Targeting neonatal ischemic brain injury with a pentapeptide-based irreversible caspase inhibitor

D Chauvier1,2, S Renolleau3,4,5, S Holifanjainaia6,7, S Ankri1,2, M Bazault1,2, L Schwendimann6,7, C Rousset6,9, R Casimir1,2,10, J Hoebeke10, M Smirnova1,2, G Debroe11,12, A-P Trichet2, Y Carlsson13,14, X Wang9, E Bernard2, M Hébert2, J-M Rauzier14,15, S Matecki16,17, A Lacampagne15,17, P Rustin6,7,13, H Hagberg8,9,13, P Gressens6,7,8, C Charriaut-Marlangue3,4 and E Jacotot1,2,6,7,8

Brain protection of the newborn remains a challenging priority and represents a totally unmet medical need. Pharmacological inhibition of caspses appears as a promising strategy for neuroprotection. In a translational perspective, we have developed a pentapeptide-based caspase inhibitor, TRP601/OPRHA133563, which reaches the brain, and inhibits caspases activation, mitochondrial release of cytochrome c, and apoptosis in vivo. Single administration of TRP601 protects newborn rodent brain against excitotoxicity, hypoxia–ischemia, and perinatal arterial stroke with a 6-h therapeutic time window, and has no adverse effects on physiological parameters. Safety pharmacology investigations, and toxicology studies in rodent and canine neonates, suggest that TRP601 is a lead compound for further drug development to treat ischemic brain damage in human newborns.

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Worldwide estimations indicate more than 2 million non-infectious neonatal brain injuries per year.1 Neonatal ischemic brain injuries, such as stroke (focal cerebral ischemia involving middle cerebral artery occlusion (MCAO)) or hypoxia–ischemia (HI) (involving systemic asphyxia), are major and untreated causes of newborn morbidity and mortality.2,3 Of the infants who survive the first few hours after intrapartum-related neonatal deaths (previously called ‘birth asphyxia’), as many as an annual 1 million may develop cerebral palsy, learning difficulties or other disabilities.4 Recent clinical trials show that therapeutic intervention by brain cooling beginning up to 6 h after perinatal asphyxia reduces cerebral injury and improves outcomes in term infants.5 Although these studies provide proof of concept that in this context cell death is both delayed and preventable, the protection is limited and there is still no treatment available for perinatal stroke or brain injury occurring in preterm infants.6

Ischemic brain injury in the developing brain involves several factors such as excitotoxicity, oxidative stress, and inflammation, which accelerate cell death through either apoptosis or necrosis, depending on the region of the brain affected and on the severity of the insult.7,8 Accumulating data suggest that apoptotic mechanisms have a more prominent role in the evolution of ischemic brain injury in neonatal rodents9–11 and humans12 than in adult brain ischemia,13,14 and that apoptosis involves the mitochondrial release of cytochrome c11 and apoptosis-inducing factor (AIF),11,15,16 which activate caspase-dependent10,16 and -independent execution pathways,13,16 respectively.

Caspases are a class of cysteine endoproteases that have strict specificity for an aspartic residue at the S1 subsite.17,18 Some caspases are important mediators of inflammation and others are involved in the apoptosis of mammalian cells, where they participate in signaling and effector pathways.19 On the basis of the optimal four-amino-acid sequence to the left of the cleavage site, caspases may be classified into three groups: group I contains caspase-1, -4, and -5 (optimal tetrapeptide: D[DE/AD]X), group II contains caspase-2 (Casp2), -3 (Casp3), and -7 (Casp7) (optimal tetrapeptide: D[DE/AD]XD), and group III contains caspase-6, -8 (Casp8), -9 (Casp9), and -10 (optimal tetrapeptide: [V/L]EXD).20–22

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Both pancaspase inhibition and Casp3-selective inhibition have been reported to be neuroprotective in various rodent models of neonatal brain injury,\textsuperscript{13,23,24} opening the possibility for pharmacological intervention.\textsuperscript{6} However, lack of protection with caspase inhibitors was also reported\textsuperscript{25,26} possibly reflecting differences between experimental models or settings (e.g., the age of animals\textsuperscript{26}), specific in vivo properties of the used inhibitors (e.g., brain penetration\textsuperscript{27}), and/or a shift to caspase-independent cell death pathways (e.g., AIF, autophagic death, necroptosis).

