TRO40303, a New Cardioprotective Compound, Inhibits Mitochondrial Permeability Transition

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ABSTRACT

3,5-Seco-4-nor-cholestan-5-one oxime-3-ol (TRO40303) is a new cardioprotective compound coming from a chemical series identified initially for neuroprotective properties. TRO40303 binds specifically to the mitochondrial translocator protein 18 kDa (TSPO) at the cholesterol site. After intravenous administration, TRO40303 tissue distribution was comparable to that of TSPO, and, in particular, the drug accumulated rapidly in the heart. In a model of 35 min of myocardial ischemia/24 h of reperfusion in rats, TRO40303 (2.5 mg/kg) reduced infarct size by 38% (p < 0.01 versus control), when administered 10 min before reperfusion, which was correlated with reduced release of apoptosis-inducing factor from mitochondria to the cytoplasm in the ischemic area at risk. Although TRO40303 had no effect on the calcium retention capacity of isolated mitochondria, unlike cyclosporine A, the drug delayed mitochondrial permeability transition pore (mPTP) opening and cell death in isolated adult rat cardiomyocytes subjected to 2 h of hypoxia followed by 2 h of reoxygenation and inhibited mPTP opening in neonatal rat cardiomyocytes treated with hydrogen peroxide. The effects of TRO40303 on mPTP in cell models of oxidative stress are correlated with a significant reduction in reactive oxygen species production and subsequent calcium overload. TRO40303 is a new mitochondrial-targeted drug and inhibits mPTP triggered by oxidative stress. Its mode of action differs from that of other mPTP inhibitors such as cyclosporine A, thus providing a new pharmacological approach to study mPTP regulation. Its efficacy in an animal model of myocardial infarctions makes TRO40303 a promising new drug for the reduction of cardiac ischemia-reperfusion injury.

Opening of the mitochondrial permeability transition pore (mPTP) within the few minutes after reperfusion is now well established as a key event contributing to myocardial ischaemia-reperfusion (I/R) injury. Mitochondrial permeability transition is triggered by the burst of reactive oxygen species (ROS) combined with calcium overload, lack of ATP, and recovery of cytoplasmic pH that follows tissue reperfusion. It is characterized by matrix swelling and rupture of the outer mitochondrial membrane (Griffiths and Halestrap, 1995). The mPTP opening is followed by mitochondrial leakage of protein factors inducing apoptosis or necrosis, depending on the ATP level and whether all or only a subpopulation of mitochondria undergo permeability transition (Halestrap, 2006). The mPTP concept first proposed by Haworth and Hunter (1979) gained prominence as an important factor in I/R and a target for cardioprotective agents when mice devoid of cyclophilin D (CyD) were found to have significantly reduced infarct size compared with wild-type mice (Baines et al., 2005). Furthermore, drugs such as cyclosporine A (CsA) that delay mPTP opening through binding, inhibition, and displacement of CyD provide cardioprotection after myocardial I/R in various animal models (Massoudy et al., 1997; Squadrito et al., 1999; Argaud et al., 2005; Gomez et al., 2007). Other procedures such as pre- and postconditioning
have also been shown to reduce I/R via inhibition of mPTP opening (Hausenloy et al., 2009). More recently, postconditioning and CsA were shown to reduce infarct size in patients undergoing percutaneous coronary intervention to establish reperfusion after an acute myocardial infarction (Staat et al., 2005; Piot et al., 2008), providing the strong clinical proof of concept that modulation of mPTP is a validated strategy to reduce myocardial damage at the time of reperfusion in humans.

Several models of the mPTP have been described previously (Baines, 2009; Javadov et al., 2009). Proteins implicated in mPTP formation include the matrix CyD, the outer membrane voltage-dependent anion channel (VDAC). However, none of these proteins is mandatory for mPTP opening as permeability transition, leading to mitochondrial-mediated cell death, even though altered for some cases, still persists after each of these proteins has been knocked-out (Kokoszka et al., 2004; Basso et al., 2005; Baines et al., 2007). Additional proteins such as the translocator protein 18 kDa (TSPO), formerly known as the peripheral benzodiazepine receptor, have been shown to interact with proteins implicated in mPTP formation and potentially regulate the pore (Veenman et al., 2007). Selective TSPO ligands, such as chlorodiazepam (Ro5-4864) (Obame et al., 2007) or 7-chloro-N,N,5-trimethyl-4-oxo-3-phenyl-3,5-dihydro-4H-pyridazino(4,5-b)indole-1-acetamide (SSR180575) (Leducq et al., 2003), were reported to provide cardioprotection in preclinical models of I/R. The pro- and antiapoptotic Bel-2 family proteins regulate mitochondrial permeability transition either by directly and independently forming a membrane channel or by interaction with mitochondrial membrane proteins such as VDAC (Shimizu et al., 1999) or adenine nucleotide translocase (Marzo et al., 1998). Other mPTP modulators include glycocon synthase kinase-3β inhibitors (Obame et al., 2008), protein kinase C-ε (Inagaki et al., 2005), and hexokinase I and II (Pastorino and Hoek, 2008). More recently, the phosphate carrier has also been shown to play an important role in mPTP regulation (Leung et al., 2008), and, finally, others have proposed that mPTP opening is the result of protein oxidation (Hossain et al., 2009) or accumulation of unfolded proteins in the outer mitochondrial membrane (He and Lemasters, 2002).

