PST 2238: A New Antihypertensive Compound that Modulates Na\(^+\),K\(^+\)-ATPase and Antagonizes the Pressor Effect of OLF

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INTRODUCTION

Over recent years, pharmacological treatment of essential hypertension (EH) has been evolving from an empirical approach based on “group therapy” to a more individual approach that addresses specific pressor mechanisms affecting certain patients (39). This evolution is also due to the huge individual variation of blood pressure response to the available antihypertensive drugs, none of which is effective throughout the whole population of hypertensive patients. One of the major problems with the treatment of hypertension is poor patient compliance, which results from the side effects and the lack of obvious immediate benefits of treatment, reducing the acceptability of drugs. The final result is that only ~30% of patients are adequately treated.

These problems arise mainly from the lack of a complete understanding of the mechanisms underlying the development of primary hypertension and the secondary involvement of major organs (mainly heart and brain). Although the antihypertensive efficacy of different classes of drugs is similar when populations are compared, there is a great deal of individual variability in the response to a given therapeutic regime. This is because hypertension develops as a consequence of the interaction between polygenic and environmental backgrounds, which differ among patients. Therefore, the future success of a new therapy for hypertension will depend upon the ability to understand the molecular and genetic mechanisms operating in a subset of patients and the ability to develop new drugs able to correct such mechanisms (23). Prassis-Sigma Tau has developed a new class of antihypertensive compounds in accordance with this line of thought and their research is aimed at correcting a genetic molecular mechanism previously demonstrated to be involved in the pathogenesis of hypertension in rats and humans.

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ETIOLOGY OF ESSENTIAL HYPERTENSION

The Endogenous Ouabain-Like Factor (OLF)

One new mechanism currently under investigation deals with the etiology of essential hypertension in a subgroup of patients. It concerns with the association between a primary defect in the ability of kidneys to excrete Na⁺ and a secondary increase in the plasma level of an endogenous inhibitor of the Na⁺-K⁺ pump (14). In fact, evidence has been provided, over recent years, that the Na⁺-K⁺ pump may be functionally modulated by endogenous compounds, including digoxin-like factors and bufadienolide derivatives (63). One of these endogenous inhibitors, the so-called ouabain-like factor (OLF), has recently been rigorously investigated in both rats (17,31,38,56) and humans (15,32,33,44,46) and has been shown to be a closely related isomer of ouabain (43,61,65).

The physicochemical characterization of OLF purified from tissues and plasma of rats (17) and humans (18) indicates that it is a non-peptide, low molecular–weight substance which, unlike ouabain, is partially inactivated by acid hydrolysis. From a biochemical point of view, OLF behaves similarly to ouabain, since both inhibit the purified Na⁺,K⁺-ATPase with high affinity (Kᵢ = 5nM) in a dose- and K⁺-dependent manner (17). OLF has the same association and dissociation rates at the receptor: it inhibits, with a potency similar to that of ouabain ⁸⁶Rb uptake into human erythrocytes and displaces [³H]ouabain from Na⁺,K⁺-ATPase without affecting membrane Ca²⁺-ATPase (17).

Despite many biochemical similarities with ouabain, OLF differs from the cardiac glycoside in its affinity towards the different isoforms of the α subunit of Na⁺,K⁺-ATPase (α₁ and α₃). In particular, OLF inhibits the rat renal α₁ Na⁺,K⁺-ATPase with 1000-fold higher potency (IC₅₀ = 10⁻⁷ M) than ouabain (IC₅₀ = 10⁻⁴ M) and has an affinity similar to that of ouabain (IC₅₀ = 1.5 x 10⁻⁸ M) for the α₃ isoform purified from rat brain (17). This biochemical difference underlies the structural difference between OLF and ouabain, as was recently confirmed (61,65), and supports the physiological and pathophysiological role of OLF as a regulator of fluid and pressure homeostasis in animal species considered resistant to ouabain, such as rats.

OLF is normally present at low concentrations in tissues and plasma, but elevated OLF levels have been demonstrated in pathological conditions, such as cardiovascular disorders (1,12,30) and some forms of human essential (33,53) and rat genetic hypertension (17,40). Furthermore, much experimental and clinical evidence suggest that OLF, through the inhibition of the Na⁺-K⁺ pump activity in particular areas, such as vasculature and nervous system, activates neurovascular reactivity (7,9) that could favor pathological complications.

A still unresolved issue concerns the precise mechanism(s) that lead to the increase in circulating levels of OLF. It has been proposed that the enhanced volume of distribution generally associated with alterations of salt and water homeostasis (14,31,46), may lead to increased synthesis and/or secretion from the target organs. No conclusive results are so far available, however, to shed light on the processes that mediate, at the renal level, the secretion and reabsorption of this endogenous compound. Since the levels of OLF may be modulated by the kidney, it is likely that the renal abnormality that accompanies the development of cardiovascular diseases may affect not only Na⁺ and water handling but...
also the clearance of endogenous compounds, such as OLF. Similarly to OLF, ouabain itself has been demonstrated to act in vivo as a hypertensive agent (34,41). The chronic infusion of low doses of ouabain in normal Sprague Dawley rats is indeed capable of inducing in 70% of animals a reversible form of hypertension (41), sustained by an upregulation of the renal Na⁺,K⁺-ATPase activity (24).

The Milan Hypertensive Rat Strain: Role of Cytoskeleton, Na⁺-K⁺ pump, and OLF in Genetic Hypertension

The above-mentioned findings suggest that ouabain sensitivity can develop because of a genetic predisposition. In fact, according to the original hypothesis of Blaustein (7) and de Wardener (14), a simultaneous peculiar renal defect in Na⁺ handling must be present in order to allow the pressor effect of OLF or ouabain to take place. In this respect, rats of the Milan hypertensive strain (MHS) represent a good example of an animal model in which a primary renal defect in the ability to excrete Na⁺ (2,3) causes hypertension and increased levels of endogenous OLF (17,18). Hypertension can be “transplanted” from MHS rats to MNS normotensive controls by kidney cross-transplantation and, vice versa, a decrease in blood pressure is observed in MHS when transplanted with MNS kidney (3,4).

