TVP1022 Protects Neonatal Rat Ventricular Myocytes against Doxorubicin-Induced Functional Derangements

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ABSTRACT

Our recent studies demonstrated that propargylamine derivatives such as rasagiline (Azilect, Food and Drug Administration-approved anti-Parkinson drug) and its S-isomer TVP1022 protect cardiac and neuronal cell cultures against apoptotic-inducing stimuli. Studies on structure-activity relationship revealed that their neuroprotective effect is associated with the propargylamine moiety, which protects mitochondrial viability and prevents apoptosis by activating Bcl-2 and protein kinase C- ϵ and by down-regulating the proapoptotic protein Bax. Based on the established cytoprotective and neuroprotective efficacies of propargylamine derivatives, as well as on our recent study showing that TVP1022 attenuates serum starvation-induced and doxorubicin-induced apoptosis in neonatal rat ventricular myocytes (NRVMs), we tested the hypothesis that TVP1022 will also provide protection against doxorubicin-induced NRVM functional derangements. The present study demonstrates that pretreatment of NRVMs with TVP1022 (1

μM , 24 h) prevented doxorubicin (0.5 μM , 24 h)-induced elevation of diastolic $[\text{Ca}^{2+}]_i$, the slowing of $[\text{Ca}^{2+}]_i$ relaxation kinetics, and the decrease in the rates of myocyte contraction and relaxation. Furthermore, pretreatment with TVP1022 attenuated the doxorubicin-induced reduction in the protein expression of sarco/endoplasmic reticulum calcium (Ca^{2+}) ATPase, $\text{Na}^+/\text{Ca}^{2+}$ exchanger 1, and total connexin 43. Finally, TVP1022 diminished the inhibitory effect of doxorubicin on gap junctional intercellular coupling (measured by means of Lucifer yellow transfer) and on conduction velocity, the amplitude of the activation phase, and the maximal rate of activation (dv/dt_{max}) measured by the Micro-Electrode-Array system. In summary, our results indicate that TVP1022 acts as a novel cardioprotective agent against anthracycline cardiotoxicity, and therefore potentially can be coadministered with doxorubicin in the treatment of malignancies in humans.

Doxorubicin or adriamycin is a quinone-containing anthracycline, and is of the most widely prescribed and effective chemotherapeutic agent used in oncology. All anthracyclines contain a common quinone moiety, readily participating in oxidation-reduction reactions that ultimately generate highly reactive oxygen species thought to be responsible for the anthracycline-induced cardiotoxicity (Sarvazyan, 1996; Menna et al., 2008). Doxorubicin is one of the most active

agents available for the treatment of breast cancer and other indications, including Hodgkin's and non-Hodgkin's lymphomas, Ewing's and osteogenic bone tumors, soft tissue sarcomas, and pediatric cancers such as neuroblastoma and Wilms' tumors. However, the clinical utility of doxorubicin is limited by its cumulative, dose-related, potentially fatal, progressive, and often irreversible cardiac toxicity that may lead to congestive heart failure (CHF) (Singal and Iliskovic, 1998; Swain et al., 2003; Takemura and Fujiwara, 2007). The chronic effects of doxorubicin expressed as CHF are invariably associated with cumulative drug exposure (Singal and Iliskovic, 1998; Swain et al., 2003; Takemura and Fujiwara, 2007). For example, at a cumulative dose not exceeding 450 to 500 mg/m^2 the incidence of CHF is ~ 4 to 5%, whereas at 550 to 600 mg/m^2 the incidence increases to 18% (Singal and

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ABBREVIATIONS: CHF, congestive heart failure; NRVM, neonatal rat ventricular myocyte; CSQ, calsequestrin; PLB, phospholamban; SERCA2, sarco/endoplasmic reticulum calcium (Ca^{2+}) ATPase 2; NCX1, $\text{Na}^+/\text{Ca}^{2+}$ exchanger 1; total Cx43, total Connexin 43; NP Cx43, nonphosphorylated Connexin 43; RyR, ryanodine receptor; MEA, Micro-Electrode-Array; hESC-CM, human embryonic stem cell-derived cardiomyocyte; ROS, reactive oxygen species; TBS, Tris-buffered saline; CBN, carbenoxolone; SR, sarcoplasmic reticulum.

Iliskovic, 1998; Takemura and Fujiwara, 2007; Menna et al., 2008). Nevertheless, despite these deleterious side effects, the benefits of anthracyclines outweigh the risks, and thus doxorubicin continues to serve as an important anticancer drug.