The role of individual caspases in the developing brain is not fully understood. Genetic analysis using constitutive deficiency revealed that Casp3 and Casp9 execute programmed (physiological) cell death in the central nervous system,\textsuperscript{28,29} whereas Casp2 does not.\textsuperscript{30} Aggravation of HI-induced lesions was reported in Casp3-null mice.\textsuperscript{31} In contrast, genetic inhibition of Casp2 is neuroprotective in newborn mice exposed to HI or excitotoxic challenges.\textsuperscript{32}

In a translational attempt to generate an efficient and safe Casp2/group-II caspase inhibitor, we have developed a potent pentapeptide-based irreversible caspase inhibitor. We report here the preclinical evaluation of this compound and present data supporting a potent neuroprotective role against perinatal ischemic brain damage in a variety of in vivo models, potentially opening an avenue for treatment.

Results

Design of a caspase inhibitor adapted for neuroprotection in neonates. We previously showed that the pancaspase inhibitor quinolyl-carbonyl-Val-Asp-diffluorophenoxymethylketone (Q-VD-OPh) has enhanced \textit{in vitro} and \textit{in vivo} pharmacological properties,\textsuperscript{33} together with potent neuroprotective effects in neonatal brain injury experimental models.\textsuperscript{10,16,34} We reasoned that an \textit{in vivo} efficient group II-selective caspase inhibitor might combine an amino-terminal quinolyl-carbonyl and a C-terminal fluorophenoxymethylketone warhead (\textit{CH}_{2}OC_{6}H_{5}F_{2}) with the Casp2-preferred pancaspase backbone VDVA\textit{D},\textsuperscript{20,33,35,36} a sequence that is also efficient as a substrate for Casp3,\textsuperscript{37} but is a weaker substrate for group-I and -III caspases (data not shown and McStay \textit{et al.}).\textsuperscript{37} Using the reported Casp2 crystallographic data,\textsuperscript{38} we modeled pentapeptide structures within the Casp2 active site (Figure 1a and Supplementary Figure S1), and chose to add methyl ester group to the lateral chain of each Asp residues to enhance lipophilicity (a well-known approach to promote blood–brain barrier penetration) and delay degradation by proteases \textit{in vivo}. The resulting substituted pancaspette quinolin-2-carboxyl-VD(Ome)VAD (OMe)-CH\textsubscript{2}-O(2,6F\textsubscript{2})Ph (referred thereafter as TRP601) was selected (Figure 1b). TRP601 is an irreversible caspase inhibitor that, similar to other VDVA\textit{D}-based inhibitors,\textsuperscript{20,21,37} is a potent inhibitor of group II caspases. Indeed, \textit{in vitro} kinetic analysis showed that TRP601 potently inhibits Casp3 (IC\textsubscript{50}/Casp3/TRP601 = 47.3 ± 11.2 nM; k\textsubscript{i} = 36.025 M/s; n = 3) and also inhibits recombinant Casp2 (IC\textsubscript{50}/Casp2/TRP601 = 479.8 ± 79.3 nM; k\textsubscript{i}/K = 1243/M/s; n = 3) (Figures 1c and d). According to its irreversible nature, TRP601 showed a fourfold IC\textsubscript{50} reduction if preincubated 45 min with Casp2 before substrate addition (IC\textsubscript{50}/Casp2/TRP601/45 min = 127 ± 25 nM; n = 3).

As expected, TRP601 had no substantial activity outside the caspase family, as found in a binding screen of 110 different receptors, transporters and ion channels, and 56 enzyme activity assays, including calpains, various cathepsins (B, H, G, L), granzyme B, and several glutamatergic sites such as N-methyl-D-aspartic acid and \textit{x}-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (Table 1). When used in cell culture assays like serum-deprived primary embryonic cortical neurons,\textsuperscript{39} TRP601 prevented caspase activation and cell death (Figure 1e). Following intravenous (i.v.) administration in adult rats, TRP601 quickly entered the brain (Figure 1f; \textit{T}_{\text{max}} = 25 min in the brain; \textit{C}_{\text{max}} = 120 ng/ml in the brain, 1 mg/kg, i.v. bolus dose). Interestingly, an active metabolite \textit{D}2Me-TRP601 (TRP601 devoid of methyl ester

