TVP1022 Attenuates Cardiac Remodeling and Kidney Dysfunction in Experimental Volume Overload-Induced Congestive Heart Failure

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Background—Despite the availability of many pharmacological and mechanical therapies, the mortality rate among patients with congestive heart failure (CHF) remains high. We tested the hypothesis that TVP1022 (the S-isomer of rasagiline; Azilect), a neuroprotective and cytoprotective molecule, is also cardioprotective in the settings of experimental CHF in rats.

Methods and Results—In rats with volume overload-induced CHF, we investigated the therapeutic efficacy of TVP1022 (7.5 mg/kg) on cardiac function, structure, biomarkers, and kidney function. Treatment with TVP1022 for 7 days before CHF induction prevented the increase in left ventricular end-diatolic area and end-systolic area, and the decrease in fractional shortening measured 14 days after CHF induction. Additionally, TVP1022 pretreatment attenuated CHF-induced cardiomyocyte hypertrophy, fibrosis, plasma and ventricular B-type natriuretic peptide levels, and reactive oxygen species expression. Further, in CHF rats, TVP1022 decreased cytochrome c and caspase 3 expression, thereby contributing to the cardioprotective efficacy of the drug. TVP1022 also enhanced the urinary Na⁺ excretion and improved the glomerular filtration rate. Similar cardioprotective effects were obtained when TVP1022 was given to rats after CHF induction.

Conclusions—TVP1022 attenuated the adverse functional, structural, and molecular alterations in CHF, rendering this drug a promising candidate for improving cardiac and renal function in this disease state. (Circ Heart Fail. 2011;4:463-473.)

Key Words: TVP1022 ■ CHF ■ fibrosis ■ heart ■ kidney

Clinical Perspective on p 473

Numerous studies have shown that key aspects of cardiomyocyte death and dysfunction in cardiovascular diseases such as ischemia and infarction are similar to those of neurodegenerative pathologies such as Parkinson disease. On the basis of the cytoprotective efficacies of propargylamines in neurodegenerative processes, we tested the hypothesis that TVP1022 (the S-isomer of rasagiline; Azilect) will alleviate cardiac and renal dysfunction in experimental CHF in rats. Previously, we demonstrated that the neuroprotective efficacies of the monoamine oxidase (MAO)-B inhibitor rasagiline are similar to those of the non–MAO inhibitor TVP1022, suggesting that cytoprotection is not due to MAO inhibition. Furthermore, we recently reported that pretreat-
The hypothesis that TVP1022 is cardioprotective in CHF was tested in rats in which the disease was induced by creating an aorto-caval fistula (ACF). We previously reported that volume-overload CHF is characterized by neurohormonal and renal manifestations that closely mimic severe CHF in patients. In support of our hypothesis, we found that TVP1022 attenuated cardiac and renal dysfunction in experimental CHF, indicating that this molecule should be considered as a novel drug for treating cardiovascular disorders resulting in CHF.

Methods

Experimental Model

Experiments were conducted in Sprague-Dawley rats (Harlan Laboratories, Ltd, Jerusalem, Israel) weighing 290–330 g, and CHF was induced by an ACF. All experiments were performed according to the guidelines of the committee for the supervision of animal experiments, Technion, IIT. Seven days before surgery and 14 to 21 days after surgery, rats were housed in individual metabolic cages for measurements of daily urine volume and urinary Na+ excretion.

Protocols for Drug Administration

TVP1022 (7.5 mg/kg per day, dissolved in drinking water) was administered daily per os beginning either 7 days before (pretreatment) or 7 days after (posttreatment) CHF induction, until 14 days after surgery. The study included 6 experimental groups (n=8 to 9 rats; group types are detailed in the online-only Data Supplement, Materials and Methods section). Because in a preliminary study we found that administration of TVP1022 to sham control rats did not affect any of the measured parameters (data not shown), we chose not to include these data.