In the present study, we investigated the cardioprotective effects of 3,5-seco-4-nor-cholestan-5-one oxime-3-ol (TRO40303), which was originally selected using a cell-based screening assay aimed to identify small molecules that maintain survival of trophic factor-deprived rat motor neurons (Bordet et al., 2007). TRO40303 binds specifically to the cholesterol site of TSPO and exhibits cytoprotective properties in various cell types correlated with prevention of mPTP opening. Here we present evidence that TRO40303 rapidly accumulates in the heart after intravenous administration and prevents cardiomyocyte death after I/R, both in vitro and in vivo. To better understand how TRO40303 provides protection against oxidative damage associated with I/R injury, we investigated its effects on isolated cardiac mitochondria and isolated cardiomyocytes subjected to oxidative stress monitoring calcium retention capacity, ROS accumulation, and mPTP opening as permeability transition, leading to mitochondrial-mediated cell death. These studies show that TRO40303 has a novel mode of mPTP modulation as it does not inhibit the calcium retention capacity of isolated mitochondria, but it reduces mPTP opening in cells, probably via reduction in ROS production.

Materials and Methods

Drugs. All compounds were purchased from Sigma-Aldrich (St. Louis, MO) unless specified. TRO40303 (Fig. 1A) was synthesized by Synkem (Dijon, France). The radiolabeled [14C]TRO40303 was synthesized by GE Healthcare (Little Chalfont, Buckinghamshire, UK) with a specific activity of 2.11 GBq/mmol. For the in vitro tests, drugs such as CsA and TRO40303 were dissolved in dimethyl sulfoxide (DMSO) to prepare 10−2 M stock solutions that were diluted to their final concentration in the appropriate buffer or medium. For in vivo studies, TRO40303 was dissolved in a solution of 30% hydroxypropyl-β-cyclodextrin in phosphate-buffered saline (HPBCD).

Animals. All animal procedures used in this study were in strict accordance with the European Community Council Directive (86-609/78-848 EEC) and recommendations of the French Ministère de l’Agriculture or in accordance with the American Veterinary Medical Association guidelines, consistent with United States federal regulations and approved by the University of Louisville Institutional Animal Care and Use Committee. Male Wistar rats (250–280 g; Janvier, Le Genest Saint Isle, France) were used for in vivo myocardial infarction studies and for preparation of adult cardiomyocytes and cardiac mitochondria. For tissue distribution studies, male Sprague-Dawley rats (250–330 g; Janvier) were used. Animals were maintained in a room with controlled temperature (21–25°C) and a reverse 12 h light/dark cycle with food and water available ad libitum.

Cell Culture and Fluorescent Probes. All cell culture medium and supplements, fetal bovine serum, Calcium Green-5N, calcein-AM, 5(and 6)-chloromethyl-2‘,7‘-dichlorodihydrofluorescein diacetate, acyl ester (DCF), tetramethylrhodamine methyl ester (TMRM), MitoTracker Red, and propidium iodide were purchased from Invitrogen (Carlsbad, CA) unless specified.

In Vitro Binding to TSPO. The affinity of TRO40303 for TSPO was evaluated by measuring its ability to compete with [3H](1-[2-(chlorophenyl])-N-methyl-N-(1-methyl-propyl)-3-isoxquinoline carbozamde (PK11195) or [3H]cholesterol binding to recombinant mouse TSPO reconstituted in proteoliposomes (Lacapère et al., 2007).
the following equation:

\[ \text{Bound} \times \frac{\text{percent}}{100} = \text{specific activity} \times \text{radioactivity} \]

Radioactive ligands were displaced by incubation in the presence of increasing concentrations of cold ligands (PK11195, cholesterol, or TRO40303). Vesicle suspensions were filtered on Whatman GF/C filters and washed, and radioactivity was measured by liquid scintillation. Nonspecific binding was estimated as the re-
analyzed as the remainder of radioactivity after washing in the presence of excess cold ligand. Binding data were fitted to a simple sigmoid curve using the following equation: \( Y = 100 - \text{amplitude} \times S/(K_d + S) \).

**Tissue Distribution.** Tissue distribution of TRO40303 was evaluated by quantitative whole-body autoradiography after intravenous administration of 2 mg/kg \[^{14}C\]TRO40303, corresponding to 3.7 MBq/kg, to six fasted male rats (Avogadro, Fontenilles, France). Blood samples were taken 1, 2, 4, 8, 24, and 48 h postdose. The total radioactivity was determined in whole blood by liquid scintillation counting. After each blood sampling, one animal was sacrificed with anesthetic and stored at \(-20^\circ\text{C}\) until slicing and whole-body autoradiography (Biotec Center, Orléans, France).