The functional alteration underlying the pressor role of MHS kidney is an enhanced Na⁺ and water tubular reabsorption (8,54). This alteration is sustained at the cellular level by an overall increase in Na⁺ transport (21,35), mainly driven by the basolateral Na⁺-K⁺ pump activity which is higher in MHS than in MNS, already before the development of hypertension (20). The enhanced Na⁺-K⁺ pump activity of MHS rats is, in turn, due to an upregulation of pump expression at mRNA and protein levels (20).

The primary genetic defect responsible for the renal functional alteration and hypertension of MHS rats has been recently identified. Two missense point mutations have been found in the α and β subunits of the MHS cytoskeletal protein adducin (5) and explain ~50% of the blood pressure difference between MHS and MNS (5). Rat renal epithelial cells (NRK), transfected with MHS adducin cDNA, show an alteration of the actin cytoskeleton structure (59) and significantly greater activity and immunohistochemical expression of the Na⁺-K⁺ pump compared with cells transfected with MNS adducin cDNA (59). These findings suggest, therefore, that the renal alteration that leads to MHS hypertension is produced by an upregulation of renal Na⁺-K⁺ pump caused by a genetic alteration of adducin.

The involvement of OLF in MHS hypertension seems to be secondary to the alteration of the renal Na⁺-K⁺ pump. It has been observed that OLF levels change with age in either MHS or MNS rats and that differences between the two strains are related to different phases of hypertension development (19). At the prehypertensive age of 21 d, hypothalamic OLF levels were lower in MHS rats than in MNS rats, while during the development of hypertension (25 to 35 d), OLF levels became higher in MHS than in MNS. This difference was maintained in adult rats (90 d) (19). A similar pattern of changes has been observed also in hypophys, but not in adrenals or kidney. Similarly to hypothalamus, plasma OLF concentrations decreased with age in either strain, but unlike in brain tissue, MHS had higher plasma OLF levels than MNS even before hypertension developed.
These findings suggest that, according to the original hypothesis (7), the primary renal alteration of tubular Na\(^+\) reabsorption in MHS rats, which causes a transient Na\(^+\) retention, could trigger increased OLF secretion from the production sites into the blood, especially during the early phase of the disease.

Since OLF is digitalis-like, its increased circulating levels in MHS rats should lead to inhibition rather than stimulation of the renal Na\(^+\)-K\(^+\) pump. The following observations suggest that it is unlikely that the renal Na\(^+\)-K\(^+\) pump of MHS rats was inhibited, however: 1) plasma OLF concentrations range from 0.1 to 2 nM in rats, a ouabain-resistant species whose \(\alpha_1\) Na\(^+\),K\(^+\)-ATPase displays an affinity for digitalis around 1 mM; 2) at low concentrations ouabain stimulates, and not inhibits, Na\(^+\)-K\(^+\) pump activity in cardiac tissue (27,28); 3) long-term treatment of cultured cells with low K\(^+\) or low concentrations of ouabain (49,57) and \textit{in vivo} chronic digitalization (62) upregulates Na\(^+\)-K\(^+\) pump pool size, measured as either activity at \(V_{\text{max}}\) or protein expression. A biphasic action of cardiac glycosides on the Na\(^+\)-K\(^+\) pump activity is, therefore, proposed: 1) at high concentrations these drugs inhibit the pump and in the long-term an upregulation of the pump expression takes place as a positive feed-back mechanism by which the cell can reestablish the equilibrium of the Na\(^+\)-K\(^+\) ion gradient altered by pump inhibition (49,51); 2) at very low concentrations (nanomolar range) prolonged exposure to ouabain (or OLF), either in the whole rat (24) or cultured rat renal cells (24), may lead to an increased pump pool size as a consequence of a positive feedback mechanism that responds to a subliminal and technically undetectable pump inhibition. A new, still unexplained, cellular effect of ouabain may stimulates the transcription machinery of the Na\(^+\)-K\(^+\) pump either through the activation of other transport systems (48,55) or by a direct mechanism.

As extensively discussed elsewhere (6), many similarities have been shown between MHS rats and a subgroup of human patients who are either hypertensive or prone to develop hypertension. These similarities concern renal function, cellular ion transport mechanisms and plasma levels of renin and OLF. The data support the notion that a primary increase in tubular Na\(^+\) reabsorption may explain hypertension also in this subgroup of patients. The involvement of adducin genetic polymorphism in human essential hypertension has been studied and the results show that: 1) there is 94% homology between human and rat adducin (10); 2) two missense point mutations in the \(\alpha\) adducin subunit have been identified also in humans (10); 3) either these mutations or DNA markers mapping close to the adducin locus are associated with hypertension (10,11,37); the adducin locus was linked with hypertension in a study of siblings (11); and 4) one of the human adducin variants was associated with alterations in the blood pressure response to acute Na\(^+\) changes (11,42).

The link among the individual steps connecting adducin polymorphism to the increase in Na\(^+\)-K\(^+\) pump activity, tubular Na\(^+\) reabsorption, OLF levels, and hypertension have not yet been fully elucidated in humans. However, very recent findings demonstrate that the “hypertensive” adducin isoforms from either rats or humans display a higher affinity for Na\(^+\),K\(^+\)-ATPase in a cell-free system than does the “normotensive” variant (22). Available findings indicate a common molecular mechanism, supported by adducin, which operates in rats as well as in a subgroup of hypertensive patients.