**Figure 1** Design and pharmacological evaluation of TRP601. (a) Substrate binding region of caspase-2 (Casp2) in complex with TRP601. The quinolin-2-carboxyl-Val-Asp(Ome)-Val-Ala-Asp(Ome)-CH\textsubscript{2} moiety is shown in sticks representation with the atoms represented as follows: gray = carbon; white = hydrogen; blue = nitrogen; and red = oxygen. The –C–covalent bond between the Cys-155 residue and the C terminus CH\textsubscript{2} of TRP601 is represented in yellow. The electric interactions (hydrogen bonds and salt bridges) are represented by the dashed green lines. The enzyme residues that interact with the inhibitor are shown by the wireframe representation. Right panel: the table shows the minimal energy (kcal/mol, \textit{E}_{\text{min}}) of the Casp2–TRP601 complex resulting from electric or Coulombic component (\textit{E}_{\text{c}}) together with repulsion–attraction component (\textit{E}_{\text{r}}). The interaction energy of the Casp2–TRP601 complex is –147.28 kcal/mol. Owing to the –C–covalent bond, the Asp residue in P1 contributes to about 30% of the interaction energy. The Asp in P4 and the Val in P5 have important non-covalent contributions to stabilization of the complex, 21% and 17%, respectively. Each of the other two residues (Ala in P2 and Val in P3) contributes to about 12% of the interaction energy. (b) Structure of TRP601. (c) Representative dose–response curve of human recombinant Casp2 and Casp3 inhibition by TRP601. Initial velocities were determined from standard colorimetric microplate assays. (d) Kinetic off rate (\textit{k}/\textit{K}) parameters of irreversible caspase inhibitors on Casp2 and Casp3. (e) TRP601 inhibits neuronal caspase activities and prevents serum deprivation (SD)-induced cell death. High-density E14 cortical neuron cultures were subjected to 24 h SD in the presence or absence of 50 \textmu M TRP601. Histograms indicate the means (± S.D.) of 15 independent experiments. (f) Representative pharmacokinetic of TRP601 after intravenous (i.v.) administration in adult rats, through liquid chromatography-mass spectrometry (LC/MS) detection in the plasma and brain homogenates. Note that following intraperitoneal (i.p.) administration of the same dose, TRP601 was detected in the brain at 0.25 h (brain \textit{C}_{\text{max}} = 25 ng/ml) and the Cmax (50 ng/ml) was not achieved in the plasma according to a plateau between 0.5 and 2 h. (g–j) TRP601 reduces excitotoxic lesions in neonates. The 5-day-old mice were subjected to intracerebral luteolate injection and killed at different time points (g = 5 days; h = 24 h; i = 4 h) following the excitotoxic challenge to determine the impact of TRP601, TRP601, and TRP901 (1 mg/kg; i.p.) on lesion severity (g), microgliosis (h), astroglisis (i), and group II caspases activity (j). Histograms show mean lesion volume (g), vehicle, \textit{n} = 16; TRP601, \textit{n} = 16; TRP901, \textit{n} = 7; TRP901, \textit{n} = 8), cell density (h and i; \textit{n} = 10 per group), or VDVA\textit{D}ase activity (j; \textit{n} = 5 per group): ± S.E.M. Asterisks indicate differences from control (\textit{P} < 0.05, \textit{P} < 0.01, \textit{P} < 0.001 in Kruskal–Wallis post hoc Dunn’s for \textit{g}, Mann–Whitney for \textit{h–j}). (k) TRP601 does not enhance protection conferred by short interfering RNA (siRNA)-mediated genetic inhibition of Casp2. The 5-day-old mice were subjected to intracerebral injection (as in e) of either an siRNA against Casp2 (si2-a) or a control siRNA (si2Co), as indicated. After 24 h, ibotenate was administered (intracerebroventricular (i.c.v.), followed immediately by vehicle (\textit{i} and \textit{j}, \textit{n} = 20, \textit{k}, \textit{n} = 20) or TRP601 (\textit{i}, \textit{j}, \textit{n} = 24) administration (i.p.). See Supplementary Table 1 for exact values and detailed statistical analysis.
Table 1: Molecular parameters.