Based on the established cytoprotective and neuroprotective efficacies of propargylamine derivatives (Youdim and Weinstock, 2001; Youdim et al., 2003) as well as on our recent study (Kleiner et al., 2008) showing that TVP1022 (the *S*-isomer of rasagiline, Azilect, Food and Drug Administration-approved anti-Parkinson drug) attenuates serum starvation-induced and doxorubicin-induced apoptosis in neonatal rat ventricular myocytes (NRVM), in the present study we tested the hypothesis that TVP1022 will provide protection against doxorubicin-induced functional derangements in NRVM. Indeed, in support of this hypothesis, we demonstrated that TVP1022 markedly attenuated the deleterious effects of doxorubicin on the $[Ca^{2+}]_i$ transients, the contractions and intercellular coupling, rendering this drug a potential cardioprotective agent against anthracycline cardiotoxicity.

Materials and Methods

Preparation of Neonatal Rat Ventricular Myocytes and the Experimental Protocols. NRVM cultures were prepared from ventricles of 1- to 2-day-old Sprague-Dawley rats as described previously (Rubin et al., 1995), with minor modifications. In brief, the ventricles of the excised hearts were dissociated with 0.1% RDB (Israel Institute for Biological Research, Ness Ziona, Israel) and resuspended in F-10 culture medium containing 1 μ M $CaCl_2$, 100 U/ml penicillin-streptomycin, 5% fetal calf serum, 5% donor horse serum, and 25 mg bromodeoxyuridine. Cell culture reagents were purchased from Biological Industries (Beit Haemek, Israel). The cells were preplated for 1 h to reduce the fibroblast content, and then seeded in 6-well plates (1.6×10^6 cells/ml). For the Micro-Electrode-Array (MEA) experiments, NRVM were plated on MEA plates at a density of 2×10^6 cells/ml. Thereafter, the cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 . Unsettled cells were washed out after 24 h, the medium was replaced, and then replaced again on alternating days. Experiments were performed on days 4 to 6 after plating, on the following experimental groups: 1) untreated cultures serving as control; 2) doxorubicin, 0.5 μ M, 24 h; 3) TVP1022 (Fig. 1) (Teva Pharmaceutical, Natanya, Israel), 1 μ M, 48 h; 4) TVP1022, 24 h, followed by TVP1022 + doxorubicin for an additional 24 h. For the MEA recordings, measurements were obtained at 0 h (serving as control) and at 48 h. For all other experiments, measurements were obtained 48 h after TVP1022 administration or 24 h after doxorubicin administration.

In the present work the doxorubicin concentration (0.5 μ M) was

chosen based on our preliminary experiments (data not shown) and on previous studies using a concentration range of 0.1 to 1 μ M (Maeda et al., 1999; Shneyvays et al., 2001; Kleiner et al., 2008). To determine the cardioprotective efficacy of TVP1022, a concentration of 1 μ M was used based on our previous work (Kleiner et al., 2008) and our findings (data not shown) illustrating that 0.01, 0.1, and 1 μ M TVP1022 similarly affected the expression of the $[Ca^{2+}]_i$ -handling proteins (see details below), total and nonphosphorylated connexin43 (Cx43), and intercellular coupling determined by Lucifer yellow transfer. It is noteworthy that because TVP1022 is being developed as a cardioprotective drug to be administered to cancer patients before and during doxorubicin treatment, NRVM cultures were pretreated with TVP1022 before exposure to doxorubicin.

Western Blot Analysis. Lysates were prepared from NRVM cultures with use of radioimmunoprecipitation assay (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.2% sodium deoxycholate, 5 mM EDTA, 1% phosphatase inhibitor) containing cocktail protease inhibitor (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined by the bicinchoninic acid assay. A 20- to 30- μ g sample of total cellular protein was loaded on 12% SDS-polyacrylamide gel electrophoresis, followed by blotting into polyvinylidene difluoride membranes (Millipore, Billerica, MA). Electrophoresis reagents were purchased from Invitrogen Corporation (Carlsbad, CA). Membranes were blocked with 5% dry milk in 0.05% Tween 20 in Tris-buffered saline (TBS) for 1 h. Antibodies against sarco/endoplasmic reticulum calcium (Ca^{2+}) ATPase 2 (SERCA2), calsequestrin (CSQ), phospholamban (PLB), and the ryanodine receptor (RyR) were purchased from Thermo Fisher Scientific (Rockford, IL); Na^+/Ca^{2+} exchanger 1 (NCX1) was from Abcam (Cambridge, UK); total Cx43 was from Millipore Bioscience Research Reagents (Temecula, CA); and nonphosphorylated Cx43 (NP Cx43) was from Zymed Laboratories (San Francisco, CA). β -Actin antibody and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies were diluted in 5% dry milk in TBS containing 0.05% Tween 20 and incubated with membranes for 24 h at 4°C followed by incubation (1 h at room temperature) in dilutions of horseradish peroxidase-conjugated secondary antibodies in the same buffer. After antibody incubations, membranes were washed in 0.05% Tween 20 in TBS. Detection was performed by use of Western blotting detection reagent, electrochemical luminescence (GE Healthcare, Little Chalfont Buckinghamshire, UK). Quantification of the 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.

