bio-Rad) for 90 min and incubated overnight at 4°C with specific primary antibodies, followed by 1h incubation with fluorescent secondary antibodies (Alexa Fluor, 680 nm, red, Invitrogen; IRDye, 800 nm, green, Rockland). The Western blotting was analyzed and quantified by Odyssey Infrared Imaging Detection System (LI-COR Biosciences). The optical densities were expressed as arbitrary units. The following antibodies were used: anti-nephrin (Progen); anti-podocin (Sigma); anti-synaptopodin (Sigma); anti-α-adducin (raised by Dr S. Salardi in Prassis sigma-tau); anti-ZO-1 (Invitrogen); anti-actin (Sigma); anti-phospho-tyrosine (clone 4G10, Upstate Millipore); anti-pTyr^{418} Src (Cell Signaling); anti-pTyr^{412} ABL1 (Cell Signaling).
Statistical Analysis

Data are reported as mean ± sem. The statistical significance was measured by ANOVA analysis and $P<0.05$ was considered statistically significant.
RESULTS

β-Adducin null mice

Effect of rostafuroxin on systolic blood pressure. SBP did not differ between β-adducin knockout (KO) and age-matched wild-type (WT) mice at any age (Fig. 1A) (Ferrandi et al., 2010b). The chronic oral treatment of WT mice, carrying the same β-adducin polymorphism (Q529R) as described in MHS rats (Tripodi et al., 1996), with 100 μg/kg/day rostafuroxin for 25 weeks, starting from weaning, did not affect either SBP (Fig. 1A) or HR (KO 641±9, n=10; WT 620±12, n=13; WT+rostafuroxin 628±15 beats/min, n=13). Analogously, rostafuroxin did not affect SBP or HR in KO mice (KO control, SBP 114±1.4, Fig. 1A, HR 641±9, n=10; KO+rostafuroxin, mean SBP 112 mmHg, HR mean 648 beats/min, n=2).

Effect of rostafuroxin on renal function. No difference in urinary parameters (volume, Na+, and K+, creatinine excretion) between KO and WT mice was observed, whereas a significant reduction of urinary protein excretion, either normalized, or not, for creatinine excretion, was present in KO as compared to WT mice (Fig. 1B) (Ferrandi et al., 2010b). The prolonged oral treatment of WT mice with 100 μg/kg/day rostafuroxin for 25 weeks normalized proteinuria to the level of KO control mice (Fig. 1B). Conversely, rostafuroxin did not affect proteinuria of KO mice (proteinuria/creatininuria: KO control, 2.6±0.16, n=10, Fig. 1B; KO+rostafuroxin, mean 2.58, n=2).

Effect of rostafuroxin on podocyte protein expression. A Western blot analysis performed on renal microsomes from KO and WT mice showed that the increased proteinuria in WT mice was accompanied by the reduction of nephrin protein expression, the key component of the podocyte slit diaphragm membrane, compared to KO mice (Fig. 1C) (Ferrandi et al., 2010b). The chronic treatment of WT mice with rostafuroxin restored nephrin protein expression to the level of KO mice (Fig. 1C), thus favoring the re-establishment of a normal podocyte filtration activity. Rostafuroxin
did not induce any nephrin modification in KO mice (KO control, 136.4±5.2, n=10, Fig. 1C; KO+rostafuroxin, mean 136 arbitrary units, n=2).

Rat models

To further investigate the effects of rostafuroxin, two rat models were studied: (i) the congenic rat substrains from MHS and MNS rats and (ii) the ouabain hypertensive rats (OHR).

(i) The congenic rat substrains from MHS and MNS rats.

Effect of rostafuroxin on systolic blood pressure. Compared to MNS rats, NA congenic substrain showed a significant raise of SBP (Table 1). Conversely, NB congenic substrain showed SBP levels similar to those of MNS rats (Table 1) (Tripodi et al., 2004; Ferrandi et al., 2010b). The prolonged oral treatment with rostafuroxin (100 μg/kg/day for 8 weeks, starting from weaning) reduced SBP in MHS and NA rats, but was inactive in MNS rats (Table 1), confirming previous data (Ferrandi et al., 2010a). HR was significantly lower in MHS as compared to MNS rats and rostafuroxin treatment did not affect HR in any rat strain (Table 1).

