

ORIGINAL ARTICLE

TRO40303, a mitochondrial-targeted cytoprotective compound, provides protection in hepatitis models

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Abstract

TRO40303 is cytoprotective compound that was shown to reduce infarct size in preclinical models of myocardial infarction. It targets mitochondria, delays mitochondrial permeability transition pore (mPTP) opening and reduces oxidative stress in cardiomyocytes submitted to ischemia/reperfusion in vitro. Because the involvement of the mitochondria and the mPTP has been demonstrated in chronic as well as acute hepatitis, we investigated the potential of TRO40303 to prevent hepatocyte injury. A first set of in vitro studies showed that TRO40303 (from 0.3 to 3 $\mu\text{mol/L}$) protected HepG2 cells and primary mouse embryonic hepatocytes (PMEH) from palmitate intoxication, a model mimicking steatohepatitis. In PMEH, TRO40303 provided similar protection against cell death due to Jo2 anti-Fas antibody intoxication. Further studies were then performed in a mouse model of Fas-induced fulminant hepatitis induced by injecting Jo2 anti-Fas antibody. When mice received a sublethal dose of Jo2 at 125 $\mu\text{g/kg}$, TRO40303 pretreatment prevented liver enzyme elevation in plasma in parallel with a decrease in cytochrome C release from mitochondria and caspase 3 and 7 activation in hepatic tissue. When higher, lethal doses of Jo2 were administered, TRO40303 (10 and 30 mg/kg) significantly reduced mortality by 65–90% when administered intraperitoneally (i.p.) 1 h before Jo2 injection, a time when TRO40303 plasma concentrations reached their peak. TRO40303 (30 mg/kg , i.p.) was also able to reduce mortality by 30–50% when administered 1 h postlethal Jo2 intoxication. These results suggest that TRO40303 could be a promising new therapy for the treatment or prevention of hepatitis.

Abbreviations

ANOVA, analysis of variance; EGTA, ethylene glycol tetraacetic acid; HAM, hepatocyte attachment media; KHB, Krebs–Henseleit buffer; DMSO, dimethyl sulfoxide; NASH, nonalcoholic steatohepatitis; PMEH, primary mouse embryonic hepatocytes; p.o., per os; AH, acute hepatitis; AIF, apoptosis-inducing-factor; ALF, acute liver failure; CES, cremophor EL/ethanol/saline; CHX, cycloheximide; CsA, cyclosporine A (Rec.INN: Ciclosporin); Cyp D, cyclophilin D; Cyt C, cytochrome C; DISC, death-inducing signaling complex; EGF, epithelial growth factor; FBS, fetal bovine serum; IMM, inner mitochondrial membrane; i.p., intraperitoneal; i.v., intravenous; LDH, lactate dehydrogenase; MEM, minimum essential medium; mPT, mitochondrial permeability transition; mPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane; ROS, reactive oxygen species; TSPO, translocator protein18 kDa.

Introduction

Acute hepatitis (AH) is a complex disease due to various etiologies with different frequencies according to geographic regions. They include drug or alcohol-induced liver injury, viral hepatitis, mushroom poisoning, autoimmune liver disease, Wilson's disease, Budd-Chiari syndrome and shock (or acute ischemic injury). AH can lead to acute liver failure (ALF), characterized by a sudden liver failure, leading to coagulopathy and in the most severe cases encephalopathy, multiple organ failure, and death (Polson and Lee 2005). In Europe and the USA, ALF is considered as an orphan disease, whereas in Asia there is a high incidence notably due to endemic chronic hepatitis B affecting 10% of the population. There is currently no treatment available apart from *N*-acetylcysteine, which is used as an antidote to acetaminophen intoxication.

The mouse model of fulminant hepatitis using Jo2 anti-Fas antibody to induce apoptosis (Ogasawara et al. 1993) is a widely used model for AH in human, in which cytotoxic T lymphocytes kill hepatocytes through several mechanisms including the engagement of Fas/CD95 to Fas ligand (Guicciardi and Gores 2005). In this model, cell death follows the opening of a mitochondrial permeability transition pore (mPTP) based on the ability of cyclosporine A (CsA) to prevent Jo2-induced hepatitis (Feldmann et al. 2000). Additionally, oxidative stress, which sensitizes mitochondria to calcium-induced permeabilization, plays a role in the acute hepatic injury both in the Jo2 mouse model (Feldmann et al. 2000; Moniaux et al. 2011) and in ALF in humans (Chung et al. 2012). Fas activation and activated mitochondrial permeabilization have also been shown to play a central role in other types of chronic hepatitis including hepatocarcinoma, chronic viral hepatitis, alcoholic hepatitis, hepatitis C virus-related fibrosis, Wilson's disease, autoimmune hepatitis, nonalcoholic fatty liver disease, and in particular nonalcoholic steatohepatitis (NASH) characterized by the accumulation of fat in the liver (steatosis) along with inflammation in patients with no history of alcohol or drug use/abuse (Guicciardi and Gores 2005; Pessayre 2007).

Clinical trials aiming at reducing liver apoptosis in patients with chronic hepatitis C (Pockros et al. 2007) or during liver transplantation (Baskin-Bey et al. 2007) have shown promising results with antiapoptotic therapies such as the orally active caspase inhibitor IDN-6556. HIP/PAP, a paracrine hepatic polypeptide growth factor that reduces oxidative damage, has been shown to protect hepatocytes and activate liver regeneration in multiple preclinical models and is now in clinical development for ALF (Simon et al. 2003; Lieu et al. 2005; Moniaux et al. 2011, 2012). These approaches confirm the potential to identify

a satisfactory treatment for ALF by targeting oxidative stress culminating in excessive pathological apoptosis in the liver.