Measurement of $[Ca^{2+}]_i$ Transients and Contractions. $[Ca^{2+}]_i$ transients and contractions were measured by means of Fura-2 fluorescence and video edge detection, respectively, routinely used in our laboratory (Dolnikov et al., 2006; Sedan et al., 2008). The following parameters were calculated: diastolic $[Ca^{2+}]_i$, the rate of $[Ca^{2+}]_i$ activation ($d[Ca^{2+}]_{i,Ac}/dt$), $[Ca^{2+}]_i$ relaxation time ($[Ca^{2+}]_{i,Relax}$), the maximal rate of contraction ($dL/dt_{Contrac}$), the maximal rate of relaxation (dL/dt_{Relax}), and contraction amplitude (L_{Amp}).

Measuring Intercellular Coupling by Lucifer Yellow Transfer. The scrape-loading technique was used to evaluate the degree of gap junctional intercellular communication (Kavanagh et al., 1987). In brief, NRVM cultures were bathed in phosphate-buffered saline containing 0.5% Lucifer yellow (Sigma-Aldrich). Fine incisions on the culture surface were made by use of a razor blade, allowing Lucifer yellow to enter the scraped NRVM. After 2.5 min of incubation, the cultures were rinsed three times with phosphate-buffered saline, fixed in 4% *p*-formaldehyde (Sigma-Aldrich), and photographed. The extent of Lucifer yellow transfer calculated by use of the Image J software, was obtained by determining the maximal distances at which Lucifer yellow fluorescence could be clearly detected perpendicular to the incision.

Fig. 1. The chemical structure of TVP1022.

Recording Extracellular Electrograms from NRVM. Extracellular electrograms were recorded from NRVM plated on the MEAs, as described previously (Meiry et al., 2001; Zeevi-Levin et al., 2005) by means of a setup by Multi Channel Systems (Reutlingen, Germany). Drugs were introduced to the cultures as described above. For the electrophysiological measurements, MEAs were removed from the incubator and placed in the recording apparatus preheated to 37°C; spontaneous electrical activity was recorded and stored for off-line analysis. Conduction velocity, the voltage amplitude of the wave form during activation (the QRS amplitude), and maximal rate of activation ($dV/dt_{\max}$) were calculated as described previously (Meiry et al., 2001; Zeevi-Levin et al., 2005).

Human Embryonic Stem Cell-Derived Cardiomyocytes. Human embryonic stem cells (hESC) from clone H9.2 were grown on

mouse embryonic fibroblast feeder, and embryoid bodies prepared as described previously (Sedan et al., 2008). The contracting areas mostly composed of cardiomyocytes were carefully dissected out by a microscalpel, and transferred to gelatin-coated 30-mm-diameter glass slides suitable for fluorescent measurements (Thermo Fisher Scientific). Contractions were recorded and analyzed as described previously (Dolnikov et al., 2006; Sedan et al., 2008).

Statistical Analysis. Raw data were analyzed and expressed as mean $\pm$ S.E.M. Statistical differences between multiple groups were determined using one-way analysis of variance, followed by the Tukey post hoc test by use of Prism v.5.00 for Windows (GraphPad Software Inc., San Diego, CA). In the MEA experiments the Student's paired t test was used. For all other experiments, independent Student's t test was used to compare the control and TVP1022 groups. A level of $P < 0.05$ was accepted as statistically significant.

Results

TVP1022 Attenuates the Deleterious Effects of Doxorubicin on the $[Ca^{2+}]_i$ Transients and Contractions. In agreement with previous reports, this study shows that doxorubicin adversely affects the $[Ca^{2+}]_i$ transients and contractions of NRVM (Maeda et al., 1998, 1999; Mijares and Lopez, 2001; Shneyvays et al., 2001; Timolati et al., 2006). As shown by a representative experiment (Fig. 2A) and by the summary of 8 to 10 experiments, doxorubicin elevated ($P < 0.0001$) diastolic $[Ca^{2+}]_i$ (Fig. 2B), decreased the maximal rate of $[Ca^{2+}]_i$ activation ($P < 0.01$) (Fig. 2C), and prolonged ($P < 0.01$) $[Ca^{2+}]_i$ relaxation time (Fig. 2D). Accordingly, doxorubicin decreased ($P < 0.05$) the maximal rates of contraction and relaxation and the contraction amplitude (Fig. 3). In support of our hypothesis, TVP1022 prevented the deleterious effects (except for the decline in the rate of $[Ca^{2+}]_i$ activation; Fig. 2C) of doxorubicin on the $[Ca^{2+}]_i$ transients (Fig. 2) and contractions of NRVM (Fig. 3).