Immunolabeling and Fluorescent Microscopy

Hearts were removed, weighed, rapidly frozen in liquid nitrogen, mounted in Tissue Tec, and cryosections 5 μm thick were prepared. Primary antibodies were applied against collagen type I and III (Rockland), dystrophin (Sigma), myosin (Sigma), complement 9 for measurements of daily urine volume and urinary Na+ excretion. Primary antibodies included anti-complement 9 antibody from BD Biosciences Pharminogen (Alexis) or 4′-6-Diamidino-2-phenylindole (DAPI; Molecular Probes). In situ reactive oxygen species (ROS) were determined using labeling with dihydroethidium. Controls and examination by laser scanning confocal microscopy (Leica TCS SP2) were carried out as reported by our group previously (see online-only Data Supplement, Materials and Methods section). Actin was labeled with phalloidin conjugated with TRITC (Sigma). Nuclei were stained with DRAQ5 (Alexis) or 4′-6-Diamidino-2-phenylindole (DAPI; Molecular Probes). In situ reactive oxygen species (ROS) was determined using labeling with dihydroethidium. Controls and examination by laser scanning confocal microscopy (Leica TCS SP2) were carried out as reported by our group previously (see online-only Data Supplement, Materials and Methods section). Collagen I and III were calculated as percent of positive labeling per tissue area. Myocyte cross-sectional area was determined as micrometers squared and myocyte diameter as micrometers. Complement 9–positive cells were calculated as number per 1000 cardiomyocytes. BNP and ROS were expressed as fluorescence intensity per unit myocardial area (AU/μm²).

Quantitative Immunofluorescence Measurements

For each measurement, cryosections from at least 2 different levels were used. All samples were immunolabeled simultaneously with identical conditions of fixation and dilutions of primary and secondary antibodies. Sections exposed to phosphate-buffered saline instead of primary antibodies served as negative controls. For each heart, at least 10 random fields of vision were analyzed with a fluorescence microscope Leica (Leitz DMRB, Wetzlar, Germany), using a ×40 Planapar objective (Leica). Immunolabeled cryosections were studied with the use of Image Analysis (Leica) and Image J software. Myocyte area and diameter were determined by delineating the dystrophin-labeled myocytes. The fluorescence intensity was measured as AU/μm² (see online-only Data Supplement, Materials and Methods section).

Measurement of Hemodynamic Parameters

Mean arterial blood pressure (MAP) was measured at baseline (1 day before TVP1022 administration in the pretreatment protocol and 3 weeks after drug administration, by using a tail-cuff method; IITC, Model 31, Woodland Hills, CA) after keeping the animals in an incubator at 37°C for 15 minutes to ensure vasodilatation. For each rat MAP was measured 3 times, and mean values were calculated.

Echocardiography

Echocardiography was performed with the use of a General Electric Vivid ultrasound imaging system equipped with a 10-MHz array transducer (Fairfield, CT). Conventional 2D imaging and M-mode recordings were performed to determine cardiac dimensions. All values were based on the average of at least 3 measurements. The person performing the echocardiographic measurements and analysis was blinded to the treatment group.

Measurements of Renal Function

Urine volume and Na+ excretion were measured daily. After completing the treatment period, animals from the different experimental groups were anesthetized, the carotid artery was cannulated, and blood samples were collected in precooled tubes. Concentrations of creatinine in plasma and urine samples were measured on the last day of the experiment, and GFR was equated with the renal clearance of creatinine (CCT or Ccr).

Preparation of Cytosolic Fractions and Western Blotting

Hearts were homogenized with a glass homogenizer in cold homogenization buffer containing a cocktail of protease inhibitors. Proteins were extracted and blotted as described previously (see online-only Data Supplement, Materials and Methods section).

Drugs and Chemicals

TVP1022 was kindly donated by Teva Pharmaceutical Co (Netanya, Israel). β-Actin antibody was purchased from Sigma Chemical Co (St Louis, MO), caspase 3 antibody from Cell Signaling (Beverly, MA), and the cytochrome c antibody from BD Biosciences Pharmingen (Oakville, Canada). Electrophoresis reagents were obtained from Invitrogen Corporation (Carlsbad, CA).

Statistical Analysis

Data are expressed as mean±SEM. For statistical analysis, 1-way ANOVA followed by Tukey post-hoc test was performed. For comparison of the graphs representing control and experimental groups, 2-way ANOVA with repeated measurements was used. A value of P<0.05 was considered significant. All data were analyzed by GRAPHPAD PRISM 5.

Results

Experimental Groups

Body weight, mean blood pressure, and heart rate at baseline before TVP1022 administration are shown in the Table. As
expected, CHF rats were characterized by low arterial pressures compared with sham control rats. These findings are in agreement with our previous reports\textsuperscript{6,12,13} and mimic the cardiac and hemodynamic alterations in patients with severe heart failure.\textsuperscript{2–4} Pretreatment or posttreatment of CHF rats with TVP1022 did not affect the reduced MAP, body weight, or plasma electrolytes. Because TVP1022 had no cardiac or renal effects on the sham group, we chose not to include this group in the Results section.