A percentage of hypertensive patients in which this molecular mechanism operates can be estimated on the basis of frequency of the \(\alpha\) adducin alleles in different human races.
This frequency varies from 65% in the Japanese population (37) to 10% in Africans (unpublished results), and around 25% in Caucasians (11).

**A NEW PHARMACOLOGICAL APPROACH TO HYPERTENSION THERAPY**

The above-mentioned alterations that lead to hypertension in either genetic models of hypertension in rats (MHS) or humans are summarized in Fig. 1. A primary genetic molecular defect of the cytoskeletal adducin may increase the Na⁺-K⁺-pump units on the cell membrane with a consequent enhancement of the overall Na⁺ transport across renal tubular cells, thus leading to an increase in renal Na⁺ reabsorption, blood pressure, and OLF levels. The link between increased OLF levels and upregulation of the Na⁺-K⁺ pump, and consequently hypertension, is still to be clarified. As mentioned above, chronic exposure to low concentrations of OLF may have a synergistic effect with adducin favoring an increase in pump units (measured in MHS rats as activity at $V_{\text{max}}$) (20). The

**FIG. 1.** Proposed sequence of mechanisms that lead to the development of hypertension in the genetic model of Milan hypertensive (MHS) rats. The genetic alteration of the cytoskeletal adducin, directly or through the modulation of actin cytoskeleton, induces an increase in renal Na⁺-K⁺ pump expression and hence an increase in tubular Na⁺ reabsorption that triggers the development of hypertension. The parallel increase of OLF levels is responsible for the sustained upregulation of the Na⁺-K⁺ pump in association with a pressor effect. The molecular mechanism of new antihypertensive compounds capable of antagonizing the pressor effect of OLF is described. This scheme may be relevant also for humans, since, in a subgroup of essential hypertensive patients, adducin modulates renal tubular Na⁺ reabsorption leading to a salt-sensitive form of hypertension.
mechanism of this increase is still unclear. Preliminary data suggest, however, that ouabain as well as adducin mutations decrease the rate of pump removal from the cell membrane.

On the basis of these findings, it can be postulated that any therapeutic maneuver able to interfere with this mechanism might also lower blood pressure in those subjects where it operates. In this respect, increased Na\(^+\)-K\(^+\) pump activity and OLF levels could represent a new pharmacological target for the treatment of hypertension dependent on this mechanism. In other genetic models of essential hypertension, such as the Dahl and the SHR strains, adducin polymorphism is not associated with hypertension (60). Although OLF may play some role in Dahl salt-sensitive (SS) rats (40), its involvement in spontaneously hypertensive rats (SHR) is doubtful (16). It is likely, therefore, that these forms of hypertension will not respond to a therapy aimed at normalizing the expression and activity of the renal Na\(^+\)-K\(^+\) pump that has been altered as a consequence of the adducin mutations. Indeed, as detailed below, SHRs do not respond to the new compound described in this manuscript (25). The possibility cannot be excluded, however, that independently from this specific genetic cause, an upregulation of the renal Na\(^+\)-K\(^+\) pump, caused by other molecular abnormalities, may represent a target for this new therapeutic approach to hypertension. The research in Prassis-Sigma Tau was aimed at synthesizing and selecting a new orally active antihypertensive compound able to interfere with the above-described sequence of events but devoid of cardiac or hormonal side effects typical of digitalis and antimineralcorticoid drugs.

In this manuscript we describe the pharmacological characteristics of PST 2238, a prototype of this new class of antihypertensive compounds.

![Chemical structure of PST 2238](image-url)

**FIG. 2.** Chemical structure of PST 2238 (17\(\beta\)-(3-furyl)-5\(\beta\)-androstan-3\(\beta\), 14\(\beta\), 17\(\alpha\)-triol).
THE NEW ANTIHYPERTENSIVE COMPOUND: PST 2238

Synthesis

As part of the program to identify novel Na⁺,K⁺-ATPase antihypertensive agents, a series of analogs of digitoxigenin was prepared. Basic structural features for the activity of digitoxigenin are the 17β-lactone and the 3β- and 14β-hydroxy substituents. The unsaturated lactone is thought to be the first point of attachment between the steroid and the receptor but is not required for the activity. The number of attractive substituents for the C-17 position is large but the structures involved fall into two very broad groups: the cyclic moieties and various open-chain forms (58). We explored the possibility of obtaining new derivatives with an affinity for Na⁺,K⁺-ATPase but with a different profile from digoxin by introducing functional groups in the 17 position, including heterocyclic substituents, different from the digitalis unsaturated lactone, and a free 17α-hydroxyl group. PST 2238 (17β-(3-furyl)-5β,14β,17α-triol) with a 3-furan ring in the 17β-position and a hydroxyl function in the 17α-position of the steroid nucleus (Fig. 2) was found to have antihypertensive activity and was chosen for an in-depth evaluation. Details of the synthesis of PST 2238 are described elsewhere (50).

In Vitro Interaction of PST 2238 with Na⁺,K⁺-ATPase

Ouabain, used as a reference compound, inhibits the purified dog kidney Na⁺,K⁺-ATPase and displaces [³H]ouabain-specific binding from the receptor with an IC₅₀ of 2.5 × 10⁻⁸ M (24). Under the same experimental conditions, PST 2238 inhibited the enzyme with an IC₅₀ of 2.5 × 10⁻⁵ M, and displaced [³H]ouabain from Na⁺,K⁺-ATPase with an IC₅₀ of 1.7 × 10⁻⁶ M (24). Therefore, there is a 15-fold difference in IC₅₀ for inhibition of the enzyme and for displacement of [³H]ouabain for PST 2238. It has been also demonstrated that this difference is due to a rapid dissociation of PST 2238 from the Na⁺,K⁺-ATPase when K⁺ is added (24). The time course of association and dissociation from the Na⁺,K⁺-ATPase for [³H]ouabain and [³H]PST 2238 was further characterized. Ouabain was found to bind to Na⁺,K⁺-ATPase slowly, with a half-time of 18.8 min, while PST 2238 binds very rapidly with a calculated half-time of 8.6 s. Similarly, the two molecules dissociate from the enzyme with different half-times, 23.2 min and 10.8 s for ouabain and PST 2238, respectively. These results indicate that binding of PST 2238 to Na⁺,K⁺-ATPase is highly reversible, while ouabain forms a pseudo-irreversible complex with the enzyme (64).