TVP1022 Attenuates the Deleterious Effects of Doxorubicin on the $[Ca^{2+}]_i$ Handling Machinery. To decipher the mechanisms underlying the protective capacity of TVP1022 on the $[Ca^{2+}]_i$ transients and contractions, we investigated the effects of doxorubicin alone, and TVP1022 + doxorubicin on the protein expression of PLB, CSQ, SERCA2,

Fig. 2. TVP1022 prevented doxorubicin-induced alterations in the $[Ca^{2+}]_i$ transients parameters in NRVM. NRVM cultures were preincubated with TVP1022 (1 μ M) for 24 h before adding doxorubicin (0.5 μ M) for an additional 24 h. $[Ca^{2+}]_i$ transients were measured by Fura-2 fluorescence as described under *Materials and Methods*. A, representative $[Ca^{2+}]_i$ transients recorded from a control culture and from cultures treated with doxorubicin alone or with TVP1022 + doxorubicin. B, diastolic $[Ca^{2+}]_i$, expressed as Fura-2 fluorescence ratio. C, the rate of $[Ca^{2+}]_i$ activation ($d[Ca^{2+}]_i/dt$). D, $[Ca^{2+}]_i$ relaxation time ($[Ca^{2+}]_i$ Relax). The values are mean $\pm$ S.E.M. of eight to nine experiments. ***, $P < 0.001$ versus control; **, $P < 0.01$ versus control. Dox, doxorubicin; TVP, TVP1022.

Fig. 3. TVP1022 prevented doxorubicin-induced alterations in the contraction parameters in NRVM. Cultures were treated as described in Fig. 2, and contraction parameters were measured by video edge detection as described under *Materials and Methods*. A, representative contractions recorded from a control culture and from cultures treated with doxorubicin alone or with TVP1022 + doxorubicin. B, the maximal rate of myocyte contraction ($dL/dt_{\text{Contraction}}$). C, the maximal rate of myocyte relaxation (dL/dt_{Relax}). D, the contraction amplitude (L_{Amp}). The values are mean $\pm$ S.E.M. of five to seven experiments. *, $P < 0.05$ versus control. Dox, doxorubicin; TVP, TVP1022.

NCX1, and RyR. As depicted in Fig. 4, doxorubicin did not affect the expression of PLB, CSQ, or RyR, but decreased the expression of SERCA2 ($P < 0.01$) and NCX1 ($P < 0.05$). In agreement with the protective efficacy of TVP1022, pretreatment for 24 h prevented doxorubicin-induced decreased expression of SERCA2 and NCX1. Hence, these beneficial effects of TVP1022 can contribute to its protection against the adverse effects of doxorubicin on the $[Ca^{2+}]_i$ transients and contractions of NRVMs.

TVP1022 Attenuates the Deleterious Effects of Doxorubicin on Intercellular Communication and Cx43 Expression. Next, we investigated the effect of doxorubicin and the protective efficacy of TVP1022 on Lucifer yellow transfer, which is indicative of intercellular coupling via gap junctions (Kavanagh et al., 1987; Matamba et al., 2006). As depicted in Fig. 5A (top), Lucifer yellow transfer can be visualized by means of fluorescence microscopy, and the distance is determined by use of the ImageJ software (Fig. 5A, bottom). As

Fig. 4. The effects of TVP1022 and doxorubicin on the expression of $[Ca^{2+}]_i$ -handling proteins in NRVM. Cultures were treated as described in Fig. 2, and the expression level of proteins was measured by Western blotting. A–E (top), representative Western blots of PLB, RyR, SERCA2, NCX1, and CSQ expression, respectively, in the control, doxorubicin and the TVP1022 + doxorubicin groups. Equivalency of loading was verified with an antibody against β-actin (A–E, middle). In A to E (bottom), bar graphs depict the quantitative densitometric analysis of each protein. Each value was divided by its corresponding β-actin value. All values are reported as mean $\pm$ S.E.M. of three to four experiments. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control. Dox, doxorubicin; TVP, TVP1022.

Fig. 5. TVP1022 attenuated doxorubicin-induced decline in the intercellular coupling in NRVM. Cultures were treated as described in Fig. 2. Intercellular coupling was measured by Lucifer yellow transfer. A (upper), representative fluorescence pictures of a control, doxorubicin-treated, and TVP1022 + doxorubicin-treated cultures, after Lucifer yellow loading. A (bottom), the Image J output analysis of the pictures shown in the top. B, Lucifer yellow transfer distance (μm). All values are reported as mean $\pm$ S.E.M. of six to eight experiments. In the CBN groups, $n = 3$ to 4 experiments. ***, $P < 0.001$ versus control. LY, Lucifer yellow; Dox, doxorubicin; TVP, TVP1022; CBN, carbenoxolone.