Effects of TVP1022 on the Histological and Pathological Characteristics of CHF Rats

**TVP1022 Prevented Ventricular Hypertrophy**

An established feature of the CHF model in rats is ventricular hypertrophy,\textsuperscript{6,12,13} which was measured by determining myocyte diameter and area (Figure 1). To ensure proper sampling, only myocytes with a nucleus were measured. As seen by the representative transverse sections and the summary of these experiments (in both the pretreatment and posttreatment

### Table. Effects of Chronic Administration of TVP1022 on Selected Physiological Parameters in the Different Experimental Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>CHF</th>
<th>TVP1022 + CHF</th>
<th>CHF</th>
<th>CHF + TVP1022</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>339.2 ± 22.0</td>
<td>355.6 ± 25.0</td>
<td>336.9 ± 8.4</td>
<td>333.6 ± 10.1</td>
<td>334.7 ± 6.5</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>130 ± 5</td>
<td>86 ± 5*</td>
<td>88 ± 3*</td>
<td>105 ± 5*</td>
<td>113 ± 2</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>420 ± 8</td>
<td>425 ± 11</td>
<td>425 ± 6</td>
<td>365 ± 14*</td>
<td>341 ± 19*</td>
</tr>
<tr>
<td>P\textsubscript{Na\textsuperscript{-}}, mEq/L</td>
<td>145 ± 1</td>
<td>142 ± 2</td>
<td>141 ± 2</td>
<td>142 ± 2</td>
<td>143 ± 1</td>
</tr>
<tr>
<td>P\textsubscript{K\textsuperscript{+}}, mEq/L</td>
<td>3.7 ± 0.1</td>
<td>4.7 ± 0.4</td>
<td>3.9 ± 0.1</td>
<td>4.2 ± 0.3</td>
<td>3.7 ± 0.3</td>
</tr>
</tbody>
</table>

BW indicates body weight; MAP, mean arterial pressure; HR, heart rate; P\textsubscript{Na\textsuperscript{-}}, plasma sodium concentration; and P\textsubscript{K\textsuperscript{+}}, plasma potassium concentration.

MAP and HR values represent the average of 3 measurements for each rat. Each group included n = 8 to 9 rats.

*P < 0.05 versus sham. Baseline refers to values of sham healthy control rats before either pretreatment or posttreatment with TVP1022.

Figure 1. Effects of pretreatment (A and B) and posttreatment (C and D) with TVP1022 on myocyte area and diameter. Diameter and area were determined in transverse ventricular sections and stained for dystrophin (green) to label the sarcolemma and blue for nuclei with 4′,6-Diamidino-2-phenylindole (A and C). To avoid prejudice and to ensure proper sampling, only myocytes with a nucleus were measured. Area and diameter of the myocytes from pretreated (B) and posttreated (D) CHF rats and their controls are expressed in micrometers squared and micrometers, respectively. *P < 0.05 versus sham; #P < 0.05 versus untreated CHF.
protocols), CHF was associated with marked hypertrophy. As the first indication of the cardioprotective efficacy of pretreatment with TVP1022 in CHF rats, the drug attenuated the hypertrophy, as indicated by a reduction in myocyte area and diameter). Similarly, posttreatment of CHF rats with TVP1022 reduced myocyte area and diameter from \(422 \pm 13.9 \times 10^{-6} \, \text{m}^2\) and \(38 \pm 4.1 \, \text{m} \) to \(267.6 \pm 10.1 \, \text{m}^2\) and \(26.1 \pm 1.4 \, \text{m} \), respectively (\(P < 0.05\) versus untreated CHF).

**TVP1022 Prevented Ventricular Fibrosis**

An important feature of the CHF heart is fibrosis, which was determined by staining for collagen I and III. Figure 2A depicts representative confocal micrographs of collagen type I and type III in myocardium of TVP1022 pretreated (A) and posttreated (C) CHF rats and their controls. A, collagen III in green; C, collagen III in red. In both panels, nuclei were stained with 4′-6-diamidino-2-phenylindole in blue. Myocardium in all sections was cut longitudinally. B, Amount of collagen I and collagen III in TVP1022 pretreated CHF rats. D, Amount of collagen I and collagen III in TVP1022 posttreated CHF rats. *\(P < 0.05\) versus sham control; \#\(P < 0.05\) versus untreated CHF.

In agreement with the cardioprotective efficacy of TVP1022, in CHF rats pretreated with TVP1022 (Figure 2A, lower row, and Figure 2B), collagen III (5.35±0.89, \(P < 0.01\) versus CHF) and I (15.22±1.99, \(P < 0.01\) versus CHF) were significantly reduced. In line with these findings, posttreatment of CHF rats with TVP1022 markedly reduced collagen III and I content (\(P < 0.05\) versus untreated CHF) (Figure 1C and 1D).