In Vitro Interaction of PST 2238 with Various Receptors

To assess the specific interaction with the Na⁺,K⁺-ATPase, the in vitro binding of PST 2238 to a panel of receptors, known to be involved in blood pressure regulation or hormonal steroid control, was studied. PST 2238, at concentrations up to 10⁻⁴ M, did not show any significant interaction with α⁻ and β-adrenergic, D₁, D₂, D₃, 5-HT₁, 5-HT₂, H₁, H₂, M₁, M₂, A₁, A₂, Ca²⁺, Na⁺, or K⁺ channel–associated receptors, AT₁, AT₂, ETₐ, ETₕ.
FIG. 3. Effect of PST 2238 on systolic blood pressure (SBP) in ouabain-hypertensive rats (OS, panel A) and in spontaneously hypertensive rats of the Milan strain (MHS) (panel B). A) PST 2238 was orally administered at doses of 0.01, 1, 10, or 100 μg/kg to OS rats for 4 w. SBP of untreated normotensive control rats (CS) is also reported. Data are means ± S.E.M. of 8 rats for each group. B) MHS rats were orally treated for 6 w with PST 2238 at doses of 0.1, 3, or 90 μg/kg or 3 mg/kg. Data are means ± S.E.M. of 7 rats per each group. Statistical differences from the untreated hypertensive group were calculated by two-way ANOVA followed by Dunnett’s t-test. *p < 0.05; **p < 0.01. From ref. 24 and 50.
GABA, thromboxane, vasopressin, angiotensin II, or the steroid-hormone receptors (andro- 
gen, progestogen, estrogen and mineralocorticoid), confirming that PST 2238 is specific 
for Na⁺,K⁺-ATPase (24).

**Interaction of PST 2238 with the Na⁺-K⁺ Pump in Cell Cultures**

**Interference with Na⁺-K⁺ Pump and Ouabain in Normal Rat Kidney Cells**

Experiments on the long-term effect of PST 2238 in cultured rat kidney cells (NRK- 
52E) were performed to assess whether this compound is able to interfere with the Na⁺-K⁺ 
pump, either alone or together with ouabain, in a system where the pump can display its 
functional role. The activity of the Na⁺-K⁺ pump in cultured NRK cells was assessed by 
ouabain-sensitive ⁸⁶Rb uptake and measured in Na⁺-loaded cells at Vmax (24), in order to 
determine the total number of pump sites capable of activation. The effect of a short-term 
exposure to PST 2238, or ouabain, on the Na⁺-K⁺ pump Vmax was measured after 5 h of 
incubation of cells with increasing concentrations of the compounds. No inhibitory effect 
was observed with PST 2238 at concentrations up to 10⁻⁴ M, while ouabain began to 
inhibit the Na⁺-K⁺ pump at concentrations above 10⁻⁵ M, with a calculated IC₅₀ of 7 × 
10⁻⁵ M (24).

To assess the long-term effect of low concentrations of PST 2238 and ouabain on the 
Na⁺-K⁺ pump activity, NRK cells were incubated with the two compounds, alone or in 
combination, for 5 d. Ouabain, at concentrations ranging from 10⁻¹⁰ M to 10⁻⁸ M, 
increased the rate constant of the Na⁺-K⁺ pump at Vmax (10⁻¹⁰ M = 37%; 10⁻⁹ M = 43%, 
p < 0.05; 10⁻⁸ M = 27%, n.s.), while PST 2238, at the same concentrations, was 
ineffective but stimulated the pump at 10⁻⁶ M (26%, n.s.) and 10⁻⁵ M (27%, p < 0.05) 
(24). The combination of PST 2238 (from 10⁻¹⁴ to 10⁻⁹ M) and ouabain at 10⁻⁹ M 
normalized pump activity at Vmax. The activity of the pump became similar to that in 
control NRK cells (24). This effect seemed to be specific for the Na⁺-K⁺ pump since PST 
2238 had no effect on either Na⁺-K⁺-Cl⁻ cotransport or the passive membrane perme-
ability under these experimental conditions. Thus, in intact cells, a 10,000- to 100,000-fold 
difference was observed between the concentrations of PST 2238 that antagonized oua-
bain and those that affected the activity of the pump.

**Interference with Na⁺-K⁺ Pump in Rat Kidney Cells Transfected with Mutated Adducin**

Normal rat kidney cells (NRK-52E) have been stably transfected with cDNA of the 
α-adducin form carrying the hypertensive rat mutation (F316Y) (59). We have previously 
demonstrated that the clone overexpressing the “hypertensive” α adducin variant called 
NRK-1, is characterized by an increased surface expression and activity of Na⁺-K⁺ pump 
at Vmax (59), as compared with the normal NRK-52E cell line expressing the normoten-
sive α-adducin variant. When both normal NRK-52E and hypertensive NRK-1 cells we 
incubated for a relatively long period of time (5 d) with very low concentrations (10⁻¹⁰ to 
10⁻⁹ M) of PST 2238, the Na⁺-K⁺ pump activity at Vmax in NRK-1, but not that in normal 
NRK-52E cells, was significantly reduced, abolishing the difference between these two 
cell lines (25).

The experiments with NRK-52E cells incubated with ouabain and with NRK-1 cells 
that overexpress the hypertensive variant of α adducin suggest that PST 2238, in nano-
molar concentrations, normalizes hyperactivation of the Na⁺-K⁺ pump altered by prolonged exposure to ouabain or by molecular alterations, such as that induced by adducin.