TRO40303 is a novel chemical entity currently in clinical development that has been shown to be a cytoprotective compound in multiple cellular and animal models involving stress-induced mitochondrial permeabilization (Schaller et al. 2010; d'Anglemont de Tassigny et al. 2013; Le Lamer et al. 2014; Richter et al. 2014). TRO40303 targets the outer mitochondrial membrane (OMM) translocator protein (TSPO), decreases oxidative stress, modulates the mitochondrial permeability transition (mPT) and as a consequence inhibits the opening of the mPTP, and reduces the release of apoptotic factors that trigger cell death (Schaller et al. 2010; Le Lamer et al. 2014).

Because of the strong implication of mitochondrial stress and permeabilization in hepatitis, these experiments aimed to investigate if TRO40303 could rescue liver cells in *in vitro* and then in *in vivo* hepatotoxicity models to investigate its potential to treat AH, ALF, or chronic disorders such as NASH. The results showed that by preventing mitochondrial permeabilization TRO40303 treatment can provide hepatoprotection.

Materials and Methods

Animals

All animal procedures used in this study were in strict accordance with the European Community Council Directive (86-609/87-848 EEC) and recommendations of the French Ministère de l'Agriculture. TROPHOS had a valid licence for experiments and surgery on rodents (agreement B 13-055-15).

Adult male (7–10 weeks old, in total $n = 605$) and pregnant CD1 mice (10 weeks old, in total $n = 6$) were purchased from Elevage Janvier (Le Genest-Saint-Isle, France). Mice were allowed to acclimate to the environment for at least 4 days before the experiments. They were individually identified by ear tags, housed five per cage except for pregnant females that were housed individually and had free access to food and water. The animals were maintained in a room with controlled temperature (21–25°C) and a 12 h light/dark cycle.

Reagents

All compounds were purchased from Sigma-Aldrich (St. Louis, MO) unless specified. TRO40303, 3,5-seco-4-norcholestan-5-one oxime-3-ol was synthesized by Synkem (Dijon, France). For all the *in vitro* tests, TRO40303 was dissolved in dimethyl sulfoxide (DMSO) to prepare

10^{-2} mol/L stock solution that was diluted to its final concentration in the appropriate medium. For in vivo studies, TRO40303 was prepared in a solution of cremophor EL/ethanol/saline (CES), (5/10/85, respective %) for intraperitoneal (i.p.) administration. The dose volume was adapted to each animal body weight (5 mL/kg).

For oral administration, TRO40303 was prepared in olive oil (30 mg/mL) and dosing (10 mL/kg) was performed by gavage using a stainless steel 22G needle.

For intravenous (i.v.) administrations, TRO40303 was prepared in liposomes as previously described (Le Lamer et al. 2014).

All cell culture media and supplements were purchased from Life Technologies (Carlsbad, CA) unless specified.

Primary cultures of mouse embryonic hepatocytes

Primary mouse embryonic hepatocytes (PMEH) were prepared as previously described (Maina et al. 2001; Moumen et al. 2007). Livers from E15.5 CD-1 mice were collected, minced, and digested first in DNase I, 14.33 μ g/mL final concentration in Krebs–Henseleit buffer (KHB) (1% NaCl, 0.035% KCl, 1.2 mmol/L KH_2PO_4 , 0.03% MgSO_4 , 0.2% NaHCO_3 , 2% glucose, containing 1% Pen/Strep [100 μ g/mL penicillin, 100 μ g/mL streptomycin]) and supplemented with 5 mmol/L ethylene glycol tetraacetic acid (EGTA) (KHB-EGTA) at 37°C, then in DNase I plus collagenase type I (83 μ g/mL final concentration) in KHB-Ca-EGTA (100 mmol/L CaCl_2 , 5 mmol/L EGTA) at 37°C. After washing with KHB-Ca (100 mmol/L CaCl_2) and filtration through a 70 μ m cell strainer, the eluate was centrifuged at 600 rpm for 5 min and the cell pellet was resuspended in 1.67 mL per liver of hepatocyte attachment media (HAM; Williams E medium with 0.4 μ g/mL dexamethasone, 1% Pen/Strep and 1% L-glutamine). PMEH were then diluted 1:10 in HAM containing 10% fetal bovine serum (FBS), 10 μ g/mL insulin, and 50 μ g/mL epidermal growth factor (EGF) and seeded into 96-well plates that had been precoated for 24 h with rat tail collagen. After 5–6 h of incubation at 37°C in a humidified atmosphere of 95% air, 5% CO_2 , medium was replaced by HAM containing 2% FBS, 10 μ g/mL insulin, and 50 μ g/mL EGF. In the morning of the following day, cells were washed twice with HAM containing 2% FBS and medium was replaced by HAM containing 2% FBS. This was repeated in the evening except that medium was replaced by HAM containing 0.01% bovine serum albumin.

HepG2 cell culture

HepG2 cells (ATCC, Manassas, Virginia) were cultivated in minimal essential media (MEM) supplemented with

2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 0.15% sodium bicarbonate, and 1% Pen/Strep. For palmitate intoxication assays, HepG2 cells were plated into 96-well plates at a density of 100,000 cells per well in a final volume of 200 μ L MEM.