| Fragment                  | $E_{\text{min}}$ (Kcal/mol) | $E_{\text{vw}}$ (Kcal/mol) | $E_{\text{c}}$ (Kcal/mol) |
|---------------------------|-------------------------------|-------------------------------|----------------------------|
| P1 Asp and C-S covalent  | -43.86                        | -17.78                        | -26.08                     |
| P2 Ala                    | -17.31                        | -6.40                         | -10.91                     |
| P3 Val                    | -17.16                        | -7.95                         | -9.21                      |
| P4 Asp                    | -30.82                        | -11.74                        | -19.09                     |
| P5 Val                    | -25.28                        | -8.52                         | -16.76                     |
| quinolin-2-carbonyl       | -12.84                        | -6.73                         | -6.11                      |
| Sum                       | -147.28                       | -59.11                        | -88.17                     |

Figures:

a) A 3D molecular model of TRP601 and Δ2Me-TRP601.
b) Chemical structures of TRP601 and Δ2Me-TRP601.
c) Initial velocity (pmoles.min$^{-1}$) of Casp2 and Casp3.
d) $IC_{50}$ (nM) and $k_3/K_i$ (M$^{-1}$s$^{-1}$) for TRP601 and Δ2Me-TRP601.

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groups on Asp lateral chains) was progressively generated in the blood. We have re-synthesized this metabolite and found that it strongly inhibits Casp3 (IC_{50}/Casp3/32Me-TRP601 against recombinant Casp3 (rCasp3) = 0.42 ± 0.16 nM; k_{d}/K_{d} = 34 782 163/M/s) and Casp2 (IC_{50}/Casp2/32Me-TRP601 against rCasp2 = 7.4 ± 3.1 nM; k_{d}/K_{d} = 182 801/M/s; n = 3) in vitro.

Table 1 Comprehensive in vitro pharmacology profile of TRP601

| Non-peptide receptors | Peptide receptors | Nuclear receptors |
|-----------------------|-------------------|-------------------|
| Adenosine             | Angiotensin-II    | Glucocorticoid    |
| Adrenergic            | Bombesin          | Estrogen alpha    |
| Cannabinoid           | Calcitonin gene-related peptide | Androgen |
| Dopamine              | Chemokine         | Ion channels      |
| GABA                  | Cholecystokinin   | Ca^{2+} channels  |
| Glutamate             | Complement 5a     | K^{+} channels    |
| Gyicine               | Endothelin        | Na^{+} channel    |
| Histamine             | Galanin           |                  |
| Imidazole             | Glucagon          |                  |
| Leukotriene           | Growth hormone secretagogue | Enzymes |
| Melatonin             | Melanin-concentrating hormone | Kinases |
| Muscarinic            | Motilin           | Phosphatases      |
| Nicotinic             | Neurokinin        | Serine proteases  |
| Purinergic            | Neuropeptide Y    | Cysteine proteases|
| Sorotinin             | Neurotensin       | Aspartate proteases|
| Sigma                 | Opioid and opioid-like | Arachidonic acid metabolism |
| Amine transporters    | Thyroid hormone   | Monoamine synthesis and metabolism |
| Choline               | Urotensin-II      | Neurotransmitter synthesis and metab. |
| Dopamine              | Vasoactive intestinal peptide | Nitric oxide synthesis |
| GABA                  | Rolipram          | Second messenger systems |
| Noradrenephrine       | Vasopressin       | ATPases           |
| Serotonin             |                   | Lipid synthesis   |
|                      |                   | Metalloproteases  |

TRP601 (10 μM) did not significantly modify the activity of 56 enzymes and did not impair or increase the binding of specific ligands to their receptors (110 receptors, channels, or transporters tested). Synthetic list of enzymes, peptide and non-peptide receptors, nuclear receptors, ion channels, or amine transporters, challenged with TRP601 (see details in Supplementary Table S3).