In Vivo Antihypertensive Activity of PST 2238

Long-Term Treatment of Experimental Hypertensive Rat Models

Ouabain-Hypertensive rats (OS rats): Chronic treatment with ouabain (50 μg/kg/d), infused through subcutaneously implanted osmotic minipumps (Alzet) to normotensive Sprague Dawley rats for 4 w, causes a significant increase in systolic blood pressure (SBP) (+15 to 26 mmHg) reaching levels of 164 ± 4 mmHg (OS rats). These levels were significantly higher than those in control rats infused with saline only (CS rats, SBP = 146 ± 3 mmHg, p < 0.001) (24).

The antihypertensive effect of PST 2238 was investigated in several experiments. OS rats were treated with different oral doses of PST 2238 (0.01 to 100 μg/kg/d) for 3 to 4 weeks. In the initial study, PST 2238 at 100 μg/kg completely abolished the ouabain-dependent increase in SBP in OS rats after 3 w of treatment (24). Furthermore, this antihypertensive effect was reversible, SBP returned to the control levels in about 2 w, after completion of the treatment (24). Further experiments were performed in OS and CS rats to study the dose dependency of the antihypertensive effect of PST 2238 and its activity in normotensive animals. At 0.01 μg/kg/d PST 2238 had no effect on the SBP of OS rats, even after 4 w of treatment (24). On the contrary, starting from the second week of treatment, PST 2238 at 0.1, 1, 10, or 100 μg/kg/d significantly reduced SBP in OS rats. At the end of week 4, SBP in OS rats was similar to that in CS controls (146 ± 3 mmHg) (Fig. 3a) (24). Four-week treatment of CS rats with PST 2238 100 μg/kg/d did not change SBP as compared to CS controls. In either OS or CS rats, heart rate was not affected by PST 2238 (24).

It can be concluded, therefore, that PST 2238 reduces the sustained pressor effect of ouabain, even at oral doses as low as 0.1 to 1 μg/kg/d.

DOCA-salt hypertensive rats: The antihypertensive effects of PST 2238 was also tested in an experimental model of volume-dependent, low-renin hypertension, associated with increased levels of OLF (38,56): DOCA-salt hypertensive rats. Sprague Dawley rats were made hypertensive by weekly subcutaneous treatments with high doses of DOCA (30 mg/kg) and replacement of drinking water with 1% NaCl. In this model, PST 2238 at 10 mg/kg/d for 3 w p.o. significantly reduced SBP (unpublished results).

Reduced renal mass (RRM) hypertensive rats: Another model of experimental hypertension characterized by volume expansion, low renin, and high plasma OLF is the reduced renal mass (RRM) rat model (50). Normotensive Sprague Dawley rats were made hypertensive by 70% reduction of total renal mass and replacement of drinking water with 1% NaCl. Hypertension developed after 3 to 4 w, reaching SBP values >180 mmHg (36). Increased levels of OLF accompanied the development of high blood pressure (13,36,47). PST 2238 given to RRM rats at 1 and 10 mg/kg/d p.o. for 2 w significantly reduced the increase in SBP as compared to untreated RRM rats (unpublished results). This effect is similar to that produced by K-canrenoate (60 to 100 mg/kg p.o.) in the same rat model (13,47). Recently Goto et al. (29) demonstrated that in RRM hypertensive rats PST 2238, at 0.1 mg/kg/d for 4 w, normalized the increased plasma K⁺ concentrations associated with
increased levels of OLF. This effect is similar to that induced in the same group of rats by anti-ouabain Fab fragments (29).

Long-Term Treatment of Genetic Hypertensive Rat Models

The MHS strain: Young (25 d-old) prehypertensive MHS rats were orally treated with PST 2238 at doses ranging from 1 to 100 \( \mu \)g/kg/d and from 1 to 10 mg/kg/d for 5 w. This experimental protocol was used to assess the effect of a long-term treatment on the time course of hypertension development. PST 2238 reduced, in a dose-dependent manner, the increase of SBP in MHS rats. The maximal antihypertensive effect was observed at 3 and 10 mg/kg/d after 5 w (−25 mmHg, p < 0.01) (25). Another study evaluated the dose dependency of the antihypertensive effect of PST 2238. The compound significantly reduced SBP in MHS rats at 3 to 90 \( \mu \)g/kg/d and at 3 mg/kg/d, with a calculated ED\textsubscript{50} of 4 \( \mu \)g/kg/d (conf. limits: 0.77–20.9). It was ineffective at 0.1 \( \mu \)g/kg/d (25) (Fig. 3b). A similar antihypertensive effect was recorded at 6 and 24 h after treatment, suggesting sustained activity of the compound. In MNS rats, PST 2238 at 90 \( \mu \)g/kg/d and 3 mg/kg/d did not affect the age-dependent SBP pattern (25). The age-dependent behavior of heart rate (HR) was not affected by PST 2238 in either strain.

Short-term treatment of spontaneous hypertensive MHS rats: To determine whether PST 2238 can lower SBP in adult already hypertensive MHS rats, 60-d-old rats received PST 2238 at 0.1, 1, or 10 mg/kg p.o. for 10 d, followed by 5 d of washout. SBP was decreased from the basal values in a dose-dependent manner by −7 ± 4.5 mmHg (p < 0.05) at 0.1 mg/kg/d, −10.7 ± 2.4 mmHg (p < 0.01) at 1 mg/kg/d, and −15.3 ± 3.4 mmHg (p < 0.01) at the highest dose of 10 mg/kg/d. The maximal hypotensive effect was achieved after 4 to 5 d of treatment and was maintained throughout the following period. HR was not affected. During the washout, SBP returned gradually to the basal values (25).