**Myocardial Infarction Model.** Using a protocol described by Obame et al. (2007), rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.), intubated, and ventilated. A fluid-filled Tygon arterial catheter was placed in the right carotid artery for measurement of arterial blood pressure with a Statham P23ID strain-gauge transducer. Heart rate was calculated by integration over time of systolic pulses issued from the arterial blood pressure signal. Tightening the snare under the left main coronary artery induced coronary artery occlusion (HAR) and releasing the suture initiated coronary artery reperfusion (CAR). In all groups of rats the coronary artery was occluded during 35 min and released for reperfusion, except in the so-called sham group in which the surgical procedure was identical to others, but the coronary artery was not occluded. In the other groups of treated rats, the vehicle (HPBCD 30% in PBS) and TRO40303 at increasing doses (0.5, 1.25, and 2.5 mg/kg) were administered at random as a 3-min infusion through the jugular vein starting 10 min before CAR. Rats were allowed to recover for 24 h before measurement of infarct size. Infarct size was determined as a percentage of the area at risk (AAR) by triphenyltetrazolium chloride staining as described previously (Obame et al., 2007). In experiments on isolated mitochondria obtained from heart tissue after CAR, rats were sacrificed after 10 min of reperfusion. To analyze the release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria, cytosolic fractions were prepared from heart tissue after 1 h of reperfusion.

**Isolation of Mitochondria and Cytosolic Fractions from Rat Heart.** To prepare mitochondria for Ca\(^{2+}\) retention capacity (CRC) experiments, the myocardium from healthy rats (noninfarcted) or from the AAR of rats that underwent I/R was excised and rapidly minced followed by homogenization with a Polytron homogenizer (low setting 3) and then with a Potter homogenizer (3 up and down strokes at 1500 rpm) in 15 ml of a cold buffer containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.04 mM fatty acid-free bovine serum albumin, pH 7.4 at 4°C. Homogenates were centrifuged at 1000g for 5 min, and supernatants were centrifuged again at 10,000g for 10 min. The final pellets were resuspended in the homogenization buffer including 0.1 mM EGTA without bovine serum albumin to obtain a protein concentration of approximately 15 mg/ml.

To measure cytochrome c and AIF release from mitochondria, cardiac homogenates were prepared according to the same protocol except that bovine serum albumin was omitted from the buffer. Homogenates were centrifuged at 1000g for 5 min, and supernatants were centrifuged at 10,000g for 10 min. The final supernatants, corresponding to the cytosolic fractions, were collected, and 20 µl of a protease inhibitor cocktail (Sigma-Aldrich) and 20 µl of 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) were added. The supernatants were immediately frozen at –80°C until determination of protein concentration and analysis of cytochrome c and AIF by Western blot.

**Evaluation of CRC of Isolated Mitochondria.** mPTP opening was assessed by monitoring mitochondrial CRC. Mitochondria were loaded with increasing concentrations of Ca\(^{2+}\) until the load reached a threshold at which mitochondria underwent a fast process of Ca\(^{2+}\) release, which was due to mPTP opening. Cardiac mitochondria...
(1 mg/ml), energized with 5 mM pyruvate/malate, were incubated in a buffer containing 50 mM sucrose, 100 mM KCl, 5 mM KH₂PO₄, 10 mM HEPES, and 1 μM Calcium Green-5N fluorescent probe. The concentration of Ca²⁺ in the extramitochondrial medium was monitored by means of an LS 50B spectrophotometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) at excitation and emission wavelengths of 506 and 532 nm, respectively. The Ca²⁺ signal was calibrated by addition to the medium of known Ca²⁺ amounts.

Western Blot Experiments. Samples of cytosolic proteins, 5 μg for cytochrome c and 15 μg for AIF, were boiled at 95°C in a buffer containing 20% sucrose, 2.4% SDS, 5% β-mercaptoethanol, and 5% bromphenol blue. They were subjected to electrophoresis on 4 to 15% (cytochrome c) and 4 to 10% (AIF) gradient SDS-polyacrylamide gel electrophoresis gels and then transferred on polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in a Tris buffer (10 mM Tris and 100 mM NaCl, pH 7.5) containing 0.05% Tween 20 overnight at 4°C. Subsequently, membranes were exposed for 1 h to either mouse cytochrome c antibody (1:1000; BD Biosciences, San Jose, CA) or goat polyclonal anti-AIF antibody (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation with horseradish peroxidase-coupled goat anti-mouse immunoglobulins (Santa Cruz Biotechnology, Inc.) or donkey anti-goat immunoglobulins (Santa Cruz Biotechnology, Inc.), proteins were revealed by enhanced chemiluminescence reaction (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) after exposure to X-ray films (GE Healthcare). Band intensities were analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

Measurement of mPTP Opening in Primary Adult Rat Cardiomyocytes Subjected to Hypoxia/Reoxygenation. Ventricular cardiomyocytes were isolated from male Wistar rats as described previously (Obame et al., 2008). The cardiomyocytes were placed into a thermostated (37°C) chamber (Warner Instruments, Hamden, CT), which was mounted on the stage of a IX71 Olympus microscope and were perfused with a Tyrode’s solution (130 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM MgCl₂, and 1.8 mM CaCl₂; pH = 7.4 at 37°C) at a rate of 0.5 ml/min. The chamber was connected to a gas bottle containing 20% sucrose, 2.4% SDS, 5% β-mercaptoethanol, and 5% bromphenol blue. They were subjected to electrophoresis on 4 to 15% (cytochrome c) and 4 to 10% (AIF) gradient SDS-polyacrylamide gel electrophoresis gels and then transferred on polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in a Tris buffer (10 mM Tris and 100 mM NaCl, pH 7.5) containing 0.05% Tween 20 overnight at 4°C. Subsequently, membranes were exposed for 1 h to either mouse cytochrome c antibody (1:1000; BD Biosciences, San Jose, CA) or goat polyclonal anti-AIF antibody (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation with horseradish peroxidase-coupled goat anti-mouse immunoglobulins (Santa Cruz Biotechnology, Inc.) or donkey anti-goat immunoglobulins (Santa Cruz Biotechnology, Inc.), proteins were revealed by enhanced chemiluminescence reaction (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) after exposure to X-ray films (GE Healthcare). Band intensities were analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