seen in Fig. 5B, doxorubicin caused a 36% reduction ($P < 0.001$) in Lucifer yellow transfer, which was mostly prevented by pretreatment with TVP1022. Finally, to confirm that the Lucifer yellow transfer method is responsive to agents that affect intercellular coupling, we tested the effects of the intercellular uncoupler carbenoxolone (CBN, 50 and 100 μM) on levels of intercellular coupling between NRVM. As shown in Fig. 5B, applying 50 μM CBN for 30 min reduced ($P < 0.001$) Lucifer yellow transfer by 54%. Because 100 μM CBN did not further decrease Lucifer yellow transfer, indicating that 50 μM is sufficient to maximally block coupling, only the effect of 50 μM is shown. To determine whether the effect of doxorubicin on Lucifer yellow transfer is additive to that of carbenoxolone, NRVM were treated with doxorubicin (0.5 μM, 24 h) and carbenoxolone (50 μM, 30 min). Figure 5B shows that, in the doxorubicin + carbenoxolone-treated group, Lucifer yellow transfer was reduced by 48% ($P < 0.001$), similarly to carbenoxolone alone, indicating that the uncoupling due to doxorubicin is less efficacious than that due to carbenoxolone, which maximally closes the channels at this concentration. Finally, as shown in Fig. 5B, TVP1022 did not affect carbenoxolone-induced reduction in Lucifer yellow transfer, indicating that the molecular mechanism by which TVP1022 maintains coupling is not affecting the carbenoxolone mechanism of channel closure. These data suggest that carbenoxolone and doxorubicin are likely to have different mechanisms of action on the closure of gap junction channels.

Because intercellular coupling largely depends on gap junctional functionality, we investigated whether doxorubicin affects total and NP Cx43. Indeed, as illustrated by the representative Western blots and the summary of six exper-

iments, doxorubicin decreased the expression of total ($P < 0.01$) and NP ($P < 0.001$) Cx43 (Fig. 6). As seen in Fig. 6B, whereas the seemingly beneficial effect of TVP1022 on the reduction in NP Cx43 did not reach a level of significance, the drug attenuated doxorubicin-induced reduction in total Cx43 (Fig. 6A), which can account for the ability of TVP1022 to attenuate the deleterious effect of doxorubicin on intercellular communication.

TVP1022 Attenuates the Deleterious Effects of Doxorubicin on Conduction Velocity and Activation of NRVM. To determine whether the decrease in intercellular coupling and Cx43 protein expression by doxorubicin and the beneficiary effects of TVP1022 are correlated with corresponding alterations in the activation properties and conduction velocity, we measured extracellular electrograms from NRVM cultures by means of the MEA setup (Meiry et al., 2001; Zeevi-Levin et al., 2005). These experiments showed (Fig. 7) that only in the doxorubicin group, conduction velocity ($P < 0.01$), QRS amplitude ($P < 0.001$), and $dV/dt_{\max}$ ($P < 0.01$) were significantly decreased at 48 h, compared with the 0-h time point. These novel findings demonstrate that, whereas TVP1022 did not affect the conduction velocity and activation properties, this drug attenuated the deleterious effects of doxorubicin, in agreement with its previously shown beneficial effects.

The Safety of TVP1022 Demonstrated in NRVM and hESC-CM. Because TVP1022 is being developed as a new cardioprotective drug against anthracycline cardiotoxicity, we tested its effects on the $[Ca^{2+}]_i$ transient and the contraction properties of human embryonic stem cell-derived cardiomyocytes (hESC-CM), an in vitro model for human cardiomyocytes. As seen in Fig. 8, TVP1022 at 0.01, 0.1, and 1 μ M did not affect the contraction amplitude (L_{Amp}), the maximal rate of contraction (dL/dt_{Contrac}), and the maximal rate of relaxation (dL/dt_{Relax}), thus demonstrating the safety of TVP1022 in this model. The safety of TVP1022 to cultured cardiomyocytes is further supported by showing that the drug did not affect the $[Ca^{2+}]_i$ transient and

Fig. 6. The effects of TVP1022 on doxorubicin-induced alterations in total and NP Cx43 expression in NRVM. Cultures were treated as described in Fig. 2, and the expression level of proteins was measured by Western blotting. A and B (top), representative Western blots of total Cx43 and NP Cx43 expression, respectively, in the control, doxorubicin, and TVP1022 + doxorubicin groups. A and B (middle), equivalency of loading was verified with an antibody against β -actin. A and B (bottom), bar graphs depict the quantitative densitometric analysis of total Cx43 and NP Cx43 expression, respectively. Each value was divided by its corresponding β -actin value. All the values are reported as mean $\pm$ S.E.M. of six experiments. **, $P < 0.01$ versus control; ***, $P < 0.001$ versus control. Dox, doxorubicin; TVP, TVP1022.

Fig. 7. TVP1022 prevented doxorubicin-induced alterations in the activation and conduction velocity in NRVM. Cultures were preincubated with doxorubicin (0.5 μ M, 24 h), with or without prior incubation with TVP1022 (1 μ M, 48 h). Untreated cultures served as controls. Activation parameters and conduction velocity of spontaneously beating NRVM were obtained by MEA recordings. Conduction velocity (A), $dV/dt_{\max}$ (B), and QRS amplitude (C) in the control, doxorubicin, TVP1022, and TVP1022 + doxorubicin-treated cultures at 0 h and at 48 h. All the values are reported as mean $\pm$ S.E.M. of five to seven experiments. For each parameter, values were normalized to 0 h. **, $P < 0.01$ versus 0 h; ***, $P < 0.001$ versus 0 h. Dox, doxorubicin; TVP, TVP1022.