Figure 2 TRP601 has neuroprotective effects in a perinatal stroke model. The 7-day-old rats underwent electrocoagulation of the left middle cerebral artery and transient homolateral common carotid artery occlusion for 50 min, followed by 48 h of recovery. (a) Pre-treatment with TRP601 confers strong cerebroprotection. Vehicle (n = 15), 100 μg TRP601 (n = 24) or 100 μg TRP601 (n = 9) was injected intraperitoneally (i.p.) before ischemia. Histograms represent mean ± S.E.M. Kruskal–Wallis (P = 0.0004), post hoc Dunn’s (P = 0.001 for TRP601 versus vehicle). (b) Dose–response of TRP601 administered 1 h after MCAO onset (n = 103, P < 0.002 Kruskal–Wallis). Histograms represent mean ± S.E.M. Asterisks indicate the level of significance in Dunn’s post hoc. (c) Representative cresyl violet-stained coronal sections from vehicle-treated and TRP601-treated (1 mg/kg i.p.; 1 h post-ischemia) animals at 48 h post-reperfusion at the level of dorsal hippocampus and anterior commissure. Dotted lines indicate infarct area. Scale bar = 500 μm. (d) Time window for treatment with TRP601 (n = 274, P < 0.0001 Kruskal–Wallis). Individual percentage infarct volumes at 48 h post-ischemia are shown in vehicle (n = 43) or TRP601-treated rats (1 mg/kg; i.p.) at 1 h (n = 51, **), 2 h (n = 45, **), 3 h (n = 40, **), 4 h (n = 48, *), and 6 h (n = 47, *) post-occlusion (asterisks indicate the level of significance in Dunn’s post hoc). Temperature and body weight were systematically monitored in ischemic animals (treated or not with TRP601) and were found unchanged during ischemia, at reperfusion, and up to 48 h post-ischemia. (e and f) TRP601 effects after intravenous injection. TRP601 was administered intragually 1 h after the ischemic onset (n = 21). (e) Representative micrographs of entire pup brains at 48 h post-stroke. Arrows indicate electrocoagulation points. Upper micrograph shows representative lesion at 48 h with visible ischemic territory. Lower micrograph is representative for low lesion score found in 33% of animals injected with 0.1 mg/kg TRP601. (f) Upper: Coryco-axial profiles (coronal sections of ipsilateral hemisphere) after intravenous (i.v.) administration of TRP601 (1 h after ischemia at 0.1 mg/kg (n = 15) or 1 mg/kg (n = 16). (g and h) TRP601 has long-term protective effects. Vehicle or 0.1 mg/kg TRP601 was i.p. injected 1 h after the ischemic onset. After 21 days of recovery, animals were killed. (g) Cresyl violet-stained coronal sections showing brain injury in a representative animal for each treatment (upper: bregma 0.7; lower: −3.3 mm). Note that TRP601 reduces the cortical cavity and tissue loss in external and internal capsule (ec, ic), caudate putamen (CPu), and amygdaloid nucleus (bregma −3.6, −4 mm; data not shown). (h) Quantification of the cavity volume (% cavitation) and hemispheric tissue loss (100% −% ipsi/contra) in the presence (n = 12) or absence (n = 6) of TRP601. Data are mean ± S.E.M. (bars) values. *P = 0.0017; **P = 0.0192 (Mann–Whitney). (i) Temperature and body weight were systematically monitored in ischemic animals (treated or not with TRP601) and were found unchanged during ischemia, at reperfusion, and up to 48 h post-ischemia. (j) TRP601 reduces the cortical cavity and tissue loss in external and internal capsule (ec, ic), caudate putamen (CPu), and amygdaloid nucleus (bregma −3.6, −4 mm; data not shown). (k) Quantification of the cavity volume (% cavitation) and hemispheric tissue loss (100% −% ipsi/contra) in the presence (n = 12) or absence (n = 6) of TRP601. Data are mean ± S.E.M. (bars) values. *P = 0.0017; **P = 0.0192 (Mann–Whitney).
TRP601 is neuroprotective in neonatal excitotoxic brain injury. We next evaluated the effect of TRP601 after excitotoxic neonatal brain injury. In 5-day-old mice intracerebrally injected with ibotenate, a single administration of TRP601 (intraperitoneal (i.p.); 1 mg/kg) significantly reduced lesion volumes of cortical ($V = 0.37 \pm 0.06 \mu \text{m}^3$, $n = 16$) and white matter ($V = 0.18 \pm 0.08 \mu \text{m}^3$, $n = 16$) versus controls ($V = 0.23 \pm 0.07 \mu \text{m}^3$, $n = 16$) regions (Figure 1g). In contrast, TRP901 (a DEVD-based preferential Casp3/Casp7 inhibitor) and TRP801 (a preferential Casp8 inhibitor), both designed with the same chemistry as TRP601 (Supplementary Figure S2), did not confer brain protection against ibotenate challenge (Figure 1g). In these
experimental conditions, TRP601 also reduced macrophage activation (43.51 ± 4.16% reduction; \( P < 0.0001 \) Mann–Whitney) and astrogliosis (37.69 ± 3.15% reduction; \( P < 0.0001 \) Mann–Whitney) as demonstrated by histological evaluation of lectine- (Figure 1h) and glial fibrillary acidic protein -positive (Figure 1i) cell densities, respectively. In addition, TRP601 strongly reduced the DVDAse-specific activity (73% reduction; \( P = 0.0079 \) Mann–Whitney) when studied 8 h after ibotenate treatment (Figure 1j).