Long-term treatment of SHR: To verify whether PST 2238 was also effective in other strains of spontaneous hypertensive rats, young prehypertensive SHR were treated with PST 2238 10 mg/kg/d p.o. in accordance with the previously described protocol for OS and MHS rats. PST 2238 had no antihypertensive activity in SHR (25). Similarly, K-canrenoate, at 60 mg/kg/d for 5 w in young rats of MHS and SHR strain, produced a significant antihypertensive effect only in MHS (−22 mmHg SBP at the 5\textsuperscript{th} week, p < 0.01), but was ineffective in SHR (25,29). These data suggest that PST 2238 selectively antagonizes the pathogenesis of hypertension in MHS rats, but not SHR.

Modulation of Na\(^+\),K\(^+\)-ATPase Activity by Chronic PST 2238 Treatment

Due to the specific interaction of PST 2238 with the Na\(^+\),K\(^+\)-ATPase \textit{in vitro} and its ability to normalize the hyperactivation of the Na\(^+\)-K\(^+\) pump in cultured renal cells by ouabain (24) or genetic alterations of the adducin gene (25), we decided to verify whether this compound could also influence the expression and the activity of renal Na\(^+\),K\(^+\)-ATPase \textit{in vivo}. These studies were performed in OS and MHS rats since they represent hypertensive animal models in which hyperactivation of renal Na\(^+\),K\(^+\)-ATPase determines the development of hypertension. The activity of renal outer medulla Na\(^+\),K\(^+\)-ATPase was determined at V\(_{\text{max}}\) in membrane microsomes in the presence of Na\(^+\), K\(^+\), Mg\(^{2+}\), and ATP (20).
Renal Na\(^+\),K\(^+\)-ATPase Activity in OS Rats

OS rats were treated orally for 4 w with PST 2238 at 0.01, 0.1, 1, 10, or 100 \(\mu\)g/kg/d. CS rats received 100 \(\mu\)g/kg/d. As previously stated, PST 2238 normalized SBP in OS rats at all doses, except for the lowest dose of 0.01 \(\mu\)g/kg/d, while it did not affect SBP in CS rats (24). Concomitantly, renal Na\(^+\),K\(^+\)-ATPase activity was significantly increased in OS control rats (1.67 ± 0.06 \(\mu\)molPil/mg/min, \(n = 8\)) as compared with CS controls (1.12 ± 0.12 \(\mu\)molPil/mg/min, \(n = 8\), \(p < 0.02\)) and it was normalized to the levels of CS controls by PST 2238, at all doses used except for the lowest one (24). In CS rats, PST 2238 at 100 \(\mu\)g/kg/d did not affect renal Na\(^+\),K\(^+\)-ATPase (24).

Renal Na\(^+\),K\(^+\)-ATPase Activity in MHS Rats

Renal Na\(^+\),K\(^+\)-ATPase activity at \(V_{\text{max}}\) was measured in MHS rats after treatment with PST 2238 0.1, 3, or 90 \(\mu\)g/kg/d and 3 mg/kg/d and in MNS rats treated with 90 \(\mu\)g/kg/d or 3 mg/kg/d for 7 w. As already reported (20), renal Na\(^+\),K\(^+\)-ATPase activity at \(V_{\text{max}}\) was significantly increased in MHS controls (2.97 ± 0.15 \(\mu\)mol/min/mg) as compared with MNS controls (2.2 ± 0.06 \(\mu\)mol/min/mg, \(p < 0.01\)) (25). This difference was abolished by PST 2238 at antihypertensive doses. PST 2238 also decreased the Na\(^+\),K\(^+\)-ATPase activity of MHS rats to the levels of MNS rats (25). PST 2238 had no effect on renal Na\(^+\),K\(^+\)-ATPase activity in MNS rats (25).

These findings indicate that, in two models of hypertension sustained by hyperactivation of the renal Na\(^+\),K\(^+\)-ATPase and elevated levels of OLF or ouabain, the antihypertensive activity of PST 2238 is accompanied by a normalization of the enhanced activity of the renal Na\(^+\),K\(^+\)-ATPase.

Effect of PST 2238 on Na\(^+\),K\(^+\)-ATPase mRNA Expression

It has previously been demonstrated that the increased renal Na\(^+\),K\(^+\)-ATPase activity of MHS rats is associated with higher mRNA levels of the enzyme than are found in MNS rats (20). We investigated, therefore, whether the downregulation of renal MHS Na\(^+\),K\(^+\)-ATPase activity at \(V_{\text{max}}\) produced by long-term administration of PST 2238 was associated with a reduction of mRNA levels of the catalytic \(\alpha_1\) Na\(^+\),K\(^+\)-ATPase subunit. MHS and MNS rats were chronically treated for 6 w with PST 2238 90 \(\mu\)g/kg/d, a dose that lowers SBP in MHS, but not in MNS rats (25). After 6 w of treatment, SBP was 164 ± 1.4 and 146.6 ± 1.26 mmHg (\(p < 0.01\)) in MHS and 141.3 ± 1.24 and 139.3 ± 0.83 mmHg in MNS receiving either vehicle or PST 2238 90 \(\mu\)g/kg/d, respectively. Renal \(\alpha_1\) Na\(^+\),K\(^+\)-ATPase mRNA levels were significantly higher in adult MHS controls (1.8 ± 0.38 arbitrary units) than in age-matched MNS controls (1 ± 0.27) (25). PST 2238 significantly reduced \(\alpha_1\) mRNA levels in MHS rats (−20%) but had no effect on mRNA levels in MNS (25).