Assessment of mPTP Opening in NRCMs after H₂O₂ Intoxication. Formation of mPTP was assessed by following the changes in calcein fluorescence using a modified cold and warm loading protocol (Nieminen et al., 1998). NRCMs were loaded with 1 μM calcein-AM for 150 min at 4°C. Medium was changed to imaging medium, and NRCMs were loaded with 2 μM MitoTracker Red FM at room temperature for 90 min. For excitation for calcein was through a 522/40-nm bandpass filter, and emission was through a 522/40-nm bandpass filter, whereas excitation for MitoTracker Red was through a 560/28-nm bandpass filter and emission was through 464/38-nm bandpass filter. Exposure duration was set at 50 ms for calcein and 100 ms for MitoTracker Red. Mitochondrial calcein fluorescence was determined using MetaMorph software in regions devoid of MitoTracker Red fluorescence to detect nonmitochondrial calcein fluorescence from the total calcein fluorescence. Calcein localization was assessed by measuring the S.D. of the intensity of all green fluorescent pixels within the same region of interest. A region of interest was defined by drawing a circle (43.61 μm²) enclosing several mitochondria. The S.D. is high when fluorescence is punctate and low when fluorescence is diffuse. Fluorescence is normalized to time 0 for each treatment.
The DCF used for these experiments is improved compared with 2',7'-dichlorodihydrofluorescein diacetate because of enhanced retention in cells. According to the manufacturer, it has been shown to detect H₂O₂ generation in the cells as well as other reactive oxygen intermediates such as OH\(^-\), HO\(_2\), and ONOO\(^-\).

NRCMs were loaded with 2 μM DCF for 30 min at 37°C. Medium was changed to imaging medium as mentioned above. DCF fluorescence was recorded through a 470/40-nm bandpass filter and emission through a 522/40-nm bandpass filter. Exposure duration was set at 100 ms. Fluorescence is normalized to time 0 for each treatment.

Assessment of Calcium Overload. Calcium levels were assessed by following the changes in Rhod-2 (Ngoh et al., 2009) and Fluo-4 fluorescence (Teshima et al., 2003a). NRCMs were loaded with 2 μM Rhod-2AM (used to assess mitochondrial calcium) or 1 μM Fluo-4AM (used to assess cytosolic calcium) for 30 min at 37°C, and media were changed to imaging media. BAPTA-AM (50 μM), a membrane permeable intracellular calcium chelator, was used as a control. Fluorescence was recorded by sequentially exciting Rhod-2 and Fluo-4 through 560/28- and 470/40-nm bandpass filters, respectively. Emission was sequentially assessed through 646/38-nm (for Rhod-2) and 522/40-nm (for Fluo-4) bandpass filters. Exposure duration was set at 100 ms for both Rhod-2 and Fluo-4.

Assessment of Mitochondrial Membrane Potential. Mitochondrial membrane potential changes was detected by following changes in TMRM fluorescence, which is rapidly and reversibly accumulated in the mitochondrial matrix in proportion to the mitochondrial membrane potential. NRCMs were loaded with 100 nM TMRM 30 min before imaging. Imaging was initiated by exciting TMRM through a 546/11-nm bandpass filter, and emission was assessed through a 567/15-nm bandpass filter. Exposure duration was set at 200 ms. Fluorescence is normalized to time 0 for each treatment.

Data Analysis. The data are reported as means ± S.E.M. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett’s post-test. Significance was accepted when \( p < 0.05 \).

Results

TRO40303 Specifically Binds to the TSPO Protein. TRO40303 (Fig. 1A) is a new chemical entity that was derived from a chemistry optimization program based on the structure of cholest-4-en-3-one, oxime (TRO19622), also called olesoxime (Bordet et al., 2007). TRO40303 was able to reduce cell death of trophic factor-deprived motor neurons as effectively as olesoxime (not shown). Because olesoxime showed specific binding to TSPO and VDAC (Bordet et al., 2007), we investigated whether TRO40303 also binds to these two proteins.

TRO40303 was studied for its ability to interact with either the PK11195- or cholesterol-binding sites on purified recombinant mouse TSPO reconstituted into proteoliposomes (Jamin et al., 2005). As shown for olesoxime, TRO40303 significantly displaced \(^{3}H\)cholesterol binding to recombinant TSPO (\( K_\text{d} = 150 \text{nM} \)) (Fig. 1B) but did not displace \(^{3}H\)PK11195 (Fig. 1C). These results demonstrate that this cholesterol-oxime family interacts with TSPO specifically at the cholesterol-binding site probably because of their cholesterol-like structure. TRO40303 was also tested for its ability to bind to the neurosteroid binding site on VDAC (Darbandi-Tonkabon et al., 2003). Unlike olesoxime, TRO40303 did not significantly displace \(^{3}H\)6-azo-pregnanolone binding to VDAC in rat brain membrane preparations (not shown).