**TVP1022 Attenuated the Increase in Plasma and Ventricular BNP Levels**

In agreement with previous reports in CHF hearts (compared with sham rats), BNP was abundantly expressed and was mainly localized in cardiomyocytes in the nuclear/perinuclear area (Figure 3A, middle row). Accordingly, tissue and plasma BNP (a hallmark of CHF) levels were higher in CHF rats (164.55±6.41 AU/\(\mu\)m\(^2\) and 223.05±55.31 pg/mL, \(P < 0.05\) versus sham) compared with sham rats (36.95±6.48 AU/\(\mu\)m\(^2\) and 14.0±7.4 pg/mL) (Figure 3B and 3C). In line with its antifibrotic effect, TVP1022 pretreatment markedly attenuated the increase in ventricular (80.41±6.18 AU/\(\mu\)m\(^2\), \(P < 0.05\)) and plasma BNP (134.13±52.71 pg/mL, \(P < 0.05\)
levels in CHF rats (Figure 3B and 3C). In agreement with these findings, posttreatment of CHF rats with TVP1022 reduced both tissue content and plasma levels of BNP from 153.43 ± 7.02 AU/µm² and 199.59 ± 46.42 pg/mL in untreated CHF to 78.89 ± 12.40 AU/µm² (P < 0.05) and 83.59 ± 24.84 pg/mL (P < 0.05) (Figure 3D, 3E, and 3F).

**TVP1022 Attenuated the Increase in Ventricular ROS Levels**

Figure 4A and 4B depict the ROS status in representative ventricular sections of the various experimental groups. Whereas in the sham tissue the moderate intensity of the red fluorescence is restricted to myocytes and the nuclei

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**Figure 3.** Effects of pretreatment (A, B, and C) and posttreatment (D, E, and F) with TVP1022 on myocardial and plasma BNP levels. Representative confocal micrographs of BNP (left columns, green) in rat ventricular myocardium of TVP1022 pretreated (A) and posttreated (D) CHF rats and their controls. The right columns are identical to the left columns and show counterstaining with F-actin (red) and 4′-6-Diamidino-2-phenylindole (blue). To facilitate evaluation of the staining intensity, all tissues were treated at the same time under identical circumstances and all pictures were taken under identical conditions regarding intensity of the laser beam and exposure time. Quantification of immunoreactive BNP of sham, untreated CHF rats, and CHF rats pretreated (B) and posttreated (E) with TVP1022. C, Plasma levels of BNP in sham rats, untreated CHF, and CHF animals pretreated with TVP1022. F, Plasma levels of BNP in sham rats, untreated CHF, and CHF animals posttreated with TVP1022. *P < 0.05 versus sham control; #P < 0.05 versus untreated CHF.
are clearly stained blue, in untreated CHF, the red fluorescence is much more intense. In contrast, in TVP1022-pretreated CHF rats, the fluorescence intensity is comparable to that of the sham sections (Figure 4A). As summarized in Figure 4B, whereas in untreated CHF rats ROS levels were much higher than in sham rats ($P<0.001$), in the TVP1022 pretreated CHF group ROS levels were similar to the sham values. Similarly, posttreatment of CHF rats with TVP1022 reduced cardiac tissue content of ROS, from $178.60 \pm 11.64$ AU/$\mu\text{m}^2$ in untreated CHF to $85.20 \pm 8.46$ AU/$\mu\text{m}^2$ ($P<0.05$) (Figure 4C and 4D). These encouraging findings suggest that TVP1022 suppresses ROS formation in CHF rats.

**TVP1022 Attenuated the Increase in Complement 9–Positive Cells**

Next, we tested the ability of TVP1022 to attenuate the expression of complement 9, an indicator of necrotic cell death. Figure 5A depicts representative confocal micrographs from the various experimental groups in the pretreatment protocol; dead myocytes are identified by red staining of complement 9, myosin is shown in green, and nuclei in blue. Whereas there were only few dead cells in sham rats (0.25±0.25 positive cells per 1000 myocytes), their number markedly increased in CHF rats (5.47±1.55, $P<0.05$ versus sham) and was reduced in TVP1022-pretreated CHF rats (1.19±0.48, $P<0.05$ versus untreated CHF) (Figure 5B).

Similarly, in the posttreatment protocol, TVP1022 significantly reduced the number of dead myocytes, from 6.16±0.36 in untreated CHF rats to 1.03±0.48 cells/1000 cells ($P<0.05$) (Figure 5C and 5D).