These results support the hypothesis that a decrease in the renal Na\(^+\),K\(^+\)-ATPase activity observed in MHS rats treated with PST 2238 (even at low doses <100 \(\mu\)g/kg p.o.) is sustained by a downregulation of the altered expression of the enzyme at mRNA and protein levels.
Effect of PST 2238 on Urinary Excretion and Renal Function

The activity of PST 2238 on urinary excretion and renal function was evaluated in different animals models under different experimental conditions. After either acute (2-d) or after long-term (10-d) treatment of MHS rats with the maximal antihypertensive dose of 10 mg/kg p.o., PST 2238 had no effect on 24-h urinary output, urinary osmolality, pH, or Na⁺, K⁺, or Ca²⁺ excretion. At the same experimental conditions, hydrochlorothiazide 40 mg/kg p.o. significantly increased urinary output in MHS rats (Ferrari P. and Duzzi L., unpublished results).

In a model of salt-dependent hypertension, such as the DOCA-salt rats, 1-m treatment with PST 2238 10 mg/kg p.o. did not cause any significant alterations in urine volumes or electrolyte excretion as compared to controls (Ferrani P. and Duzzi L., unpublished results). Repeated (15 d) administration of high doses of PST 2238 (300 mg/kg/d) also had no effect on creatinine clearance or urine composition in normotensive SD rats (Ferrari P. and Duzzi L., unpublished results).

Effect of PST 2238 on Cardiac and Vascular Contractility

Ex-Vivo Study on Right Ventricles and Aortic Strips of MHS Rats

To assess whether prolonged treatment with PST 2238 can affect Na⁺,K⁺-ATPase in cardiac and vascular tissues, where it might lead to unwanted side effects, MHS rats were treated with PST 2238 1 and 10 mg/kg/d for 7 w, starting in the prehypertensive age. The responsiveness of isolated right ventricular strips to increasing ouabain concentrations was determined under basal conditions. The responsiveness of cardiac tissue was compared with that of vascular preparations. Right ventricular strips from treated and control rats displayed similar basal tension and responded to ouabain with comparable increases in developed tension. The concentration-response curves were superimposable (Giacalone G. and Micheletti R., personal communication). Also aortic strips responded to ouabain in a similar way in either treated or control groups (Giacalone G. and Micheletti R., personal communication). These results indicate that chronic treatment of MHS with PST 2238, at doses far in excess of those shown to lower blood pressure and downregulate renal Na⁺,K⁺-ATPase, does not affect the basal tension of cardiac preparations, nor their sensitivity to ouabain, thus suggesting that PST 2238 might selectively modulate renal Na⁺,K⁺-ATPase.

Inotropic Activity on Guinea Pig Atria

The potential inotropic and arrhythmogenic properties of PST 2238 were studied in anesthetized guinea pigs. PST 2238 was administered by slow intravenous infusion and its effects were compared with those of ouabain. PST 2238, at a mean dose of 6.45 ± 0.32 mg/kg, did not significantly increase dP/dt (30% ± 12%), while under the same conditions, ouabain at 0.148 mg/kg increased dP/dt by 284% (Schiavone A., Rossi R., personal communication). PST 2238 caused neither arrhythmias nor any other toxic effects up to the maximal tested dose of 6.52 mg/kg, while ouabain induced arrhythmias at 0.148 mg/kg and death of the animals at 0.363 mg/kg (Schiavone A., Rossi R., personal communica-
tion). These results indicate that PST 2238, administered by slow intravenous infusion, is devoid of inotropic or toxic effects.

**Toxicology**

*Acute and Subchronic Toxicity*

Acute oral toxicity of PST 2238 was studied in rats and mice. Its LD$_{50}$ exceeded 200 mg/kg. PST 2238 was well tolerated and neither death nor clinical changes were observed throughout the experiment. The post-mortem examination did not show any treatment-related change (data on file, Prassis-Sigma Tau).

A subchronic study was conducted in normal rats by treating animals for 15 d with PST 2238 at oral doses of 100, 300, or 900 mg/kg. No death, organ damage, or hematological or urinary changes were observed during the study in either female or male animals. The only exception was for the group of males treated with the highest dose of 900 mg/kg, where decreases in body weight, food and water consumption, and white blood cell number were observed. There were no significant changes in various parameters in plasma (data on file, Prassis-Sigma Tau).

*Chronic Toxicity*

One and three-month chronic toxicological studies have been performed in rats and monkeys (data on file, Prassis-Sigma Tau). These studies indicate that administration to rats of PST 2238 at doses up to 100 mg/kg p.o. for 3 m does not induce mortality or significant clinical or hematological alterations. The only exception was a slight decrease in serum GOT and an increase in alkaline phosphatase in males. Similarly, 3-m repeated oral administration of PST 2238 at doses up to 180 mg/kg to monkeys caused no toxicological alterations.

These data indicate that in rats the ratio between therapeutic and toxic doses is higher than 1 to 25,000, considering that the ED$_{50}$ for the antihypertensive effect in rat is 4 μg/kg p.o.

*Pharmacokinetics*

Plasma, urinary, and fecal levels of PST 2238 have been determined in rats and monkeys. Preliminary results indicate that PST 2238 is absorbed and metabolized, since both the original compound and some metabolites have been found in the urine of both species (Longo A. and Pace S., personal communication). Further pharmacokinetic studies are currently in progress.

*Mutagenesis*

PST 2238 had no mutagenic activity in the Ames test, with and without metabolic activation (data on file, Prassis-Sigma Tau). PST 2238 was assayed for chromosomal aberration in human lymphocytes at concentrations ranging from 3 to 500 μg/ml. No chromosome aberrations were caused by PST 2238 (data on file, Prassis-Sigma Tau). In V79 Chinese hamster lung cells, PST 2238 at concentrations up to 150 μg/ml did not induce any gene mutation (data on file, Prassis-Sigma Tau). Preliminary fertility and early
embryonic development studies were performed in rats and rabbits showing that PST 2238 at doses up to 135 mg/kg p.o. in rats and at 400 mg/kg in rabbits is devoid of any adverse effects on reproduction or embryonic development (data on file, Prassis-Sigma Tau).