Effect of rostafuroxin on podocyte protein expression. MNS rats develop an age-dependent glomerulosclerosis and proteinuria (Ferrandi et al., 2010b), that is sustained by an increased glomerular production of thromboxane compared to MHS (Brandis et al., 1986; Pugliese et al., 1986; Salvati et al., 1990). Although the prostanoid alteration is the main cause of this dysfunction, the presence of β-adducin in NB rats contributes to a further deterioration of the glomerular damage with age, as documented by the enhanced proteinuria over MNS rats, that is independent from BP levels (supplementary Table S1) (Ferrandi et al., 2010b). On the contrary, MHS and NA rats, carrying the mutant α-adducin, appear protected over time (supplementary Table S1) (Ferrandi et al., 2010b).
Due to the interference exerted by the genetic background of MNS rats, in the present study we did not investigate the effect of rostafuroxin in vivo in congenic rats since the excessive prostanoid production could mask the effect of the compound on the portion of proteinuria related to the adducin-dependent mechanism.

To overcome this difficulty and to directly assess the effect of rostafuroxin on the podocyte protein expression, glomerular podocytes were isolated and cultured in the presence of the compound.

Previous studies have indicated that MNS and NB, compared to MHS and NA rats, show an early reduction of the expression of some podocyte proteins, including nephrin, that precedes the development of proteinuria (Ferrandi et al., 2010b). Here, we confirmed by Western blot analysis that in glomerular podocytes from newborn rats the protein expression of nephrin, podocin, synaptopodin, α-adducin and ZO-1, but not actin, was significantly down-regulated in MNS and NB as compared to NA rats (Fig. 2A). The incubation of cultured podocytes from NB rats with 10^{-9} M rostafuroxin for 5 days favored the re-expression of all these proteins, except for actin, counteracting podocyte protein loss and damage caused by the presence of mutant β-adducin. Conversely, the effect of rostafuroxin was almost absent in NA rat podocytes, since the presence of mutant α-adducin already exerted a protective role from podocyte injury (Fig. 2A).

Furthermore, a fluorescence analysis for phalloidin, a high affinity probe for filamentous actin, of glomerular podocytes from NB rats showed that mutant β-adducin induced a cytoskeletal damage associated with a shortening of actin filaments, while the incubation of podocytes with 10^{-9} M rostafuroxin was able to restore actin filament length (+38%, P<0.05) (Fig. 2B).

Collectively, the findings in cultured podocytes suggest that rostafuroxin protects NB rats from podocyte protein loss and cytoskeletal alterations associated with the presence of mutant β-adducin genetic variant.

(ii) The ouabain hypertensive rats (OHR).
OHR rats were obtained by a chronic ouabain infusion with osmotic minipumps releasing 15 μg/kg/day for 12 weeks, as previously described (Ferrari et al., 1998).

**Effect of rostafuroxin on systolic blood pressure.** Ouabain infusion in rats increased SBP and rostafuroxin treatment at 100 μg/kg/day for 8 weeks antagonized this ouabain-induced effect (Fig. 3A), confirming previous data (Ferrari et al., 1998). Neither ouabain nor rostafuroxin affected HR (control, 350±6.3, n=15; OHR, 355±7.5, n=15; OHR+ rostafuroxin, 358±9 beats/min, n=15).

**Effect of rostafuroxin on renal function.** Ouabain and rostafuroxin did not induce variations of creatinine excretion (control, 31.8±1.16, n=15; OHR, 30.63±0.95, n=15; OHR+rostafuroxin, 30.98±1.14 mg/24h, n=15). Conversely, ouabain infusion in rats induced a significant increase of urinary protein excretion (mg/24h) (Bignami et al., 2013), that was normalized by the prolonged treatment with rostafuroxin (Fig. 3B). A similar picture was observed when proteinuria was normalized for creatinine excretion (control, 1.19±0.1, n=15; OHR, 1.95±0.2, n=15, P<0.05 vs control; OHR+rostafuroxin, 1.2±0.11, n=15, P<0.05 vs OHR).

**Effect of rostafuroxin on podocyte protein expression.** The significant increase of proteinuria induced by ouabain in OHR rats was associated with the down-regulation of nephrin protein expression (Fig. 3C,E) but not synaptopodin (Fig. 3D,E) (Bignami et al., 2013), as measured by Western blot in renal microsomes and immunofluorescence analysis. Rostafuroxin prolonged treatment of OHR rats restored nephrin protein expression (Fig. 3C,E), but did not affect synaptopodin (Fig. 3D,E).