**TVP1022 Attenuated Cytochrome C and Caspase 3 Levels**

During the apoptotic process, the loss of mitochondrial membrane potential leads to release of cytochrome c from mitochondria, which in turn activates downstream caspases including caspase 3, to cause apoptosis.19,20 To determine whether TVP1022 can attenuate the apoptotic cascade, which is an important contributor to cell death in CHF,21 we investigated its effects on the expression of the key proapo-
ptotic proteins cytochrome c and caspase 3. As shown in Figure 6, the representative Western blots and the summary of these experiments illustrate that whereas in CHF hearts the expression of both proteins was increased (P < 0.05 versus sham), their level was attenuated by TVP1022 (P < 0.05 versus CHF), thus establishing the antiapoptotic efficacy of this drug.

**TVP1022 Improved Cardiac Dysfunction in CHF Rats**

To determine whether the attenuation of the deleterious structural and molecular characteristics of CHF by TVP1022 was associated with improvement of cardiac performance, left ventricular function was measured by means of echocardiography in the pretreatment protocol. As predicted from the experimental model, in CHF rats, left ventricular end-diastolic area and end-systolic area were markedly increased and fractional shortening was decreased, compared with sham rats (P < 0.05) (Figure 7). In agreement with its beneficial antihypertrophic and antifibrotic effects, pretreatment with TVP1022 prevented the increases in left ventricular dimensions and the decrease in fractional shortening measured 14 days after surgery (Figure 7).

**Effects of TVP1022 on Renal Dysfunction in CHF Rats**

As previously described by us and others in CHF rats, both cumulative urinary volume and Na^+^ excretion were reduced compared with sham rats (P < 0.05) (Figure 8A and 8B). As seen in Figure 8A and 8B, TVP1022 increased (P < 0.05) cumulative urine volume and Na^+^ excretion. For example, the cumulative urine volume on day 18 was increased by 44%, from 176.6 ± 7.6 (in CHF + vehicle) to 254.2 ± 21.7 mL (in TVP1022 + CHF). Similarly, TVP1022 increased (P < 0.05) cumulative Na^+^ excretion (calculated on day 18) by 49%, from 19645.2 ± 418.7 (in vehicle + CHF) to 31177.1 ± 118.2 µEq, P < 0.05. Finally, as previously reported in CHF rats, GFR was reduced from 1.66 ± 0.26 (in sham control rats) to 0.73 ± 0.23 mL/min, P < 0.01 (Figure 8C). In accordance with the improvement of the diuretic and natriuretic parameters by TVP1022 in CHF rats, TVP1022 increased (P < 0.05) GFR from 0.73 ± 0.23 to 1.49 ± 0.26 mL/min (P < 0.05). The beneficial effects of TVP1022 on GFR could not be attributed to changes in blood pressure because the drug did not alter MAP (Table). Collectively, these findings suggest that TVP1022 can improve renal function in CHF without affecting blood pressure.

**Discussion**

Our goal was to test the hypothesis that TVP1022 will provide cardioprotection in CHF. We demonstrated that in CHF rats, TVP1022 (1) attenuated cardiac fibrosis and myocyte hypertrophy; (2) decreased plasma and ventricular BNP levels; (3) decreased ROS expression and the number of dead myocytes; (4) prevented the increase in left ventricular end-systolic area, left ventricular end-diastolic area, and the decrease in fractional shortening; (5) increased urine flow, Na^+^ excretion, and GFR; and (6) reduced the expression of cardiac cytochrome c and caspase 3. Collectively, we demonstrated that through multiple mechanisms of action, TVP1022 attenuated the adverse structural, molecular, and functional alterations in CHF, making this compound a
promising candidate for improving cardiac and renal function in this disease state. Importantly, the cardioprotective effects were evident when TVP1022 was given as posttreatment after CHF induction or as a pretreatment before disease induction.

**Experimental Model of CHF**

Rats with experimental CHF displayed cardiac dysfunction along with profound cardiac remodeling characterized by increased myocardial collagen content (i.e., fibrosis) and elevated heart and myocyte size. This model mimics clinical CHF, with similarities in structural abnormalities such as increase in heart mass and cellular hypertrophy.

These changes constitute a general adaptive response to states of ventricular hyperfunction and are observed in a wide range of physiological and pathological states, including volume overload and excessive exposure to neurohumoral stress/stimulation. When subjected to one of these negative stimuli, the heart undergoes remodeling with increased cardiomyocyte dimensions. This results in left ventricular dilatation and a concurrent elevation in stroke volume. Permanent structural changes including reorganization of sarcomeres and microtubules occur in response to these hypertrophic stimuli and may be the key to the phenotypic and functional changes. In line with these observations, the ACF model of CHF is characterized by volume overload, cardiac remodeling, and activation of neurohormonal systems such as the RAAS, SNS, endothelin, and antidiuretic hormone (ADH), which play a dominant role in the development of myocardial enlargement and remodeling in this model.