Fig. 8. The effects of TVP1022 on the contraction parameters of hESC-CM. A, a representative experiment demonstrating that TVP1022 did not affect the contraction amplitude of a 32-day-old hESC-CM paced at 0.5 Hz. EB, embryoid body. B, a summary of the effects of TVP1022 on the contraction parameters on hESC-CM ($n = 5$ embryoid bodies). In A and B, the effects of TVP1022 are presented as the percentage of change of their respective controls. For each drug concentration, the mean $\pm$ S.E.M. is presented.

contraction parameters (Fig. 9A), the expression of the $[Ca^{2+}]_i$ handling proteins (Fig. 9B), intercellular coupling (Fig. 9C), total Cx43 and NP Cx43 (Fig. 9D), as well as conduction velocity and activation (Fig. 7). These findings are of importance because of the therapeutic potential of TVP1022.

Discussion

In the present work we investigated in NRVM the cardioprotective efficacy of TVP1022 against functional derangements caused by doxorubicin. The major findings are: 1) pretreatment with TVP1022 prevented doxorubicin-induced elevation of diastolic $[Ca^{2+}]_i$, the slowing of $[Ca^{2+}]_i$ relaxation kinetic, the decrease in the maximal rates of con-

Fig. 9. The safety of TVP1022 is demonstrated in NRVM. Cultures were treated with TVP1022 (1 μ M) for 48 h, and the effects were compared with control cultures. A, $[Ca^{2+}]_i$ transients ($n = 8$ –10 cultures) and contraction parameters ($n = 5$ –6 cultures). B, the protein expression of the $[Ca^{2+}]_i$ -handling proteins (see details in Fig. 4) ($n = 3$ –4 cultures). C, Lucifer yellow ($n = 6$ –8 cultures). D, total Cx43 and NP Cx43 expression ($n = 6$ cultures). In A through C, the effects of TVP1022 are presented as the percentage of change of their respective controls. LY, Lucifer yellow.

traction and relaxation, and the decrease in contraction amplitude; 2) TVP1022 attenuated doxorubicin-induced reduction in the protein expression of SERCA2 and NCX1; 3) TVP1022 attenuated doxorubicin-induced reduction in intercellular coupling measured by Lucifer yellow transfer; accordingly, TVP1022 prevented doxorubicin-induced decrease in total Cx43 protein expression; 4) TVP1022 attenuated the doxorubicin-induced reduction in conduction velocity, QRS amplitude, and dV/dt_{max} of the extracellular electrograms; and 5) TVP1022 did not affect the $[Ca^{2+}]_i$ transients and contractions of hESC-CM and NRVM, the expression of the Ca^{2+} -handling proteins, and the MEA-recorded conduction and activation properties.

TVP1022 Attenuated the Deleterious Effects of Doxorubicin on the $[Ca^{2+}]_i$ Transients and Contractions of NRVM. In the present study we showed that doxorubicin elevated diastolic $[Ca^{2+}]_i$, decreased the maximal rate of $[Ca^{2+}]_i$ activation, and prolonged $[Ca^{2+}]_i$ relaxation time. Concomitantly, doxorubicin decreased the maximal rates of contraction and relaxation and contraction amplitude. These findings are in agreement with previous reports demonstrating that doxorubicin adversely affects $[Ca^{2+}]_i$ handling and contractions in NRVM (Maeda et al., 1999; Shneyvays et al., 2001) and in adult cardiomyocytes from different species (Maeda et al., 1998; Mijares and Lopez, 2001). In agreement with our recent report showing that TVP1022 attenuated apoptosis induced by doxorubicin in NRVM (Kleiner et al., 2008), this study shows that TVP1022 diminished doxorubicin-induced deleterious effects on $[Ca^{2+}]_i$ transients and contractions.

TVP1022 Attenuated the Deleterious Effects of Doxorubicin on the $[Ca^{2+}]_i$ Handling Proteins. To decipher the mechanisms underlying the adverse effects of doxorubi-