This finding was reproduced *ex vivo* by incubating primary cultures of normal rat podocytes with 10⁻⁹ M ouabain, without or with 10⁻⁹ M rostafuroxin, for 5 days. In this setting, ouabain lowered nephrin protein expression (Fig. 3F) (Bignami et al., 2013), but did not affect synaptopodin (Fig.
3G), as documented by a Western blot analysis. As observed in vivo, rostafuroxin, simultaneously incubated with ouabain, blunted the effect of ouabain, favouring the re-expression of nephrin (Fig. 3F), without affecting synaptopodin expression (Fig. 3G).

Collectively, the present findings suggest that rostafuroxin counteracts ouabain-induced effects on glomerular podocytes, preventing nephrin protein loss and consequently restoring podocyte function.

**Effect of rostafuroxin on SH2-domain interactions**

We attempted to investigate the molecular mechanism through which rostafuroxin antagonizes mutant β-adducin and ouabain-induced effects on podocytes. We based our hypothesis on previous findings indicating that rostafuroxin antagonizes mutant α-adducin and ouabain-activated Na-K ATPase interaction at the Src-SH2 domain, resulting in BP normalization through the modulation of renal tubular Na-K ATPase activity and its related signaling (Ferrandi et al., 2004; Ferrandi et al., 2010a).

Here, we hypothesized that the compound may recognize other SH2-domain interactions, besides that at Src. This finding may be relevant to address the effect of rostafuroxin in nephrin-signaling network, since evidence has indicated that SH2-domain interactions are involved in the complex signaling cascade originating from the slit diaphragm (Verma et al., 2006). The tyrosine kinases on which we evaluated the effect of rostafuroxin were selected on the basis of a score matrix assisted ligand identification analysis (SMALI) performed on nephrin and β-adducin protein sequences. According to this analysis, nephrin may be phosphorylated in tyrosine residues by Fyn, Src and Yes (supplementary Fig. S1), as already published (Verma et al., 2003, Verma et al., 2006, and Quack et al., 2006), but also by ABL1 while β-adducin appears phosphorylated by Fyn, as known (Gotoh et al., 2006), but also by Yes (supplementary Fig. S2).
Interestingly, these identified kinases share a specific sequence for the SH2-binding motif, that is R-E-FLVRESE in Src, Yes and ABL1 and R-E-FLIRESE in Fyn and Lyn (Zhao et al., 2013).

Therefore, we set up experimental conditions in a cell-free system to activate the selected tyrosine kinases by using also the recombinant wild-type and mutant β-adducin to evaluate whether rostafuroxin may prevent interactions at a selective SH2-domain.

We showed that rostafuroxin inhibited the phosphorylation of ABL1 and Yes when these tyrosine kinases were activated by Src (supplementary Fig. S3A,B), with a peak effect at $10^{-11}$-10$^{-12}$ M, but not by Lyn (supplementary Fig. S3C,D). Furthermore, rostafuroxin antagonized the tyrosine phosphorylation of mutant, but not wild-type, β-adducin induced by Yes (Fig. 4A), with a peak effect at $10^{-11}$ M, but not by Fyn (Fig. 4B). We also showed that rostafuroxin antagonized the activation of Yes (supplementary Fig. S4A), with a peak effect at $10^{-10}$-10$^{-11}$ M, but not by Fyn (supplementary Fig. S4B) induced by another substrate, VEGFR1/Flt-1.

We started to validate these findings in vivo. In particular, we studied ABL1 membrane localization and its protein expression in congenic rats. We showed that ABL1 is compartmentalized with caveolin1 and nephrin in caveolae microdomains isolated from rat kidneys (supplementary Fig. S5A,B). Preliminary data indicated that ABL1 is localized in glomerular podocytes (supplementary Fig. S5C) and its expression level appears down-regulated in MNS and NB congenic rats compared to MHS and NA, consensually to nephrin (supplementary Fig. S5D). A positive correlation between nephrin and ABL1 protein expression in glomerular podocytes is shown (supplementary Fig. S5E).