**TVP1022 Provides Cardioprotection in CHF**

Our findings demonstrate that pretreatment with TVP1022 starting 7 days before or 7 days after the surgical induction of CHF attenuated the development of ventricular remodeling (measured 14 to 21 days after CHF induction) at the molecular, structural, and functional levels. One of the putative mechanisms contributing to the cardioprotective effects of TVP1022 is BNP, a member of the natriuretic peptide family that is activated in heart failure and plays a compensatory role in this disease state as the result of its diuretic, natriuretic, and vasodilating actions and inhibitory effects on the RAAS and endothelin systems. In agreement with these reports, our results demonstrate that whereas both plasma and ventricular BNP levels were augmented in untreated CHF rats, pretreatment or posttreatment with TVP1022 in CHF rats markedly attenuated these increases. In this regard, it was previously shown that in contrast to angiotensin II, endothelin, ADH, and other vasoconstrictors that promote myocardial fibrosis, BNP has an antifibrogenic activity. Likewise, apart from acting as a circulating hormone, BNP exerts local effects at its myocardial production site and inhibits cardiomyocyte hypertrophy in heart failure. Therefore, it is hypothesized that the elevated expression of myocardial BNP acts as a counterregulatory...
response to the development of cardiac remodeling and fibrosis. In addition, besides their antifibrotic and hypertrophic effects, natriuretic peptides can reduce preload and afterload in normal and failing hearts by reducing blood volume as a short-term effect and by promoting renal salt and water excretion as a long-term effect, in addition to antagonizing the RAAS at many levels.32 Each of these actions affords indirect benefit to a volume-overloaded heart, a hallmark feature of the ACF model of CHF. This leads us to the second major beneficial effect of TVP1022 on renal function.

**Beneficial Effect of TVP1022 on Renal Function**

It is well established that the kidney plays a major role in the pathophysiology of CHF,4,5 and impaired renal function and reduced GFR are considered to be strong independent predictors of death in patients with CHF.5 Several neurohumoral systems with renal vasoconstrictor properties such as RAAS and the sympathetic nervous system are thought to mediate the alterations in renal function in CHF.4,5 At the early stages, the increased activity of these neurohormonal systems in CHF restores the hemodynamic abnormalities including hypotension, diminished plasma volume, and impaired cardiac function, by producing vasoconstriction and Na⁺ reabsorp-

tion.3,4 However, chronic excitation of these systems induces several long-term adverse myocardial and renal effects. Of special interest are our findings demonstrating that both urine flow and renal Na⁺ excretion were increased after chronic (7 days) administration of TVP1022 in rats with CHF compared with untreated CHF animals. The mechanisms underlying the natriuretic effect of TVP1022 in rats with CHF are not known yet. However, this phenomenon may stem from either direct inhibitory action of this drug on the tubular Na⁺ reabsorption or indirectly by improving GFR and/or cardiac performance. In addition, because TVP1022 is deprived of MAO inhibitory action,33,34 its diuretic/natriuretic and hyperfiltration actions cannot be attributed to interference with MAO activity. However, such actions can be due to the fact that TVP1022 but not the R-isomer rasagiline has no hypotensive effect, as we previously showed in normal rats.35 In addition, the stimulatory effects of TVP1022 on GFR may contribute to the enhanced urinary flow and Na⁺ excretion.

**TVP1022 Exerts Antiapoptotic and Antioxidant Effects in CHF**

Various mechanisms such as oxidative stress,36 activation of proteases,37 and alterations in death and survival signals38 were suggested to play a role in cardiomyocyte apoptosis in
heart failure caused by volume overload. Mitochondrial damage, cytochrome c, and the final effector of the caspase cascade, caspase 3, are increasingly recognized as modulators of cardiomyocyte apoptosis. The current findings show that pretreatment or posttreatment with TVP1022 decreased ROS levels and inhibited the apoptotic process by attenuating the expression of cardiac cytochrome c and caspase 3. It is unlikely that the cardioprotective effects of TVP1022 involved MAO inhibition because TVP1022 lacks MAO inhibitory activity. The mechanism of action of TVP1022 may be partly related to its N-propargyl moiety. Indeed, structure-activity studies provide evidence that the N-propargyl moiety of TVP1022 and rasagiline promotes cellular survival through similar antiapoptotic pathways. In addition, TVP1022 was previously found to exert neuroprotective effects in cell culture and in animal models similar to its R-isomer, rasagiline, suggesting that the MAO inhibition properties are not a prerequisite for their neuroprotective activity.

In summary, in CHF rats TVP1022 attenuated ventricular remodeling and the decline in renal function. The mechanisms underlying these beneficial effects appear to involve inhibition of collagen deposition in the myocardium as well as prevention of cytochrome c release from the mitochondria and caspase 3 activation. Thus, TVP1022 is a novel therapeutic compound that may be advantageous in patients with compromised cardiac and renal function such as in heart failure.