cin on the $[Ca^{2+}]_i$ transients and contractions, we measured its effect on key $[Ca^{2+}]_i$ -handling proteins. Doxorubicin decreased the expression of SERCA2 and NCX1, but did not affect the expression of RyR, calsequestrin, or phospholamban. Accordingly, previous studies showed that doxorubicin decreased SERCA2 mRNA and protein expression in cultured NRVM (Arai et al., 2000), adult rat ventricular cardiomyocytes (Timolati et al., 2006), and rabbit in vivo model of doxorubicin cardiotoxicity (Arai et al., 1998; Olson et al., 2005). Furthermore, doxorubicin reduced NCX1 expression (Fig. 4D), in agreement with previous findings showing similar effects in atrial and ventricular preparations isolated from doxorubicin-treated rabbits (Olson et al., 2005) and in dog heart sarcolemmal vesicles (Caroni et al., 1981). Collectively, the reduction in SERCA2 and NCX1 expression can explain the elevated diastolic $[Ca^{2+}]_i$ and the slowing of $[Ca^{2+}]_i$ relaxation kinetics, as well as reduced rate of myocyte relaxation caused by doxorubicin. The increased diastolic $[Ca^{2+}]_i$ can also be accounted for by the findings that doxorubicin increased $[^3H]$ ryanodine binding to the RyR, and the increased RyR channel open probability (Pessah et al., 1990; Saeki et al., 2002). These observations indicate that doxorubicin alters RyR function, and not necessarily protein content, which is in agreement with our finding that doxorubicin did not affect RyR protein expression [but contrasted with the decrease in RyR protein expression in rabbit hearts (Olson et al., 2005)]. Hence, the increased diastolic $[Ca^{2+}]_i$ and reduced rate of $[Ca^{2+}]_i$ activation may lead to the reduction in the contraction rate of myocytes. Our findings that doxorubicin did not affect calsequestrin or phospholamban expression are in partial agreement with those of Olson et al. (2005) who found that the protein level of phospholamban was unchanged and that of calsequestrin was decreased by doxorubicin in rabbit heart model of cardiotoxicity. The dissimilarities between our findings and those of Olson et al. can be due to the difference in the experimental models: in vivo versus in vitro, adult versus neonatal hearts, and rabbit versus rat. Furthermore, Burke et al. (2002) reported that neonatal rabbit sarcoplasmic reticulum (SR) is less sensitive to anthracycline cardiotoxicity, which can further explain the differences in the effects of doxorubicin in the diverse experimental models. In agreement with the ability of TVP1022 to attenuate the adverse effects of doxorubicin on the $[Ca^{2+}]_i$ transients and contractions, the drug also prevented the decline in SERCA2 and NCX1 expression.

TVP1022 Attenuated the Adverse Effects of Doxorubicin on Intercellular Coupling, Cx43 Expression, Activation, and Conduction Velocity. A key ventricular function adversely affected by doxorubicin is intercellular coupling. As shown by the Lucifer yellow transfer experiments, doxorubicin reduced intercellular coupling, as indicated by a 36% decline in the Lucifer yellow transfer distance (Fig. 5). It is noteworthy that doxorubicin did not amplify the uncoupling effects of carbenoxolone, suggesting that carbenoxolone maximally uncouples the cells and the doxorubicin effect is swamped out. Furthermore, because TVP1022 did not rescue the uncoupling due to carbenoxolone, but it rescued the doxorubicin-mediated loss of coupling, it is likely that TVP1022 and carbenoxolone operate by different mechanisms.

Hence, the decrease in intercellular coupling by doxorubicin probably results from reduced Cx43 protein content, open

probability, and/or gap junctional permeability (Ek-Vitorin et al., 2006). Furthermore, Valiunas et al. (2002) showed a linear relation between gap junctional permeability and Lucifer yellow transfer and conductance in Cx43-expressing HeLa cells. Hence, the 39 and 56% reduction in total and NP Cx43 protein expression, respectively (Fig. 6), shown here can account (at least, in part) for the reduction in Lucifer yellow transfer by doxorubicin.

As illustrated in Fig. 7, the reduction in the intercellular coupling and Cx43 protein content was associated with a 50 to 60% decrease in the QRS amplitude, $dV/dt_{\max}$, and conduction velocity at the cell network level. In agreement with the alleviating effects of TVP1022 on NRVM function shown previously, the drug attenuated doxorubicin-induced reduction in Lucifer yellow transfer, total Cx43 (but not NP CX43), as well as the decrease in conduction velocity, QRS amplitude, and $dV/dt_{\max}$. In this regard, it was reported that the relations between Cx43 phosphorylation state and intercellular coupling are controversial, and that phosphorylation modulates gap junctional conduction in both positive and negative fashions, depending on the activity of different kinases (Hervé and Sarrouilhe, 2002; Lampe and Lau, 2000; Lampe et al., 2000). The increase in total Cx43 in the TVP1022 + doxorubicin group (compared with the doxorubicin group), but not in NP Cx43, led us to conclude that TVP1022 elevated phosphorylated Cx43. Hence, the TVP1022-induced augmentation in phosphorylated Cx43 probably contributed to the increased intercellular coupling, resulting in increased activation and propagation parameters. This conclusion is supported by the following findings: 1) an increase in phosphorylated Cx43 protein expression with no parallel increase in NP Cx43 is positively correlated with synchronized contraction, reflecting better activation and propagation patterns in NRVM (Oyamada et al., 1994); 2) we previously reported that, in NRVM, increased Cx43 density is associated with higher conduction velocity, QRS amplitude, and $dV/dt_{\max}$ (Meiry et al., 2001); 3) slower conduction velocity was demonstrated in myocytes from the Cx43 homozygote neonatal (Beauchamp et al., 2004) and heterozygote neonatal and adult (Guerrero et al., 1997) knockout mice compared with wild-type mice; 4) the conduction velocity in Cx43 mutant gene-modified NRVM was more than 3-fold slower relative to controls, indicating the central role of Cx43 in electrical impulse conduction (Kizana et al., 2007). However, in contrast with these findings, it should be noted that Thomas et al. (2003) showed that a 50% reduction in Cx43 levels did not result in a reduced conduction velocity. In summary, based on our current findings as well as on other reports, we suggest that by increasing phosphorylated Cx43, TVP1022 alleviated the adverse effects of doxorubicin on intercellular coupling, activation, and conduction velocity. The mechanism whereby TVP1022 increased phosphorylated Cx43 is yet to be determined.