Having found recently that genetic inhibition of caspase-2 (Cas2) protects newborn mice from ischemic brain injury,\(^{32}\) we further investigated the protective effect of TRP601 during Casp2 silencing. When TRP601 was administered in mice neonates treated with Casp2-specific siRNA, no significant additional protection was found against ibotenate, neither in the white matter nor in the cortical plate (Figure 1k and Supplementary Table S1). This suggests that, at least in this experimental setting, TRP601-mediated neuroprotection is Casp2 dependent.

**TRP601 is neuroprotective in neonatal rats after ischemic stroke.** We then investigated whether TRP601 might be operative in perinatal arterial stroke. The 7-day-old rat pups were subjected to a permanent occlusion of the MCAO and subsequent transient unilateral carotid ligation,\(^{41}\) a model of neonatal ischemia with reperfusion that induces ipsilateral cortical injury associated with initiator and effector caspase processing,\(^{42}\) extensive neuronal loss, inflammatory responses, nitric oxide production and the evolution of a cortical cavitory infarct.\(^{41}\)

A single dose of TRP601 (5 mg/kg; i.p.) administered before occlusion induces a highly significant reduction (59%) of infarction (8.83 ± 1.8%; \( n = 24 \)) compared with the control (21.67 ± 1.65%; \( n = 15 \)) (Figure 2a). In contrast, the Casp3/ Casp7 inhibitor TRP901 does not confer significant protection (22.2 ± 5.67%; \( n = 9 \)). TRP601 also produces significant reductions in cortical infarction when administered i.p. 1 h after the ischemic onset (i.e., at reperfusion) at doses between 1 \( \mu \)g/kg and 10 mg/kg (\( n = 103; \) \( P < 0.002 \) Kruskal–Wallis), with an optimal dose of 1 mg/kg (\% infarction: 9.73 ± 1.9%; \( n = 18, \) \( P < 0.001 \) post hoc Dunn’s; Figures 2b and c).

To determine the therapeutic time window of TRP601 in this perinatal stroke rat model, we designed a large protocol (\( n = 274 \) rat pups, two independent experimenters) with randomized litters in each group (\( P < 0.0001 \) Kruskal–Wallis; Figure 2d). When injected 2 h post-ischemia at 1 mg/kg (i.p.), TRP601-induced reduction of infarction was around 40% (\% infarction: 11.78 ± 1.01%; \( n = 47, \) \( P < 0.001 \) post hoc Dunn’s; Figure 2d) and remained significant (19.18% reduction) when TRP601 was added up to 6 h post-ischemia (\% infarction: 16.01 ± 0.92%; \( n = 47, \) \( P < 0.05 \) post hoc Dunn’s; Figure 2d).

The most clinically relevant administration route being i.v. injection, we set up similar experiments with post-ischemia intrajugular bolus of TRP601. Lesion scores on the entire brain and also section-based infarction quantifications converged to conclude that i.v. injected TRP601 (0.1–1 mg/kg; 1 h post-ischemia) considerably reduces ischemia-induced brain lesions along the rostro-caudal axis (Figures 2e and f), correlating with a significant neurological score amelioration in sensory and motor profiling assays (Table 2). We further investigated if cerebroprotection was long-lasting. At 21 days post-ischemia, the ipsilateral hemisphere of vehicle-treated animals exhibited a large cavity in the full thickness of the frontoparietal cortex (\% cavitation: 12.5 ± 3.53%; \( n = 6 \)) and a tissue loss (32.33 ± 5.56%), which were markedly reduced in TRP601-treated rats (\% cavitation: 1.42 ± 0.68%; \( n = 12; \) \% tissue loss: 12.1 ± 3.9%; Figures 2g and h), correlating with reduced astrogliosis (Supplementary Figure S3). Hence, in 7-day-old rats subjected to perinatal stroke, TRP601 provided sustained neuroprotection and neurological improvement, with a 6 h time window for administration.