To identify other potential molecular targets of TRO40303, it was screened on a large panel of pharmacologically important targets (Diversity profile; CEREP, Poitiers, France) at concentrations up to 30 μM. Among the 69 receptors, channels, or transporters and 16 enzymes tested, TRO40303 did not show any reproducible or specific binding or functional interference (not shown). Thus, the binding of TRO40303 to the cholesterol site of the TSPO protein is extremely specific, and this protein might represent its cellular target.

Tissue Distribution of TRO40303. Tissue distribution of TRO40303 was evaluated after a single intravenous administration of 2 mg/kg \(^{14}C\)TRO40303 to fasted male rats. After 1 h \(^{14}C\)TRO40303 was highly distributed throughout the body with the highest affinities for the liver, lungs, hypophysis, kidneys, adrenals, intestines/contents, bone marrow, and spleen and lowest for brain (Fig. 1D). TRO40303 accumulated in the heart by a factor of 2 to 4 times that in total blood, depending on time (Fig. 1E). The distribution pattern of TRO40303 was similar to that of TSPO described for ligands such as chlorodiazepam or PK11195, which showed the highest levels in steroidogenic tissues such as adrenal cortex but also substantial levels in tissues with a high concentration of mitochondria such as heart, brown fat, and regions of the kidney, lung, liver, and spleen but only low levels in brain (Anholt et al., 1985).

Effects of TRO40303 in a Myocardial Infarction Model in Rats. Because TRO40303 accumulates in heart and possesses cytoprotective activity similar to that of olesoxime, which appears to involve a reduction in mitochondrial permeability transition (Bordet et al., 2007), and because mPTP appears to be a key step in I/R cardiotoxicity, TRO40303 was tested for its effect on infarct size in a model of I/R in rats. As shown in Fig. 2A, TRO40303 at 2.5 mg/kg, administered just before reperfusion, reduced infarct size by 38% (\( p < 0.01 \)), whereas the doses of 0.5 and 1.25 mg/kg were not active. At 2.5 mg/kg, TRO40303 had no effects on either heart rate or mean arterial pressure (not shown).

TRO40303 Did Not Modify CRC of Isolated Cardiac Mitochondria but Inhibited Apoptotic Factor Release after I/R In Vivo. In a first step, we investigated whether TRO40303 could increase CRC in isolated cardiac mitochondria from a normal myocardium. As seen in Fig. 3, 12.5 ± 1.1 pulses of 10 μM Ca\(^{2+}\) (125 nmol/mg mitochondrial protein) were required to induce mPTP opening in control mitochondria. The presence of 3 μM TRO40303 in the medium did not increase CRC (11.0 ± 1.5 pulses: 110 nmol/mg mitochondrial protein), whereas 1 μM CsA, a known mPTP and CRC inhibitor, significantly increased it (16.3 ± 0.4 pulses: 163 nmol/mg mitochondrial protein).

In a second step, to determine whether the effects of TRO40303 on CRC would only be observed when mitochondria were challenged in vivo with a more complex mitochondrial stress, the effects of TRO40303 on CRC were then evaluated on cardiac mitochondria isolated from rats subjected to 35 min of CAO and 10 min of CAR treated or not with 2.5 mg/kg TRO40303, 10 min before CAR. In addition, administration of TRO40303 in vivo might allow the compound to interact with its physiological target more easily than when incubated in vitro with isolated mitochondria from a normal heart. The amount of Ca\(^{2+}\) required to trigger mPTP opening in mitochondria isolated from sham-operated rats reached 92.5 ± 16.0 nmol/mg mitochondrial protein, similar to that seen in the previous study. After I/R, CRC was
reduced (35.8 ± 9.0 nmol/mg mitochondrial protein) (p < 0.05), and this was unchanged by TRO40303 administration (38.9 ± 11.6 nmol/mg mitochondrial protein), again demonstrating that the drug did not modify isolated mitochondrial sensitivity to Ca²⁺-induced mPTP opening sensitized by I/R.

However, in the same series of experiments, mitochondria and cytosol were separated and analyzed by Western blot to observe the effect of TRO40303 treatment on the release of proapoptotic factors. As shown in Fig. 2, B and C, cytochrome c and AIF were increased in cytosolic fractions isolated from AAR of rats after 35 min of CAO and 1 h of CAR. Treatment with TRO40303 (2.5 mg/kg) 10 min before reperfusion limited the release of AIF (p < 0.05) although the release of cytochrome c was not significantly affected. These results...
show that although TRO40303 treatment had no effect on Ca\(^{2+}\)-induced mPTP as measured by CRC in mitochondria once they have been isolated from their intracellular environment, TRO40303 reduced the release of AIF and further cell death in the AAR after I/R in rats.

TRO40303 Delayed mPTP Opening after Hypoxia/Reoxygenation in Isolated Cardiomyocytes. TRO40303 binds to TSPO, reduces infarct size, and limits apoptosis by reducing the release of AIF from the mitochondria to the cytosol after in vivo ischemia reperfusion, but TRO40303 showed no effect on the CRC of isolated mitochondria. To reconcile these effects that could seem paradoxical, we hypothesized that TRO40303 might inhibit mPTP opening only in a whole-cell environment, because it has an effect via the TSPO located at the outer membrane of the mitochondria. To investigate this hypothesis, we used a model of adult rat cardiomyocytes challenged by hypoxia and reoxygenation in vitro, and mPTP opening was followed using the calcein-cobalt staining method. As shown in Fig. 4 and Table 1, 3 \(\mu\)M TRO40303 delayed mPTP opening, as measured by an increase in the time to detect a 50% decrease in mitochondrial calcein fluorescence, in a manner similar to that of 1 \(\mu\)M CsA.