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**Disclosures**

None.

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CLINICAL PERSPECTIVE

TVP1022 (the S-isomer of rasagiline; Azilect) is an antiapoptotic cytoprotective drug by virtue of its action on the mitochondria and its ability to improve the balance between the antiapoptotic and proapoptotic proteins toward the former. Specifically, TVP1022 increases the expression of the key antiapoptotic protein kinase C-ε, which is a major regulator of cell life/death cascades operating at the level of mitochondria. The aim of this research was to explore the use of TVP1022 as a cardioprotective drug in a preclinical model of heart failure (HF). In a rat HF volume-overload model, we demonstrate that TVP1022 attenuated the remodeling process when given either before or after HF induction and attenuated HF-induced cardiomyocyte hypertrophy, fibrosis, B-type natriuretic peptide elevation, and reactive oxygen species expression. TVP1022 also enhanced urinary Na⁺ excretion and improved the glomerular filtration rate. Because cardiac dysfunction in patients with HF is a major cause for morbidity and mortality, these promising initial findings suggest that TVP1022 is a candidate for further exploration ultimately aimed at improving cardiac function and patient status in the large population of patients with HF.
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Supplemental Material

Materials and Methods

The experimental model

Experiments were conducted in Sprague Dawley rats (Harlan Laboratories, Ltd., Jerusalem, Israel) weighing 290–330 g. The animals were kept in a temperature-controlled room and fed standard rat chow containing 0.5% NaCl and tap water ad libitum. All experiments were performed according to the guidelines of the committee for the supervision of animal experiments, Technion, IIT. To induce CHF, the abdominal aorta and inferior vena-cava were exposed through a mid-abdominal incision under pentobarbital anesthesia (60 mg/kg i.p.), and an aorto-caval fistula (ACF) was surgically created in the common wall of the two vessels (side to side, 1.2 mm O.D.). Seven days prior to surgery and 14-21 days after surgery rats were housed in individual metabolic cages for measurements of daily urine volume and urinary Na⁺ excretion.

Protocols for drug administration

To test the therapeutic efficacy of TVP1022, the drug (7.5 mg/kg/day; dissolved in drinking water) was administered daily per os beginning either 7 days prior (“pre-treatment”) to or 7 days after (“post-treatment”) CHF induction, until 14 days post-surgery. The study included 6 experimental groups (n=8-9 rats in each group): (1) Control: sham-operated rats treated with vehicle (water); (2) Control: sham-operated rats treated with TVP1022; (3) CHF rats pre-treated with vehicle; (4) CHF rats pre-treated with TVP1022; (5) CHF rats post-treated with vehicle; (6) CHF rats post-treated with TVP1022. In a preliminary study, we found that administration of TVP1022 to sham control did not affect any of the measured parameters below (data not shown), therefore we chose not to include these data.
Immunolabeling and fluorescent microscopy

Hearts from the different experimental groups were removed, weighed and rapidly frozen in liquid nitrogen. The hearts were mounted in Tissue Tec and cryosections 5 µm thick were prepared. Cryosections were air-dried and fixed for 10 min in acetone (−20°C), 4% paraformaldehyde, or Carnoys’ solution (at room temperature). After a wash with phosphate buffered saline (PBS), sections were incubated with 1% bovine serum albumin (BSA) for 30 minutes to block non-specific binding sites. Following a second wash in PBS the samples were incubated overnight with primary antibodies against collagen type I and III (Rockland, Gilbertsville, PA, USA), dystrophin (Sigma, St. Louis, USA), vinculin (Sigma) and BNP (Biozol, St. Charles, MO, USA). After incubation with the primary antibodies, the sections were thoroughly washed in PBS and incubated with secondary biotin-conjugated anti-mouse or anti-rabbit IgG (Dianova, Rodeo, CA, USA) for 1 hour followed by incubation with Cy2 or Cy3 conjugated streptavidin. Actin was labeled with phalloidin conjugated with TRITC (Sigma). Nuclei were stained with DRAQ5 (Alexis, Lausen, Switzerland) or 4'-6-Diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen Corporation, CA, USA). In situ reactive oxygen species (ROS) were determined using labeling with dihydroethidium. Tissue sections were examined by laser scanning confocal microscopy (Leica TCS SP2, Mannheim, Germany). A series of confocal optical sections were obtained using a Leica Planapo x40/1.00 or x63/1.32 objective lens. Each recorded image was obtained using dual-channel scanning and consisted of 1024 x 1024 pixels. To improve image quality and to obtain a high signal to noise ratio, each image from the series was signal-averaged. After data acquisition, the images were transferred to a Silicon Graphics workstation (Silicon Graphics, Fremont, CA, USA) for restoration and three-dimensional reconstruction using Imaris 4.5 multichannel image processing software (Bitplane, Zurich, Switzerland). For quantification of complement 9-positive myocytes, three sections 5 µm thick cut at a distance of 100 µm, were stained for complement 9 (mouse monoclonal, clone
NCL-CCC9, Novocastra, Wetzlar, Germany) and quantitatively evaluated. Counter staining for identification of myocytes was done with FITC-phalloidin (Sigma).