Collectively, the present findings suggest that rostafuroxin antagonizes selective SH2-domain interactions of tyrosine kinases at the binding motif R-E-FLVRESE, common to Src, ABL1 and Yes, but not R-E-FLIRESE, common to Fyn and Lyn. Although not yet clarified, these results may indicate a possible protective effect of rostafuroxin on podocyte derangement mediated through the modulation of specific SH2-containing proteins/adaptors that may be downstream to nephrin signaling networks.
DISCUSSION

The present study uncovers a novel pharmacological application of rostafuroxin in glomerular diseases mediated by mutant β-adducin and ouabain. By combining mouse and rat models (mice carrying mutant β-adducin, congenic rats for mutant α- and β-adducin derived from MHS rats and ouabain hypertensive rats) and rat podocyte cultures, we produced consistent data indicating that rostafuroxin antagonizes podocyte protein loss and the increased urinary protein excretion induced by mutant β-adducin and ouabain. These effects occur independently from blood pressure modulation.

In particular, studies in mice and NB congenic rats, both carrying the same β-adducin polymorphism (Q529R) described in MHS rats (Tripodi et al., 1996), or in ouabain hypertensive rats (OHR), have shown that the development of glomerular podocyte damage and proteinuria is associated with an early down-regulation of nephrin. Conversely, conditions that protect from podocyte injury, such as those observed in the presence of mutant α-adducin genetic variant in MHS and NA rats, restore proteinuria and nephrin expression to normal levels. Similar alterations of podocyte protein expression have been reproduced by culturing podocytes from NB congenic rats (Ferrandi et al., 2010b) or by incubating podocytes in the presence of nanomolar concentrations of ouabain (Bignami et al. 2013). These data imply that β-adducin mutation and ouabain induce direct changes in podocyte architecture and activity.

However, the novelty of the present study is the effect of rostafuroxin. For the first time, we show that the in vivo treatment of mice carrying β-adducin mutation and OHR rats with rostafuroxin normalizes podocyte nephrin expression and proteinuria. The effect of rostafuroxin in vivo in NB congenic rats has not been investigated, due to the interference of the genetic background of MNS rats, which favors the development of a form of glomerulosclerosis associated with an excessive glomerular production of prostanoids (Pugliese et al., 1986; Salvati et al., 1990). However, in
cultured podocytes, where the effect of tromboxane is removed, rostafuroxin indeed restores podocyte protein expression to the normal levels more in NB, where mutant β-adducin induces a more pronounced damage, than in NA rats, which already appear protected. Analogously, rostafuroxin antagonizes ouabain-induced down-regulation of nephrin in ouabain-cultured podocytes as well as in vivo in OHR rats.

The definition of the genetic-molecular mechanisms underlying the different forms of podocytopathies appears as an useful approach to target treatments and to optimize the response to the pharmacological therapy. From this study, rostafuroxin has emerged as an important tool to study and identify the genetic-biochemical abnormalities underlying glomerular diseases triggered by adducin mutations and EO/ouabain in genetic and experimental animal models. The compound appears as a novel drug that may target these altered molecular mechanisms also in patients. Indeed, previous findings have indicated that rostafuroxin is a potent and safe drug (Ferrari et al., 1999a, Ferrari et al., 1999b) able to counteract hypertension development, and the related hypertensive effects, induced by mutant α-adducin and ouabain at renal and vascular level, preventing in the long run cardiac and renal hypertrophy in animal models (Ferrandi et al., 2004, Zhang et al., 2005). More recently, a pharmacological clinical trial has shown that rostafuroxin normalizes BP levels also in patients carrying a selective genetic profile that includes genes encoding for adducin variants and enzymes controlling ouabain synthesis and transport (Lanzani et al, 2010).

Rostafuroxin molecular mechanism underlying the anti-hypertensive effect has been identified in our previous studies. It relies on its ability to inhibit the interaction of mutant α-adducin or ouabain-activated-Na-K ATPase at the Src-SH2-domain, preventing Src activation and the Src-dependent Na-K ATPase phosphorylation and activation at renal tubular level (Ferrandi et al., 2010a). The result is the disruption of the complex signaling cascade downstream to Src-Na-K ATPase induced by rostafuroxin, that is associated with the normalization of BP in hypertensive rats and patients (Ferrandi et al., 2010a; Lanzani et al, 2010).
Definite protein interaction networks that involve SH2-domain containing proteins appear to control a wide spectrum of physiological activities, that include cardiac and renal function, and influence cardiovascular remodeling (Grahammer et al., 2013; Liu et al., 2012). However, also podocyte activities and actin cytoskeletal dynamics are known to be integrated in a dynamic signaling hub at the slit diaphragm membrane (Grahammer et al., 2013; New et al., 2013). Alterations in these signaling pathways, frequently caused by genetic mutations within these networks that are responsible for biochemical dysfunctions in proteins and in their related signaling cascades, may progress to glomerular diseases (Mathieson et al., 2008; Wiggins, 2007). One of the key components of the podocyte signaling hub is nephrin, which appears to be controlled at multiple levels, by the modulation of its phosphorylation state via Src family tyrosine kinases (Fyn, Yes, Src) and trafficking (Quack et al., 2006) or by a direct degradation process mediated by calpain (Peltier et al., 2006; Tian et al., 2014).