Two types of fluorescent signal kinetics were observed: either a gradual decrease or a complete and rapid drop in calcein fluorescence. These phenomena probably correspond to the sequence of these events in comparison with mPTP opening induction.

TRO40303 greatly increased the percentage of cardiomyocytes that displayed gradual mPTP openings compared with that in control cells (Table 1). Concomitantly, TRO40303 delayed nuclear labeling by propidium iodide, indicating that the compound also delayed the hypoxia/reoxygenation-induced cardiomyocyte cell death. In conclusion, although TRO40303 had no effect on Ca\(^{2+}\)-induced mPTP opening in isolated mitochondria, TRO40303 reduced mPTP opening in cardiomyocytes stressed by hypoxia/reoxygenation in vitro.

**TABLE 1**

| Effects of TRO40303 and CsA on mPTP opening after hypoxia/reoxygenation |
|-----------------------------|-----------------|-----------------|-----------------|
| Cells with Rapid mPTP Opening | Cells with Gradual mPTP Opening | Average 1\% mPTP Opening |
| Control (\(n = 5\)) | 95.2 ± 2.8 | 4.8 ± 2.8 | 34.5 ± 4.3 |
| 1 \(\mu\)M CsA (\(n = 3\)) | 35.4 ± 13.1** | 64.6 ± 13.1** | 67.9 ± 3.7*** |
| 3 \(\mu\)M TRO40303 (\(n = 3\)) | 54.3 ± 6.3** | 65.7 ± 6.3** | 69.3 ± 9.4*** |

**Note:** The results were averaged.

**Fig. 4.** TRO40303 delays mPTP opening and cell death after hypoxia/reoxygenation in isolated adult rat cardiomyocytes. The time course of mPTP opening and cell death triggered by reoxygenation of hypoxic cardiomyocytes was evaluated in the absence or presence of 3 \(\mu\)M TRO40303. Cardiomyocytes were coloaded with calcein, CoCl\(_2\), and propidium iodide (PI).

Fluorescence was normalized to 100% of the initial value for calcein and 100% of the final value for PI. Traces from three to five experiments were averaged.

TRO40303 Reduced H\(_2\)O\(_2\)-Induced ROS Production, Ca\(^{2+}\) Overload, and Loss of Mitochondrial Membrane Potential. ROS and Ca\(^{2+}\) overload are key contributors to I/R injury. We used the H\(_2\)O\(_2\) intoxication model to investigate the mechanism of action of TRO40303 through the assessment of its effects on ROS production and Ca\(^{2+}\), as well as the sequence of these events in comparison with mPTP opening induction.

DCF was used to measure ROS production. H\(_2\)O\(_2\) induced an immediate significant increase in ROS generation in NRCMs as reflected by increased DCF fluorescence (Fig. 6A; Table 2). Pretreatment of NRCMs with 3 \(\mu\)M TRO40303 before H\(_2\)O\(_2\) reduced the induction of ROS production start-
ing between 10 and 20 min after H$_2$O$_2$ exposition and reaching a significant reduction 60 min after the addition of H$_2$O$_2$. Pretreatment with 1 µM CsA slightly decreased the rate of H$_2$O$_2$-induced ROS production, but the effect on the DCF fluorescence decrement 60 min after addition of H$_2$O$_2$ was not statistically significant.

To determine whether TRO40303 had an effect on H$_2$O$_2$-induced Ca$^{2+}$ overload, we measured cytoplasmic Ca$^{2+}$ levels

| Table 2 | Quantitative effects of TRO40303 and CsA at 60 min after H$_2$O$_2$ exposition in newborn rat cardiomyocytes on ROS production (DCF), Ca$^{2+}$ overload in cytosol (Fluo-4) and mitochondria (Rhod-2), mPTP opening (Δ calcein S.D.), and loss of mitochondrial membrane potential (TMRM) Data are expressed in arbitrary units of fluorescence or percent variation ± S.E.M. Statistical analysis was performed by one-way ANOVA versus H$_2$O$_2$ treatment followed by Dunnett’s post-test. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Control | H$_2$O$_2$ | 3 µM TRO40303 | 1 µM CsA |
| DCF (% increase from time 0 to time 60 min) |         |         |             |             |             |         |         |             |
| Fluo-4 (AU)     | 15 ± 9*** | 126 ± 28 | 53 ± 15* | 89 ± 9 NS |
| Rhod-2 (AU)     | 26 ± 2*** | 255 ± 27 | 121 ± 26*** | 117 ± 21*** |
| Δ calcein S.D. (% reduction from time 0 to 60 min) | 11 ± 14*** | 303 ± 40 | 133 ± 15** | 134 ± 13*** |
| TMRM (% reduction from time 0 to time 90 min) | 0 ± 0*** | 39 ± 4 | 10 ± 6* | 5 ± 11** |
| AU, arbitrary units. |         |         |             |             |             |         |         |             |
| * p < 0.05.     |         |         |             |             |             |         |         |             |
| ** p < 0.01.    |         |         |             |             |             |         |         |             |
| *** p < 0.001.  |         |         |             |             |             |         |         |             |