Quantitative immunofluorescence measurements

Cryosections from at least two different levels in each case were used. All samples were immunolabeled simultaneously with identical conditions of fixation and dilutions of primary and secondary antibodies. Sections exposed to PBS instead of primary antibodies served as negative controls. For each heart at least 10 random fields of vision were analyzed with a fluorescent microscope Leica (Leitz DMRB, Wetzlar, Germany) using a x40 Planapo objective (Leica). Immunolabeled cryosections were studied using image analysis (Leica) and Image J software. Myocyte area and diameter were determined by delineating the dystrophin-labeled myocytes. The fluorescence intensity was measured as AU/μm². For each quantification procedure a specific setting was established and kept constant in all measurements. Quantification of BNP and ROS were performed by measuring the fluorescence intensity using a range of 0 to 255 gray values. Arbitrary units of the fluorescence intensity were calculated per unit myocardial area (AU/μm²). The areas of collagen I and III were calculated as percent of positive labeling per tissue area. Myocyte cross-sectional area was determined from at least 100 myocytes per section by delineating vinculin-stained myocytes. Complement 9-positive cells were counted in the entire section. The number of myocyte nuclei per 5 randomly chosen fields of vision (x400) was counted and calculated per μm². From these data and the size of the tissue section, the total number of myocytes was determined and complement 9-positive cells were expressed as number per 1000 cardiomyocytes.
Measurement of hemodynamic parameters

Mean arterial blood pressure (MAP) was measured at baseline (one day prior to TVP1022 administration in the pre-treatment protocol and 3 weeks after drug administration, by using a tail-cuff method (IITC, Model 31, Woodland Hills, CA, USA) after keeping the animals in an incubator at 37°C for 15 min to ensure vasodilatation. For each rat MAP was measured 3 times and mean values were calculated.

Echocardiography

Echocardiography was performed using a General Electric Vivid ultrasound imaging system equipped with a 10-MHz array transducer (Fairfield, CT, USA). Conventional two-dimensional imaging and M-Mode recordings were performed to determine cardiac dimensions. All values were based on the average of at least three measurements. Left ventricular (LV) end diastolic area (DA) and end systolic area (SA) were measured. Percent fractional shortening (FS) was calculated as $FS = [(DA-SA)/DA] \times 100$. The person performing the echocardiographic measurements and analysis was blinded to the treatment group.

Preparation of lysates and cytosolic fractions from rat hearts

Rat hearts were homogenized with a glass homogenizer in cold homogenization buffer (5 mMTris-HCL, 250 mM sucrose, 1 mM EDTA, pH 7.5) containing a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany). Homogenates were mixed with 1% sodium dodecyl sulfate (SDS) and 4% Triton-100 and further centrifuged at 1000 x g for 10 min at 4°C to remove crude nuclear material. The supernatant represented the lysate. The cytosolic fraction was separated by further centrifugation of the supernatant at 10,000 x g for 30 min at 4°C and followed by ultra-centrifugation at 100,000 x g for 60 min at 4°C. The protein content of
the lysates and cytosolic fractions was determined using the Bradford method with BSA as standard.

Immunoblotting analysis

Equal amounts of the samples were resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidenedifluoride membranes (Millipore). Membranes were treated with a blocking buffer (5% dry milk, 0.05% Tween 20 in TBS). Primary antibodies against cytochrome c, caspases 3, and β-actin were diluted in TBS containing 5% dry milk and 0.05% Tween 20 and incubated with membranes for 24 h at 4°C followed by incubation (1 hr at room temperature) in dilutions of horseradish peroxidase-conjugated secondary antibodies in the same buffer. Following antibody incubation, membranes were washed in 0.5% Tween 20 in TBS. Detection was achieved using the Western blotting detection reagent, ECL (Amersham, Pharmacia, Little Chalfont Buckinghamshire, UK). Quantification of results was accomplished by measuring the optical density of the labeled bands from the autoradiograms, using the computerized imaging program Bio-1D (Vilber Lourmat Biotech. Bioprof., France). The values were normalized to β-actin intensity levels.