In vitro intoxication with palmitate

PMEH or HepG2 cells were pretreated for 1 h with TRO40303 or the same concentration of DMSO (0.1%) ($n = 8$) prior to and during intoxication with 0.2 mmol/L palmitate for 24 h or 48 h, respectively. Each 96-well plate contained eight positive control wells treated by DMSO only without palmitate intoxication, used to assess the maximum hepatocyte number and viability, and eight negative control wells treated with DMSO and palmitate to assess maximal cell death. The dose of palmitate and timing of intoxication was selected to result in at least 50% cell death based on lactate dehydrogenase (LDH) or calcein endpoints.

In vitro intoxication with Jo2 antibody

PMEH were intoxicated with 100 μ g/mL Jo2 antibody (BD Biosciences Pharmingen, San Diego, CA) in the presence of 2 μ g/mL cycloheximide (CHX) for 24 h (Ni et al. 1994) ($n = 8$). Cells were pretreated for 1 h with TRO40303 or the same concentration of DMSO (0.1%) ($n = 8$). Each plate contained eight positive control wells used to assess maximum hepatocyte number and viability when exposed to DMSO only, and eight negative control wells treated by DMSO and intoxicated with Jo2 and CHX.

Cell viability measurement using calcein

PMEH were incubated with 2 μ g/mL calcein-AM for 30 min at 37°C and residual fluorescence in the medium was quenched by addition of 6.6 mg/mL hemoglobin. Automatic fluorescent image acquisitions of all living cells in individual wells of a 96-wells plate were performed using the Plate-Runner[®] (Trophos, Marseille, France) with 40 msec exposition time at 520 nm. Cell survival was quantified in each well using dedicated software (Tina V4.9[®]; Trophos, Marseille, France) in global fluorescence mode.

Cell mortality assessment by LDH release

LDH release was measured using the cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Optical readings were performed by spectrophotometry (μ Quant; Biotek, Winooski, VT) at 492 nm for the formazan prod-

uct generated and 690 nm for background and the arithmetic difference between readings at 492 and 690 was calculated.

In vivo hepatotoxicity model in mice

The Jo2 antibody was administered i.p. at various lethal or sublethal doses, depending on the objective of the experiment. TRO40303 was administered by i.p., p.o., or i.v. routes. Control mice received the same amount of the vehicle.

Twenty mice per group were used in each experiment group aside from the establishment of a survival curve where 10 mice per group were used.

In sublethal dose experiments (Jo2, 125 $\mu\text{g}/\text{kg}$ i.p.), blood was taken 24 h after intoxication from the ophthalmic venous plexus into lithium heparin tubes and kept on ice until plasma was prepared by centrifugation (1500g, 10 min, 4°C) and stored at -20°C. ALAT activity was assayed in plasma by Idexx Laboratories (Eragny sur Oise, France).

In lethal doses experiments (Jo2 175, 200, and 250 $\mu\text{g}/\text{kg}$ i.p.), mortality was assessed either by counting the number of surviving animals 24 h post-Jo2 antibody injection or by tracking mouse movements that were recorded every 30 min over a 24 h period using a video system (Retiga 1300; Q Imaging, Surrey, Canada). Mice that did not move between two consecutive images (at time T and $T + 30$ min) were considered dead.

Pharmacokinetics of TRO40303

Blood samples for TRO40303 analysis were taken from mice administered with 30 mg/kg i.p. or 300 mg/kg p.o. Blood was drawn from the ophthalmic venous plexus under isoflurane gas anesthesia 1, 2, 4, 8, and 24 h after drug administration (one mouse per time point). Anesthesia was verified by checking that no reaction occurred after pinching the toes. The blood samples were cooled on ice and plasma samples were prepared within 60 min of sampling by centrifugation for 10 min at 1500g, under refrigeration (+4°C) and stored at -20°C until analysis. Quantification of TRO40303 in plasma samples was performed by liquid chromatography-mass spectrometry.

Subcellular fractionation

Cytosolic fractions were prepared from liver tissue collected after 24 h Jo2 intoxication. Livers were perfused with saline before removal. They were conserved on ice and minced into small pieces before deep freezing in liquid nitrogen and conserved at -80°C before thawing on ice to prepare homogenates. Tissue from livers were suspended in 500 μL of buffer A (20 mmol/L Hepes pH

7.5, 210 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L MgCl_2 , 1 mmol/L EDTA, 1 mmol/L DTT and 1 mmol/L PMSF [added extemporaneously]) and homogenized with 20 strokes of a dounce homogenizer (pestle B) to prepare the lysate. After two cycles of centrifugations for 10 min at 750g at 4°C, the resulting supernatant was then centrifuged first for 10 min at 10,000g at 4°C and then for 1 h at 100,000g at 4°C. The resulting supernatant containing the cytosolic fraction was then frozen and conserved at -20°C before measurement of protein concentration using BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL) and Western blotting.