Fig. 5. TRO40303 inhibits mPTP opening in newborn rat cardiomyocytes intoxicated with H$_2$O$_2$. Assessment of sensitivity to formation of mitochondrial permeability transition pore (mPTP) in NRCMs treated with DMSO (0.1%, control), TRO40303 (3 µM), or CsA (1 µM) before H$_2$O$_2$ treatment using calcein and MitoTracker Red. Loss of calcein fluorescence was used to indicate mPTP (n = 6 per group). H$_2$O$_2$ treatment induced mPTP opening, which was significantly attenuated by TRO40303 and CsA. A, representative montages showing change in calcein and MitoTracker Red fluorescence over time. B, time-dependent changes in S.D. of mean calcein fluorescence intensity in NRCMs exposed to nothing (control, □), H$_2$O$_2$ (■), H$_2$O$_2$ + TRO40303 (○), and H$_2$O$_2$ + CsA (gray circles) for one representative experiment (from n = 6). C, quantitative bar graph showing the change in S.D. of mean calcein fluorescence intensity between 0 and 60 min. Average of n = 6 experiments. *, p < 0.05; **, p < 0.01 versus H$_2$O$_2$ by ANOVA followed by Dunnett’s post-test.
using Fluo-4 fluorescence and mitochondrial Ca\(^{2+}\) levels using Rhod-2 fluorescence. Treatment with 100 \(\mu\)M \(H_2O_2\) caused a significant rise in cytosolic and mitochondrial Ca\(^{2+}\) levels as measured by Fluo-4 and Rhod-2 fluorescence, respectively (Fig. 6, B and C), with the onset of the rise between 20 and 30 min after \(H_2O_2\) addition. The increase in both cytosolic and mitochondrial Ca\(^{2+}\) levels were significantly attenuated by pretreatment with 3 \(\mu\)M TRO40303 or 1 \(\mu\)M CsA (Table 2). BAPTA-AM, an intracellular Ca\(^{2+}\) chelator, completely blocked the \(H_2O_2\)-mediated increases in both Fluo-4 and Rhod-2 fluorescence, confirming that these increases in fluorescence were due to increased \([Ca^{2+}]_i\) (not shown).

We examined the effects of TRO40303 treatment on oxidative stress-induced loss of mitochondrial membrane potential using TMRM fluorescence. \(H_2O_2\) treatment significantly reduced TMRM fluorescence by 90 min, and this decrease was reduced by 3 \(\mu\)M TRO40303, although not significantly, indicating a tendency for preservation of \(\Delta\Psi_m\) (Fig. 6D; Table 2). CsA pretreatment significantly attenuated the \(H_2O_2\)-mediated decrease in TMRM fluorescence and loss of \(\Delta\Psi_m\) at 90 min.

Discussion

We report here the identification and characterization of a new cardioprotective compound, TRO40303, selected from a chemical optimization program beginning with olesoxime based on the ability to protect trophic factor-deprived primary rat motor neurons. TRO40303 retains a cholesterol-like structure explaining its binding to the cholesterol site on TSPO. The compound has a tissue distribution that corresponds very well to the known expression pattern of TSPO, most probably accounting for its uptake and higher relative concentration in heart compared with plasma.

Binding of TRO40303 to TSPO was very specific as no displacement or competition with ligands or substrates was found for a large panel of physiologically important receptors, enzymes, transporters, and ion channels that could mediate its pharmacological action or interfere with further in vivo studies. Although TSPO is the target of a number of compounds previously shown to have neuroprotective and/or cardioprotective properties such as PK11195, chlorodiazepam (Obame et al., 2007), or SSR180575 (Leducq et al., 2003),...
these compounds bind to a promiscuous drug-binding site on TSPO that appears to influence cholesterol uptake and steroidogenesis, which is initiated by CYP11A1 (or P450scc), located in the inner mitochondrial membrane (Papadopoulos et al., 2006). Even though TRO40303 competes for cholesterol binding to TSPO, the drug had no major effect on plasma cholesterol levels in preclinical safety and toxicology studies (not shown) and so is unlikely to have an effect on cholesterol metabolism or steroidogenesis. This may be explained by the fact that TRO40303 has a lower affinity than cholesterol for TSPO. The full significance of the interaction of TRO40303 with the cholesterol site of TSPO remains to be investigated; however, it might account for the targeting of TRO40303 to the mitochondria as for cholesterol and olesoxime.

A tissue distribution study of TRO40303 in rat showed that it had a relatively high concentration in the heart, whereas it penetrated poorly in brain and that its distribution was similar to that of TSPO, highlighting the interest to test this cytoprotective compound in a myocardial infarction model in which the mitochondrial permeability transition has been shown to play a major role in I/R injury. We show that TRO40303 reduced myocardial infarct size in rats and delayed mPTP opening and mortality triggered by hypoxia/reoxygenation of adult primary rat cardiomyocytes in vitro. Cardiac protection by TRO40303 was correlated with a reduction in AIF release in cytosolic fractions of cardiac tissue isolated from the ischemic area, whereas the effect on cytochrome c release was variable and not significant. Independent release of AIF versus cytochrome c has been shown in isolated heart after hypoxia/reoxygenation, and this phenomenon was blocked by CsA (Kim et al., 2003). In addition, an antibody against TSPO has also been shown to selectively prevent AIF release but not that of cytochrome c after Ca\(^{2+}\)-induced mPTP opening in rat brain mitochondria (Azarashvili et al., 2007), providing evidence that the release of these two proapoptotic factors can be dissociated. Although the effect of TRO40303 on AIF alone might be sufficient to account for the 38% reduction in infarct size in rats treated with 2.5 mg/kg TRO40303, it must also be noted that AIF and cytochrome c release were measured 1 h after reperfusion, and it cannot be ruled out that an effect on cytochrome c might be more apparent at another time point.