Interestingly, although we have not yet fully addressed the molecular mechanism through which rostafuroxin prevents nephrin loss at podocyte level and proteinuria, we have two additional sets of data indicating that:

(i) rostafuroxin appears to recognize and antagonize selective interacting substrates at the SH2-binding motif R-E-FLVRESE, common to Src, Yes and ABL1 kinase, but not at the SH2-binding motif R-E-FLIRESE, common to Fyn and Lyn, where a valine (V) is substituted with an isoleucine (I) (Zhao et al., 2013). Interestingly, it has been documented that mutant, but not wild-type, β-adducin tyrosine phosphorylation induced by Yes is reduced by rostafuroxin as well as Src and Flt-1 activation of Yes. Since also nephrin is under the control of Yes, it remains to be established whether rostafuroxin may affect Yes-mediated nephrin interaction and phosphorylation with potential consequences on its functional activity.

We started to investigate one of the selected kinases, ABL1, in \textit{in vivo} studies. In rat kidneys, preliminary results indicate that ABL1 localizes with nephrin in caveolae subdomains, known to
functionally organize signaling pathways (Simons et al., 2001), is present in rat glomerular podocytes and its expression level is down-regulated in MNS and NB glomerular podocytes, as well as nephrin, compared to MHS and NA.

Although the relevance of this finding in the context of the nephrin-signaling network still remains to be elucidated, ABL1 kinase appears of particular interest since a SMALI analysis suggests that nephrin contains three possible SH2-consensus sequences for ABL1 (supplementary Fig. S1), not yet functionally identified, that, if verified, may indicate the involvement of this kinase in the modulation of nephrin signaling function.

Indeed, nephrin-associated kinases are implicated in the recruitment of SH2–SH3 domain–containing adaptor proteins and in the phosphorylation of substrate proteins belonging to the podocyte junctions, favoring the assembly of actin filaments and the integrity of the podocyte signaling network (Verma et al., 2006).

The results of the present study may imply, but still needs to be verified, that mutant β-adducin and EO/ouabain may affect signaling pathways downstream to nephrin, which include interactions with SH2-containing substrate proteins, or adaptors, responsible for podocyte derangements.

Rostafuroxin may exert a selective activity on specific SH2-containing domains preventing the sequence of events leading to deleterious podocyte lesions.

(ii) Mutant β-adducin and ouabain increase calpain activity in cultured podocytes and this effect associates with nephrin cleavage (Ferrandi M, personal communication). This hypothesis is further reinforced by the evidence that, in mesenteric arteries of OHR rats, ouabain increases the activity of α2 Na-K ATPase, NCX1 and TRPC6 channels, in a Src-dependent manner, being responsible for the increased intracellular Ca++ (Zulian et al., 2013). Since rostafuroxin blocks adducin and ouabain-induced effects, we can envisage that the compound may affect Ca++ signaling also in glomerular podocytes, thus preventing mutant β-adducin and ouabain-mediated nephrin
cleavage induced by calpain or, possibly, the TRPC6-PLC-γ1-mediated pathway for nephrin signal transduction regulation.

Certainly, the identification of a successful treatment of glomerular diseases is still a great medical need and new therapeutic strategies are highly desirable. Clinical evidence supports the notion that once the glomerular disease is established, it may progress to terminal renal failure (Levey et al., 2012). Therefore, in the clinical practice, it is crucial to find a drug which can block the initial triggering events but also delay, or prevent, the progression to end-stage renal disease. Although not completely understood, the molecular mechanisms that operate in chronic renal failure causing glomerular damage, loss of active nephron, proliferation of mesangial cells and thickening of basal lamina are frequently associated with tubule-interstitial fibrosis, renal tissue inflammation and matrix deposition that may ultimately induce a compensatory hypertrophy of the remaining nephrons and of the whole kidney (Turner et al., 2012). Peptides, such as angiotensin II, or the increased hydrostatic pressure, responsible for mechanical stress, and the rearrangement of actin cytoskeleton mediated by Rho family GTPases and reactive oxygen species (ROS) (Babelova et al., 2013) have been considered the most likely cause of podocyte injury. From the present study, mutant β-adducin and increased levels of EO/ouabain emerge as additional causes of podocytopathies.