**Safety Pharmacology**

PST 2238 had no effect on hemodynamics in anesthetized dogs (data on file, Prassis-Sigma Tau). In rats at single doses up to 90 mg/kg p.o., it did not affect spontaneous locomotion or respiratory activity, gastrointestinal motility, body temperature, or hexobarbital induced sleep (data on file, Prassis-Sigma Tau). PST 2238 was devoid of androgenic, estrogenic, or corticomimetic effects *in vivo*, and had no effect on ACTH or prolactin secretion or steroidogenesis *in vitro* (Lucreziotti R., personal communication). The compound did not modify the pressor responses to vasoactive substances (noradrenaline, acetylcholine, angiotensin II, renin) in rats even after prolonged oral treatment at doses of up to the maximal tested dose of 10 mg/kg (24).

**DISCUSSION**

In this manuscript we describe the *in vitro* and *in vivo* pharmacological activities of a new antihypertensive compound that selectively interacts with the Na⁺,K⁺-ATPase and antagonizes both the molecular effect produced by a genetically determined alteration of the α adducin protein and the sustained pressor effect of ouabain.

Previous studies have shown a link between a primary genetic renal alteration (i.e., adducin polymorphism), the upregulation of the renal Na⁺-K⁺ pump as a consequence of mutations of adducin, and increased levels of circulating OLF as possible causes of hypertension. Therefore, these mechanisms have been proposed as possible targets to be addressed in the search for a pharmacogenomic therapy to cure hypertension, at least in a subgroup of patients in which these mechanisms are operative (23). Prassis-Sigma Tau has started a pharmacological project aimed at discovering a new class of antihypertensive compounds that would act by interacting with Na⁺-K⁺ pump and effects of ouabain or OLF without causing the cardiac or hormonal side effects of digitalis or mineralcorticoid-antagonists.

A new compound, PST 2238, has been selected for *in vivo* studies. *In vitro* it displaces ouabain from Na⁺-K⁺ pump receptor and antagonizes at nanomolar concentrations, increases in the activity of the Na⁺-K⁺ pump induced either by low concentrations of ouabain or by a genetic alteration (i.e., adducin). This compound at very low doses lowers blood pressure and normalizes upregulation of the renal Na⁺-K⁺ pump in genetic hypertensive MHS rats carrying the adducin mutation (25), as well as in rats made hypertensive by ouabain infusion (24).

The ability of PST 2238 to normalize upregulation of renal Na⁺,K⁺-ATPase in MHS, ouabain-induced hypertensive rats, NRK cells transfected with hypertensive adducin, and NRK cells in which Na⁺,K⁺-ATPase activity is increased as a consequence of incubation with ouabain, raises the question of whether both mutated adducin and ouabain (or OLF) affect Na⁺,K⁺-ATPase expression and activity (and consequently cause hypertension) through a common mechanism that can be corrected by PST 2238. Since ouabain affects the half-life of cell membrane Na⁺-K⁺ pumps (52) by a transient increase in the rate of synthesis (57) as well as a decrease of the pump degradation rate (52), an alteration in
cell-membrane Na⁺,K⁺-ATPase cycling may be proposed as the common mechanism triggered either by adducin or ouabain that is affected by PST 2238. The antihypertensive activity of PST 2238 seems to be selective for some forms of genetic hypertension, considering, as we have demonstrated, that this compound has no effect on blood pressure in SHR. This may be explained by the fact that in SHR adducin polymorphism (60), renal Na⁺,K⁺-ATPase (45) and OLF levels (16) do not seem to be involved in the development of hypertension.

PST 2238 is devoid of the undesired cardiac or hormonal effects typical of digitalis or antimineralcorticoid drugs, and it does not bind to other receptors involved in blood-pressure regulation or hormonal homeostasis (24). It also has no intrinsic cardiac inotropic or proarrhythmogenic activities and shows a safe toxicological profile. Phase I clinical studies of PST 2238 in healthy volunteers have been completed, and the compound will be entering Phase II clinical studies in hypertensive patients during 1999. According to its pharmacological profile, PST 2238 may represent the prototype of a new class of anti-hypertensive agents that control blood pressure by specifically correcting a biochemical alteration (upregulation of the Na⁺-K⁺ pump) determined by a genetic defect (adducin polymorphism) and further sustained by an increase in (OLF levels) triggered by the same genetic cause.

The relevance of these findings to human essential hypertension stems from the following considerations. Adducin gene mutations have been demonstrated in humans and were associated with hypertension (10,11) and with pronounced changes in blood pressure and Na⁺ levels (11,42). Increased circulating OLF levels have been found to accompany the development of high blood pressure in a subgroup of essential hypertensive patients (18,53). When these findings are considered in view of a causal approach to the therapy of essential hypertension, it appears that compounds like PST 2238 should be targeted toward patients in which, as in MHS rats, alterations of the renal Na⁺ handling and OLF levels are associated with specific genetic molecular mechanisms, such as adducin polymorphism or any other protein alteration that affects renal Na⁺,K⁺-ATPase activity.

SUMMARY

The genetic approach to the study of the mechanisms underlying hypertension has led to the identification of a polymorphism of genes for the cytoskeletal protein, adducin, which is genetically linked with rat as well as human hypertension. In MHS rats, adducin mutations are associated with an upregulation of the renal Na⁺,K⁺-ATPase and increased levels of the endogenous OLF. We describe here the pharmacological profile of a new antihypertensive compound, PST 2238, that antagonizes the pressor effect of ouabain (or OLF) in vivo, normalizes ouabain-dependent upregulation of the renal Na⁺-K⁺ pump, and lowers blood pressure and renal Na⁺,K⁺-ATPase activity also in MHS genetic hypertension.

REFERENCES


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