Potential Protective Mechanisms of TVP1022 Against the Adverse Effects of Doxorubicin. The molecular basis of the cardiotoxicity induced by doxorubicin remains a matter of debate, and has been attributed to a large number of effects. The proposed mechanisms of doxorubicin cardiotoxicity include the generation of a secondary alcohol metabolite doxorubicinol (doxol), which was found to deregulate calcium and iron homeostasis in cardiomyocytes (Mi-

notti et al., 2004; Menna et al., 2008), receptor-mediated and direct effect on proteins (Pessah et al., 1990; Saeki et al., 2002), and apoptosis (Kleiner et al., 2008; Ueno et al., 2006). Although a number of mechanisms that lead to doxorubicin cardiotoxicity have been proposed, most studies support the view that a common trigger of doxorubicin cardiotoxicity is linked to an oxidative stress caused by the production of reactive oxygen species (ROS) (Doroshov, 1983; Sarvazyan, 1996; Singal et al., 2000; Takemura and Fujiwara, 2007). In this regard, Kim et al. (2006) demonstrated in rat cardiomyocytes that doxorubicin-mediated ROS formation increased SR Ca^{2+} release, which resulted in further ROS generation. In addition, $[Ca^{2+}]_i$ overload in cardiac cells may render mitochondrial Ca^{2+} overloading, resulting in modulated mitochondrial permeability by opening the mitochondrial permeability transition pores. This in turn leads to cytochrome *c* release, which may culminate in apoptosis. Kim et al. (2006) also showed that increased caspase 3 activity was doxorubicin/ROS-mediated, via SR Ca^{2+} release, indicating the important role of Ca^{2+} in cardiomyocyte apoptosis. Respecting the cytoprotective efficacy of TVP1022, this molecule was shown to induce superoxide dismutase and catalase in neuronal cultures and in vivo (Youdim et al., 2001), to inhibit the apoptotic cascade and to protect mitochondrial viability in NRVM and neuronal cells (Youdim et al., 2001, 2003; Youdim and Weinstock, 2001; Kleiner et al., 2008). Collectively, these activities probably caused ROS reduction and improved myocyte viability, thereby attenuating doxorubicin-induced cardiotoxicity. However, despite this supporting evidence, the interaction between TVP1022 and ROS resulting in attenuation of doxorubicin cardiotoxicity was not determined directly in this study, and other possible mechanisms of action may account for the beneficial effects of TVP1022.

The Safety of TVP1022. As clearly depicted in this study, TVP1022 did not affect the measured functional properties of NRVM or hESC-CM. These findings bare significant clinical relevance because they imply that TVP1022 may potentially be safely administered to the patients treated with anthracyclines. In further support of the suitability of TVP1022 for providing cardioprotection to doxorubicin-treated cancer patients, we recently found that TVP1022 (0.01, 0.1, and 1 μ M) neither caused proliferation of the human cancer cell lines HeLa and MDA-231 nor diminished the anticancer efficacy of doxorubicin (Kleiner et al., 2008).

Study Limitation. This study demonstrated the cardioprotective efficacy of TVP1022 against doxorubicin toxicity in vitro in NRVM. A key limitation of this, as well as many other in vitro studies investigating disease settings, is that most pathologies in humans develop over time, whereas in vitro experiments in cell culture are naturally short-termed (in our study, 24 h). In contrast to the in vitro scenario, the clinical signs of anthracycline-induced cardiotoxicity are associated with cumulative drug exposure, and develop on a time scale of months/years. In this regard, even animal models of doxorubicin-induced cardiotoxicity are somewhat limited because cardiac damage develops within days/weeks. Hence, in view to these limitations, the results of this study should be carefully interpreted.

In summary, this study demonstrated that TVP1022 attenuated doxorubicin-induced adverse effects on functional properties of NRVM. Based on these encouraging findings,

TVP1022 can be considered as a novel cardioprotective molecule against anthracycline cardiotoxicity, and may thus be administered in combination with doxorubicin in the treatment of different malignancies in humans.

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