Interestingly, rostafuroxin counteracts adducin-mediated cytoskeletal alterations and ouabain pro-hypertrophic effects (Ferrandi et al., 2004) and prevents proteinuria occurrence in animal models. Therefore, additional studies should be undertaken to establish whether the observations in animal models may be translated to patients, thus indicating if rostafuroxin may be a drug of choice to antagonize mutant β-adducin and ouabain-mediated effects in human glomerular diseases. This observation, if verified, becomes of particular relevance since it opens a novel therapeutic option for patients who experience glomerular disease progression associated with these two mechanisms.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Ferrandi, Ferrari, Bianchi and Manunta

Conducted experiments: Ferrandi, Molinari and Rastaldi

Performed data analysis: Ferrandi and Rastaldi

Wrote or contributed to the writing of the manuscript: Ferrandi, Ferrari and Bianchi
REFERENCES


FOOTNOTES

No financial support is declared.
FIGURE LEGENDS

Figure 1 - Effect of rostafuroxin treatment in mice. Wild-type mice (WT), carrying the same β-adducin polymorphism of MHS rats were orally treated with 100 μg/kg/day rostafuroxin (WT+rostaf, n=13) or with vehicle (WT control, n=13), starting from weaning. β-Adducin null mouse model (KO, n=10), were used as controls. A. Effect of rostafuroxin on systolic blood pressure (SBP). SBP was recorded weekly in conscious mice. Data are mean±sem after 25 weeks of treatment. B. Effect of rostafuroxin on proteinuria. Urinary parameters were measured in conscious mice. Data of proteinuria, normalized for creatinine excretion, are mean±sem after 25 weeks of treatment. C. Effect of rostafuroxin on podocyte nephrin expression. After 25 weeks of rostafuroxin treatment, all mice were sacrificed to prepare renal microsomes for immunoblotting quantification of nephrin (10 μg protein/lane). The densitometric analysis for nephrin was expressed as arbitrary units and normalized for actin content. Data are mean±sem. $P<0.05$ WT vs KO mice; * $P<0.05$ WT vs WT+rostaf.

Figure 2 - Effect of rostafuroxin treatment on podocyte protein expression in congenic rat substrains. Glomerular podocytes were isolated from neonatal MNS, NA and NB rats. NA and NB podocytes were cultured without (control) or with 10^{-9} M rostafuroxin for 5 days for immunoblotting quantifications and immunofluorescence analysis. A. Western blot analysis of cultured podocytes for nephrin, podocin, synaptopodin, α-adducin, ZO-1 and actin. The densitometric analysis was expressed as arbitrary units and normalized for actin content. Data are mean±sem. MNS, n=12; NA control, n=9; NA+rostafuroxin (rostaf) n=6; NB control, n=14; NB+rostafuroxin n=12. $P<0.05$ vs MNS; * $P<0.05$ NB control vs NB+rostafuroxin. B.
Immunofluorescence analysis of phalloidin. NB podocytes were cultured alone or with $10^{-9}\text{ M}$ rostafuroxin for 5 days. Phalloidin was used to evaluate the protective effect of rostafuroxin on cytoskeletal damage associated with actin filament shortening. Two rats per each group were analyzed. Magnification x400.