Western blotting

Hepatic extracts containing 60 μg protein were subjected to SDS-PAGE and then transferred on polyvinylidene difluoride membranes (Pierce, Rockford, IL). Membranes were blocked with 5% skimmed dry milk in a Tris buffer (10 mmol/L Tris, 100 mmol/L NaCl, pH 7.5) containing 0.05% Tween-20 overnight at 4°C under gentle stirring. Subsequently, membranes were exposed for 1 h to either cleaved caspase 3 antibody (rabbit polyclonal from Cell signaling, Danvers, Massachusetts), cleaved caspase 7 antibody (rabbit polyclonal from Cell signaling, Danvers, MA), cytochrome C (Cyt C) (mouse monoclonal from Sigma-Aldrich), or actin antibody (mouse monoclonal from Sigma-Aldrich) used as a loading control after stripping the membranes. After incubation with horseradish peroxidase coupled goat anti-rabbit or goat anti-mouse immunoglobulins (Santa Cruz Biotechnology, Dallas, TX), proteins were revealed by enhanced chemiluminescence reaction (GE Healthcare, Little Chalfont, United Kingdom) after exposure to X-ray films (GE Healthcare).

Data analysis

The data are reported as means \pm SEM. Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by Dunnett's test when multiple groups were compared and by unpaired t -test when two groups were compared. In vivo, effect on final survival (number of surviving mice 24 h after intoxication) was evaluated by Fisher test and survival curves were compared by using log-rank test. Significance was accepted when $P < 0.05$.

Informed consent

All appropriate steps have been taken in obtaining informed consent of any and all human subjects participating in the research comprising the manuscript submitted for review and possible publication.

Results

TRO40303 protects hepatocytes in vitro hepatotoxicity models

Palmitate intoxication of PMEH or human HepG2 cells has been proposed as a model of hepatic steatosis (Gomez-Lechon et al. 2007; Srivastava and Chan 2007). Both cell models were used to evaluate the hepatoprotective effects of TRO40303 in vitro against palmitate intoxication in a first set of experiments. The effect of palmitate was analyzed by looking at PMEH cell survival using the calcein assay using the Plate-Runner[®] in the Global fluorescent mode, whereas HepG2 cell death was analyzed using LDH release assessment. TRO40303 at doses from 0.3 to 3 $\mu\text{mol/L}$ protected both PMEH and human HepG2 cells intoxicated with palmitate (Fig. 1A and B).

In a second set of experiments, a more general model of inflammation-induced fulminant hepatitis was performed by treating PMEH with anti-Fas antibody Jo2 in the presence of CHX. Cell survival was assessed using the calcein assay. TRO40303 at doses from 0.3 to 3 $\mu\text{mol/L}$ protected PMEH intoxicated with Jo2 (Fig. 1C).

These experiments provided evidence of TRO40303's potential hepatoprotective effects, which could be useful for treating AH, ALF, and steatohepatitis.

TRO40303 pretreatment reduces Fas-induced hepatotoxicity in mice, a model of acute (fulminant) hepatitis

Since TRO40303 was able to protect PMEH from Fas-induced hepatotoxicity in vitro, we evaluated its effects in

mice subjected to sublethal and lethal treatment with Jo2 anti-Fas antibody. TRO40303 has been developed to allow administration as a single i.v. bolus in acute, emergency indications (The MITOCARE Study Group 2012). For initial studies of the in vivo hepatoprotective potential of TRO40303, the compound was administered to mice via the i.p. route 1 h before Jo2 intoxication. The i.p. route was chosen for practical reasons in the mouse model, knowing that the pharmacokinetic profile of the compound (Fig. 2A) was very similar to what was found with i.v. administration in previous studies (Le Lamer et al. 2014). In a second stage, TRO40303 was administered by i.v.

Using a sublethal dose of Jo2 antibody (125 $\mu\text{g/kg}$), pretreatment with TRO40303 was able to significantly reduce ALAT levels in plasma measured 24 h postintoxication at the doses of 10 and 30 mg/kg administered by i.p. route (ALAT levels reduced by 80%, $P < 0.01$; Fig. 2B), whereas the dose of 3 mg/kg was not effective.

Further studies to investigate the effects of TRO40303 on Fas-induced mortality were performed using the effective doses of TRO40303 found in the "sub-lethal study" (i.e., 10 and 30 mg/kg, i.p.). A dose of 250 $\mu\text{g/kg}$ Jo2 antibody resulted in about 80% mortality, and 1 h pretreatment with 10 or 30 mg/kg TRO40303 significantly reduced mortality by about 50 and 90%, respectively (Fig. 2C and Table 1). A video tracking system was used to study the time course of mortality and protective effect of 30 mg/kg TRO40303 administered 1 h prior to injecting 200 $\mu\text{g/kg}$ Jo2 antibody, a dose resulting in 70% mortality at 24 h; TRO40303 pretreatment significantly reduced mortality in this study (Fig. 2D).