The specific binding of TRO40303 to TSPO, a protein structurally related to TspO, a bacterial oxygen sensor (Yeliseev et al., 1997), may play a role in the modulation of ROS-induced mPTP opening. Indeed TSPO expression levels are positively correlated with survival of hematopoietic cells exposed to H\(_2\)O\(_2\) (Carayon et al., 1996). To investigate this possibility, we have closely examined the effect of TRO40303 on oxidative stress-induced mPTP opening using H\(_2\)O\(_2\)-intoxicated newborn rat cardiomyocytes. When looking carefully at the onset of events and effects of both TRO40303 and CsA as well as the extent of these effects in these experiments, it seems that both compounds have slightly different effects: the effects of TRO40303 on ROS and mPTP opening occurs slightly earlier than the effects of CsA on these two events, whereas CsA inhibition of mPTP opening appears to coincide with its effects on ROS production and calcium overload. Interestingly, the effects of TRO40303 and CsA on both mitochondrial and cytosolic calcium increases are nearly identical. This finding suggests that the massive calcium increase is a secondary effect after mPTP opening, which is in agreement with the effect observed by Lemasters et al. (2009) in studying hypoxia reoxygenation in adult cardiomyocytes. The different modes of mPTP inhibition by TRO40303 and CsA could correspond to the ROS and calcium triggers of mPTP opening as described by Hossain et al. (2009). Interestingly, whereas TRO40303 significantly reduced ROS production, at 60 min H\(_2\)O\(_2\)-induced ROS production in the presence of CsA showed only a trend to reduction, yet both TRO40303 and CsA reduced mPTP opening by approximately 50%. This could be understood as follows: the inhibitory effects of TRO40303 on mPTP may be mediated primarily through reduction of ROS production, whereas CsA inhibits mPTP opening mainly by binding to CsD and inhibiting its prolyl-isomerase activity, resulting in the displacement of CyD from the mPTP.

Together, these studies demonstrate the ability of TRO40303 to modulate mPTP opening in intact cells after hypoxia/reoxygenation or H\(_2\)O\(_2\) intoxication. Yet, unlike CsA, TRO40303 has no effect on CRC, a measure of Ca\(^{2+}\)-triggered mPTP in isolated mitochondria, highlighting the different mechanisms that can be modulated to inhibit mPTP in cells. This finding is consistent with the different proteins targeted by CsA and TRO40303. CsA targets CyD, located in the mitochondrial matrix where Ca\(^{2+}\) overload triggers mPTP opening. TRO40303 binds to TSPO in the outer mitochondrial membrane where it prevents mPTP opening in intact cardiomyocytes induced by I/R and by H\(_2\)O\(_2\)-activated ROS production. The difference between TRO40303 and classic TSPO ligands such as PK11195, chlorodiazepam, or SSR180575 is that TRO40303 binds to the cholesterol site and is potentially a substrate of TSPO, whereas classic TSPO ligands modulate cholesterol uptake and steroidogenesis, which may mediate the role of TSPO in protection from oxidative stress. The effects of TRO40303 on mitochondrial permeability transition could also be due to an effect on the interaction of TSPO with other putative members or modulators of the mPTP such as VDAC (McEnery et al., 1992). Alternatively, because of its cholesterol-like structure, TRO40303 may, like cholesterol, favor the VDAC-hexokinase interaction, increasing glycolysis and reducing the need for oxidative phosphorylation to maintain ATP levels (Pastorino and Hoek, 2008). Such an effect would be beneficial under ischemic conditions and protect tissue by reducing ROS formation via the electron transport chain once oxygen supply is restored. These indirect effects may prevent or delay mPTP opening in cardiomyocytes subjected to I/R or H\(_2\)O\(_2\) intoxication and explain the cytoprotective effects of TRO40303 in intact cells even though it has no effect on CRC as a measure of Ca\(^{2+}\)-induced mPTP opening in isolated mitochondria. It must be noted that even CsA is unable to increase CRC in the absence of phosphate and does not inhibit ROS-triggered mPTP opening in mitochondrial assay systems (He and Lemasters, 2002; Basso et al., 2008; Hossain et al., 2009). Thus, the fact that a compound does not inhibit CRC under particular conditions does not mean that it is not an inhibitor of mPTP opening in a cellular context; and this is what is observed with TRO40303.

In conclusion, we have identified TRO40303 as a new cardioprotective molecule that binds to TSPO, inhibits the mitochondrial permeability transition in intact cells, and reduces infarct size in a rat model of myocardial infarction when administered at reperfusion. Therefore, TRO40303 represents both a promising new tool to further investigate...
mechanisms involved in mPTP opening as well as a new class of drugs to treat reperfusion injury to the heart.

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