**Figure 3 - Effect of rostafuroxin treatment in ouabain hypertensive rats (OHR).** Ouabain (15 $\mu$g/kg/day) was subcutaneously infused into normotensive Sprague-Dawley rats for 12 weeks (OHR rats). After 4 weeks, a group of OHR rats received orally 100 $\mu$g/kg/day rostafuroxin (R) for 8 weeks (OHR+R, n=15) while OHR control received vehicle (OHR, n=15). Normotensive controls received subcutaneously saline solution (control, n=15). **A.** Effect of rostafuroxin on systolic blood pressure. SBP was recorded weekly in conscious rats. Data are mean±sem after 12 weeks of ouabain infusion. $$P<0.01\text{ control vs OHR; } **P<0.01\text{ OHR vs OHR+rostafuroxin. }$$ **B.** Effect of rostafuroxin on proteinuria. After 12 weeks of ouabain infusion, rats were housed in single metabolic cages and 24h urine collected for urinary parameter measurements. Proteinuria was expressed as mg/24h. $P<0.05\text{ control vs OHR; } *P<0.05\text{ OHR vs OHR+rostafuroxin.}$ **C,D.** Effect of rostafuroxin on nephrin and synaptopodin expression in renal microsomes. At sacrifice, kidneys were excised and used to quantify nephrin (C) and synaptopodin (D) protein expression in renal microsomes by immunoblotting. The densitometric analysis was expressed as arbitrary units and normalized for actin content. Data are mean±sem; nephrin n=15 rats each group, synaptopodin n=6 rats each group. $P<0.05\text{ control vs OHR; } *P<0.05\text{ OHR vs OHR+rostafuroxin.}$ **E.** Immunofluorescence analysis for nephrin and synaptopodin in kidney sections from control, OHR and OHR treated with rostafuroxin. Two rats per each group were analyzed. Magnification x400. **F,G.** Effect of rostafuroxin on nephrin and synaptopodin expression in cultured podocytes. Glomerular podocytes from neonatal Sprague Dawley rats were incubated for 5 days with $10^{-9}\text{ M}$ ouabain, alone or in association with $10^{-9}\text{ M}$ rostafuroxin (R), and samples were analyzed for
immunoblotting. The densitometric analysis was expressed as arbitrary units and normalized for actin content. Data are mean±SEM; nephrin n=12 each group, synaptopodin n=6 each group. $P<0.05$ control vs ouabain; * $P<0.05$ ouabain vs ouabain+rostafuroxin.

**Figure 4 - Effect of rostafuroxin on in vitro phosphorylation of tyrosine kinases containing SH2-domains.** Recombinant β-adducin (0.75 μg) was incubated in a cell-free system for 10 min at 30°C with tyrosine kinases (50 ng). A tyrosine kinase containing the SH2-binding motif R-E-FLVRESE (Yes) and one containing the SH2-binding motif R-E-FLIRESE (Fyn) were chosen. The effect of increasing concentrations of rostafuroxin (range from $10^{-12}$ to $10^{-9}$ M), simultaneously added to the kinases, was investigated. The reaction was stopped by adding Laemmli sample buffer and samples were analyzed by Western blotting for adducin phosphorylation by using an antiphospho-tyrosine antibody. **A.** Effect of rostafuroxin (rost) on mutant (left) and wild-type (right) β-adducin (Add) tyrosine phosphorylation induced by Yes. **B.** Effect of rostafuroxin on mutant (left) and wild-type (right) β-adducin tyrosine phosphorylation induced by Fyn. Data are mean±SEM of 4 independent experiments. *$P<0.05$ plus rostafuroxin vs minus rostafuroxin (black bar).
Table 1

Effect of rostafuroxin on systolic blood pressure and heart rate in MNS, MHS and NA congenic rats

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<th>CONTROLS</th>
<th>+100 μg/kg/day ROSTAFUROXIN</th>
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<tr>
<td>Rats</td>
<td>MNS n=8</td>
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<td>BW g</td>
<td>350 ± 4</td>
<td>375 ± 6</td>
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<td>SBP mmHg</td>
<td>139 ± 1</td>
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<td>HR beats/min</td>
<td>398 ± 12</td>
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Data are mean±sem of parameters measured in male rats at 3 months of age. N, number of rats per each group. BW, body weight; SBP, systolic blood pressure; HR, heart rate. The statistical significance among groups was measured by ANOVA. $$ P<0.01 \text{ vs MNS}; \text{** } P<0.01 \text{ controls vs rostafuroxin.}
FIGURE 2

A

[Graphs showing the optical density of Nephrin, Podocin, Synaptophysin, α-Adducin, ZO-1, and Actin in control and +rostaf conditions for MNS, NA, and NB.]

B

[Images showing fluorescence microscopy of NB control and NB + rostaf conditions, with red fluorescence indicating the distribution of the proteins.]
FIGURE 3

A Systolic blood pressure

B Proteinuria

C Nephrin

D Synaptopodin

E control OHR OHR + rostaf

F Nephrin

G Synaptopodin
FIGURE 4

A  Yes + β-Adducin

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B  Fyn + β-Adducin

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