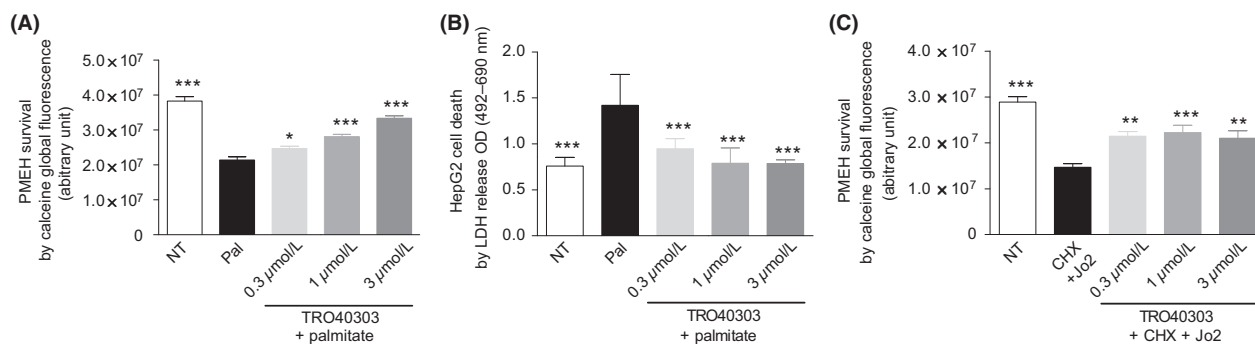


Figure 1. TRO40303 provides protection in hepatocyte intoxication models in vitro. Primary mouse embryonic hepatocytes (PMEH) were isolated as described in material and methods section. The cells (PMEH or HepG2) were pretreated for 1 h with compounds at a final constant DMSO concentration (0.1%) ($n = 8$ for all conditions), then intoxicated in the presence of compounds as follows. For palmitate intoxication, PMEH were intoxicated for 24 h at 0.2 mmol/L and viability was assessed using calcein staining (A), whereas HepG2 were subjected to 48 h 0.2 mmol/L palmitate intoxication for LDH cell death assessment (B). For anti-Fas antibody intoxication, PMEH were intoxicated for 24 h with 100 $\mu\text{g/mL}$ Jo2 and 2 $\mu\text{g/mL}$ CHX and viability was assessed using calcein staining (C). Results are presented as mean \pm SEM and statistical analysis was performed by one-way analysis of variance followed by Dunnett's posttest compared to the toxic control (** $P < 0.01$, *** $P < 0.001$). Survival of control, nontreated cells (NT), except for DMSO, is shown for comparison. Data shown are for a representative example of three independent experiments performed using three different cell culture preparation. CHX, cycloheximide; DMSO, dimethyl sulfoxide.

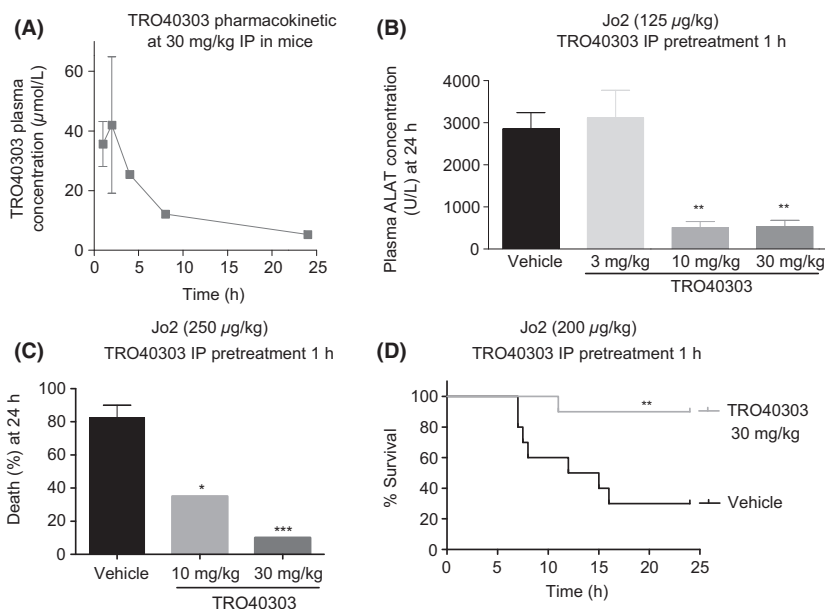


Figure 2. Pretreatment with TRO40303 protects mice from Jo2 intoxication. (A) TRO40303 was administered i.p. to mice at 30 mg/kg in CES. Blood samples ($n = 3$ mice per time point) were taken at 1, 2, 4, 8, and 24 h after drug administration and quantification of TRO40303 in plasma samples was performed by LC-MS-MS to analyze the pharmacokinetic profile of the compound. Results are presented as mean \pm SEM. (B) The dose of 125 $\mu\text{g}/\text{kg}$ Jo2 antibody was administered i.p. to mice 1 h after TRO40303 treatment (i.p. doses of 3, 10 and 30 mg/kg in CES compared to vehicle). ALAT activity was assayed in plasma 24 h postintoxication. Results are presented as mean \pm SEM and statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's posttest compared to vehicle (** $P < 0.01$). (C) The dose of 250 $\mu\text{g}/\text{kg}$ Jo2 antibody was administered i.p. to mice 1 h after TRO40303 treatment (i.p. doses of 10 and 30 mg/kg in CES compared to vehicle). Mortality was assessed by counting the number of surviving animals 24 h after intoxication. Results are presented in percent of initial mice surviving ($n = 20$ per TRO40303-treated group and $n = 40$ for vehicle) and statistical analysis was performed using Fisher test ($P = 0.024$ and $P < 0.0001$ for the 10 and 30 mg/kg doses, respectively). TRO40303 at 10 and 30 mg/kg improved viability of the mice by 47% and 90%, respectively. Results are presented as mean \pm SEM for the vehicle group (mean of two experiments) and statistical analysis was performed by one-way ANOVA followed by Dunnett's posttest compared to vehicle (* $P < 0.05$, *** $P < 0.001$). (D) The dose of 200 $\mu\text{g}/\text{kg}$ Jo2 antibody was administered i.v. to mice 1 h after TRO40303 treatment (30 mg/kg i.p. in CES compared to vehicle). The survival plot was established by video tracking for 24 h, starting just after intoxication. Results are presented in percent of initial mice surviving ($n = 10$ per group) and statistical analysis was performed using log-rank Mandel-Cox test (** $P < 0.01$). i.p., intraperitoneally; CES, cremophor EL/ethanol/saline; i.v., intravenous.

In order to further investigate the mode of protection afforded by TRO40303 in the Fas-intoxicated mice, additional experiments were performed to evaluate TRO40303 efficacy when administered p.o. using the sublethal dose of Jo2 of 125 $\mu\text{g}/\text{kg}$. In these experiments, 300 mg/kg TRO40303 was administered 4 h before intoxication, as the plasma concentration at this time, is similar to what was found 2 h after administering 30 mg/kg i.p. (Fig. 3A).

Using this protocol, alteration of hepatic damage was assessed by measuring plasma ALAT levels. Despite a high interindividual variability in this biomarker, oral administration of TRO40303 significantly reduced the level of ALAT in plasma after 24 h Jo2 antibody intoxication (Fig. 3B, 1937 ± 349 U/L versus 642 ± 172 U/L in vehicle and TRO40303 groups, respectively; $P = 0.0025$).

In this experiment, markers of apoptosis in liver cytosolic fractions were evaluated by western blot using antibodies for cleaved caspase 3 or 7 as well as Cyt C. This

analysis showed that oral pretreatment with TRO40303 greatly reduced the amount of activated caspase 3 and 7 as well as the amount of Cyt C released from mitochondria to the cytosol (Fig. 3C).

Additional experiments were performed with i.v. administration of TRO40303 in a liposomal formulation developed for clinical use. Doses of 1 and 3 mg/kg of TRO40303 were given by i.v. 15 min prior to injecting 200 $\mu\text{g}/\text{kg}$ Jo2 antibody, a dose resulting in 90% mortality at 24 h. Treatment with 3 mg/kg TRO40303 significantly reduced mortality by 67% ($P = 0.0002$) whereas the dose of 1 mg/kg was not effective (Table 1). TRO40303 peak plasma concentration at 15 min after i.v. administration was 14.9 and 61.2 $\mu\text{mol}/\text{L}$ for the 1 and 3 mg/kg doses, respectively, confirming that a plasma concentration of 40 $\mu\text{mol}/\text{L}$ or higher at the time of intoxication provides protection as observed following administration using the i.p. and p.o. routes in this severe hepatic intoxication model.

Table 1. Mortality reduction by TRO40303 using various doses, routes, and time of administration in mice intoxicated with Jo2 antibody.

Jo2 ($\mu\text{g}/\text{kg}$)	Protocol	TRO40303 dose (mg/kg)	Death		Mortality		Mortality reduction	Fisher test	P value
			Vehicle	TRO40303	Vehicle	TRO40303			
175	Posttreatment 1 h	30 i.p.	8/20	0/20	40%	0%	↓ 100%	**	0.003
175	Posttreatment 2 h	30 i.p.	7/20	5/20	35%	25%	↓ 29%	NS	0.731
200	Pretreatment 1 h	30 i.p.	7/10	1/10	70%	10%	↓ 86%	*	0.010
200	Pretreatment 15 min	1 i.v.	18/20	18/20	90%	90%	↓ 0%	NS	1
200	Pretreatment 15 min	3 i.v.	18/20	6/20	90%	30%	↓ 67%	***	0.0002
200	Posttreatment 1 h	10 i.p.	14/20	8/20	70%	40%	↓ 43%	NS	0.111
200	Posttreatment 1 h	30 i.p.	16/20	8/20	80%	40%	↓ 50%	*	0.022
200	Posttreatment 2 h	30 i.p.	15/20	13/20	75%	65%	↓ 13%	NS	0.731
250	Pretreatment 1 h	10 i.p.	15/20	7/20	75%	35%	↓ 53%	*	0.024
250	Pretreatment 1 h	30 i.p.	18/20	2/20	90%	10%	↓ 89%	****	<0.0001
250	Posttreatment 1 h	10 i.p.	18/20	17/20	90%	85%	↓ 5.5%	NS	1
250	Posttreatment 1 h	30 i.p.	19/20	13/20	95%	65%	↓ 32%	*	0.043

i.p., intraperitoneally; i.v., intravenous.

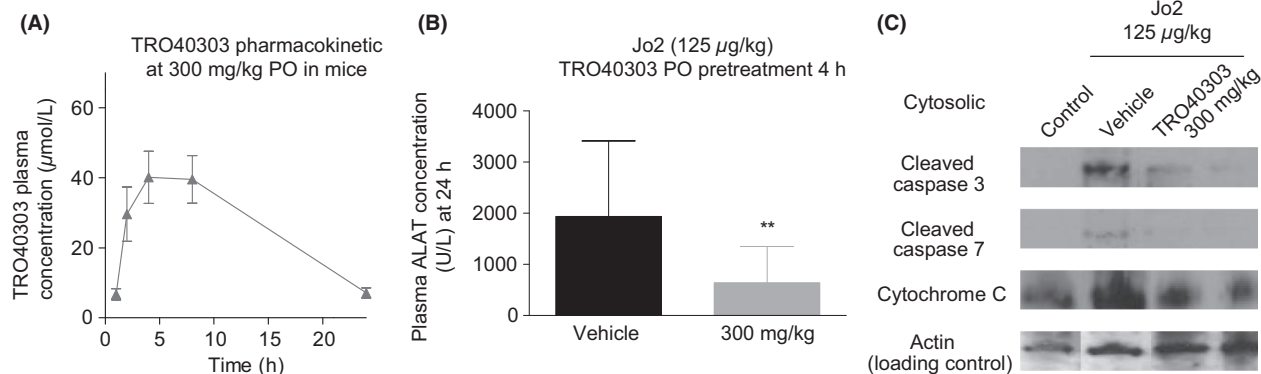


Figure 3. TRO40303 reduces apoptosis in mice intoxicated with Jo2. (A) TRO40303 was administered p.o. to mice at 300 mg/kg in olive oil. Blood samples ($n = 3$ mice per time point) were taken at 1, 2, 4, 8, and 24 h after drug administration and quantification of TRO40303 in plasma samples was performed by LC-MS-MS to analyze the pharmacokinetic profile of the compound. (B) The dose of 125 $\mu\text{g}/\text{kg}$ Jo2 antibody was administered i.p. to mice 4 h after TRO40303 treatment (p.o. dose of 300 mg/kg in olive oil compared to vehicle). ALAT activity was assayed in plasma 24 h postintoxication. Results are presented as mean \pm SEM and statistical analysis was performed by t -test (** $P < 0.01$). (C) Western blot analysis of cleaved caspase 3, cleaved caspase 7, and cytochrome C on 60 μg of cytosolic liver extracts from mice treated as described in B with 125 $\mu\text{g}/\text{kg}$ Jo2 \pm 300 mg/kg TRO40303 p.o. compared to cytosolic liver extracts prepared from a control untreated mouse. Western blot actin levels were used as a loading control ensuring that the same amount of protein is loaded in each lane. p.o., per os.

TRO40303 reduces mortality as a curative treatment in the mouse model of acute (fulminant) hepatitis

In order to further assess the potential of TRO40303 as a treatment for ALF, it was of utmost importance to assess if the protection afforded by TRO40303 in the mouse model would also be effective as a posttreatment in a curative manner.

Increasing doses of Jo2 antibody (175, 200, and 250 $\mu\text{g}/\text{kg}$) were given by i.p. to mice leading to increasing cell death from 37.5% to 75% and 92.5%, respectively in vehicle-treated mice. TRO40303 administered i.p. at

the dose of 30 mg/kg 1 h post-Jo2 intoxication was able to significantly reduce mortality (Fig. 4 and Table 1). The dose of 10 mg/kg of TRO40303 was also able to slightly reduce mortality when doses of 200 and 250 $\mu\text{g}/\text{kg}$ of Jo2 antibody were used, although the effect was not statistically significant (Table 1). Further studying the effect of 30 mg/kg TRO40303 as a posttreatment 2 h after administering 175 or 200 $\mu\text{g}/\text{kg}$ Jo2 antibody showed that delaying treatment further is no longer protective (Fig. 4 and Table 1).

In conclusion, TRO40303 was able to provide curative protection in a model of AH in mice in a dose-related manner. The beneficial effect was also related to the dose

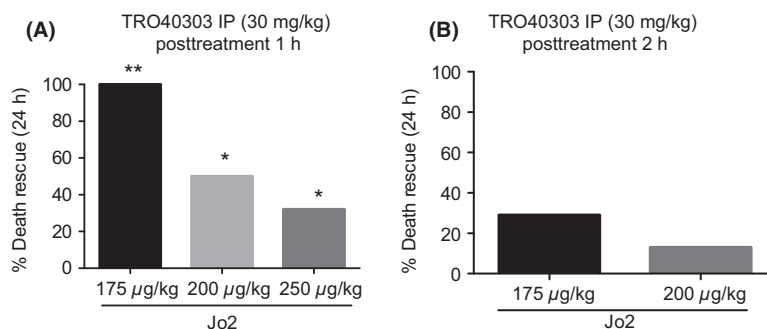


Figure 4. Posttreatment with TRO40303 protects mice from Jo2 intoxication. The dose of 175, 200, and 250 $\mu\text{g}/\text{kg}$ Jo2 antibody was administered i.p. to mice 1 h before TRO40303 treatment (i.p. dose 30 mg/kg) (A). The dose of 175 and 200 $\mu\text{g}/\text{kg}$ Jo2 antibody was administered i.p. to mice 2 h before TRO40303 treatment (i.p. dose 30 mg/kg) (B). Mortality was assessed by counting the number of surviving animals 24 h after intoxication in comparison with vehicle (CES). Percent of death rescue in the TRO40303 group compared to the vehicle group is calculated at each dose and time and presented in the bar graphs. Statistical analysis was performed for each TRO40303 group compared to its vehicle group at each dose and time, using a Fisher test on final survival (number of surviving mice 24 h after intoxication) (* $P < 0.05$, ** $P < 0.01$). Animal numbers, survival per group is given in Table 1. CES, cremophor EL/ethanol/saline.

and timing of Jo2 antibody administration, that is, the severity or extent of hepatitis damage induced by Jo2. TRO40303 was effective at lower doses when Jo2-induced damage was less severe and when administered soon after the intoxication. The most efficient protection (100% reduction in mortality) was obtained when 30 mg/kg TRO40303 was given i.p. 1 h postintoxication with 175 $\mu\text{g}/\text{kg}$ of Jo2 antibody.

Discussion

TRO40303 is a cytoprotective compound that has been extensively studied in models of cardiotoxicity (Schaller et al. 2010; Le Lamer et al. 2014). Here, we demonstrate that this mitochondrial-targeted compound is also active in hepatotoxicity models, validating its use to prevent various types of stress-induced mitochondrial permeabilization leading to cell death. In vitro, TRO40303 was protective in both human-derived and mouse hepatocyte models mimicking NASH and further explored in a murine model of AH. In vivo, the AH model induced with Jo2 anti-Fas antibody further demonstrated the potent protection provided by TRO40303 to both prevent and cure hepatotoxicity as shown by the reduction in plasma ALAT levels as well as in mortality induced by Jo2 antibody. These results support a new therapeutic potential for TRO40303 as a treatment for AH.

The Jo2-induced Fas-mediated hepatotoxicity model mimics a range of acute and chronic liver diseases (Guicciardi and Gores 2005). This initial investigation was oriented to investigate the dose, routes of administration, the therapeutic window and the mode of action of TRO40303 to provide hepatoprotection. These results allow further investigations of TRO40303 in other more

specific preclinical models and eventual clinical investigations in acute or chronic forms of hepatitis.

Mitochondria have been shown to play a major role in hepatotoxicity both in steatohepatitis (Pessayre 2007) and AH (Feldmann et al. 2000). Excessive apoptosis and oxidative stress are indeed the mechanisms targeted by compounds tested in hepatitis clinical trials: IDN-6556 (Pockros et al. 2007) and HIP/PAP (Moniaux et al. 2012). TRO40303 targets the mitochondria and provides cytoprotection from stressed cells by inhibiting mitochondrial permeability transition and reducing oxidative stress as demonstrated in cardiac cells (Schaller et al. 2010; Le Lamer et al. 2014). This provided a good rationale for the compound to be protective against AH and thus against ALF.

The mode of protection afforded by TRO40303 in hepatocytes was confirmed to be related to the reduction in Cyt C release from the mitochondria to the cytosol after Jo2 intoxication in mice, further reducing caspase 3 and 7 activation. Indeed, Fas signaling can induce apoptosis via either extrinsic or intrinsic death pathways leading to mitochondrial permeabilization triggered by BH3 proteins. TRO40303's activity in hepatoprotection models further validates the proposed mechanism of action of TRO40303 in cardiotoxicity models that involve mitochondria permeabilization and mPTP-triggered apoptosis (Schaller et al. 2010; Le Lamer et al. 2014).

The efficacy of TRO40303 was related to the dose of Jo2 used and the timing of administration. It was efficient by different routes of administration with pretreatment times corresponding to peak plasma concentrations: 4 h after 300 mg/kg p.o., 1 h after 10 or 30 mg/kg i.p., and 15 min after 3 mg/kg I. The dose of 30 mg/kg TRO40303 i.p. was also protective with posttreatment administration

1 h after Jo2 intoxication but protection was lost when the compound administration 2 h postintoxication. When comparing TRO40303 plasma exposure with efficacious doses via these routes of administration, it can be concluded that a plasma level of 40 $\mu\text{mol/L}$ or higher at the time of intoxication provides protection. Indeed, 40 $\mu\text{mol/L}$ was the maximal TRO40303 plasma concentration in the i.p. and p.o. experiments at the start of Jo2 intoxication. However, previous studies have shown that TRO40303 accumulates in liver (Schaller et al. 2010), therefore it is possible that lower plasma concentrations sustained over 24 h would also be effective. Adapting formulations or repeated dosing to provide more sustained plasma levels and evaluating tissue accumulation in future studies could explore this possibility.

It must be noted that the Jo2 antibody induces rapid and severe hepatotoxicity (mice die within 24 h following intoxication) and that in patients, AH can last over several days. Because TRO40303 was still effective when administered 1 h after Jo2 intoxication it is possible that TRO40303 could be administered after onset of AH and prevent progression to AHF; this would probably have to be studied a clinical trial. For such further development for AH or ALF, in order to maintain TRO40303 levels, i.v. infusion or repeated i.v. administrations could be investigated using an available liposomal formulation (Le Lamer et al. 2014). Additionally, TRO40303 could also be investigated in other models of chronic hepatitis such as steatohepatitis and in particular nonalcoholic forms based on the preliminary positive in vitro results obtained in the palmitate intoxication model. For clinical development to treat this or other chronic liver diseases, oral TRO40303 administration would be preferred and this would require developing an oral formulation.

In conclusion, by preventing mitochondrial permeabilization, TRO40303 has the potential to provide hepatoprotection and treat acute and chronic liver diseases. Indeed, this compound could be beneficial in many other clinical indications involving activated mitochondrial permeabilization, with or without the participation of a specific mPTP.

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Author Contributions

Schaller, Michaud, Clémançon de Bellefois, and Pruss participated in research design. Latyszenok, Robert, Hocine, Arnoux, Gabriac, Codoul, Bourhane, and Clémançon de Bellefois conducted experiments. Schaller, Michaud, Clémançon de Bellefois, and Afxantidis performed data analysis. Schaller, Michaud, and Pruss contributed to the writing of the manuscript.

Disclosures

None declared.

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