**TRP601 prevents apoptosis, caspase activation and cytochrome c release in vivo.** Both in vivo propidium iodide staining and ex vivo terminal transferase dUTP nick-end labeling analysis of ipsilateral regions in brain sections from ischemic pups revealed a massive TRP601-sensitive cell death with apoptotic phenotype (Figures 2i and j). When administered at reperfusion, TRP601 prevents Casp2 processing (Figure 2k) and activation of all caspase-like activities, whereas TRP901 only reduces the DEVDase activity (Figures 2k and l). Previous reports had suggested that Casp2 may act directly and/or through Bid/Bax on mitochondrial membranes.\(^{43,44}\) Using the mitochondria isolated from neonatal brain, we found that a mixture containing rCasp2 and full-length Bid induce TRP601-sensitive cytochrome c release, correlating with Casp2-induced Bid cleavage (Supplementary Figure S4). In addition, cytochrome c detection at 24 h post-ischemia in the ipsilateral cytosolic versus mitochondrial brain fraction indicates that TRP601 (added at reperfusion; i.p.) prevents the mitochondrial release of cytochrome c in vivo (Figure 2m), correlating with TRP601-mediated prevention of matrix swelling (Figure 2n).

**TRP601 reduces HI brain injury.** We next decided to determine whether the TRP601 pharmacological spectrum of use might include the most widely used neonatal HI model, where a focal brain injury occurs in 8-day-old rats after unilateral carotid ligation and exposure to 7.8 % oxygen for 50 min.\(^{46}\) Indeed, a single dose of TRP601 (1 mg/kg; i.p.) administered just after hypoxia induces a significant reduction of infarct volume (30.3%; \( P = 0.0239 \) Student’s \( t \)-test; \( V = 60.22 ± 8.47 \) mm\(^3\); \( n = 24 \) versus control \( V = 86.43 ± 6.54 \) mm\(^3\); \( n = 19 \)), percentage infarction (48.48 ± 3.13% versus 34.17 ± 4.69%; \( P = 0.0287 \) Mann–Whitney; Figure 3a), and tissue loss (27.5%; \( P = 0.0412 \) Mann–Whitney; \( V = 88.64 ± 11.17 \) mm\(^3\); versus control \( V = 122.29 ± 6.84 \) mm\(^3\); Figures 3b–d). An early TRP601-sensitive, but TRP901-insensitive, DVDAse activity (12 pmol/min per mg) was detected as early as 1 h post-HI in the cortex (Figure 3e). TRP601 also reduced various aspects of HI-induced white matter injury, including neurofilament degradation (NF68: 64.24 ± 8.5% versus controls 87.66 ± 5.32%; \( P = 0.049 \) Mann–Whitney; Figure 3f), oligodendroglial loss (CNPase: 69.56 ± 8.37% versus controls 91.29 ± 2.59%; \( P = 0.0307 \) Student’s \( t \)-test) (Figure 3g) and hypomyelination (MBP: 87.66 ± 8.8% versus controls 91.03 ± 2.6%; \( P = 0.041 \) Student’s \( t \)-test) (Figures 3h and i).
Table 2 Neurological benefit at 48 h: effect of TRP601 (i.v., 1 mg/kg, 1 h post-ischemia) on general behavioral profiles

|                     | Naive (n = 20) | Isch.+vehicle (n = 20) | Isch.+TRP601 (n = 20) | P-values ($\chi^2$) (n = 20) |
|---------------------|----------------|------------------------|-----------------------|-----------------------------|
| Spontaneous activity| 1.8 ± 0.4      | 0.3 ± 0.6              | 1.1 ± 0.6             | 0.026 × 10^-6               |
| Walking (circle versus straight) | 0.3 ± 0.4      | 1.6 ± 0.6              | 0.8 ± 0.6             | 1.092 × 10^-6               |
| Reaction to pain    | 1.7 ± 0.6      | 0.7 ± 0.6              | 1.2 ± 0.6             | 95.33 × 10^-6               |
| Paw withdrawal      | 1.6 ± 0.6      | 0.3 ± 0.6              | 1.2 ± 0.6             | 0.261 × 10^-6               |
| Mean lesion volume (mm$^3$) | 0              | 24.3 ± 2.5             | 9.2 ± 2.3             | NA                          |

Sensorimotor neurological deficits were assessed in a blinded manner in 7-day-old rat pups. Animals were subjected to ischemia–reperfusion (as in Figures 2d–f) and treated with 1 mg/kg TRP601 (i.v., 1 h post-ischemia). At 48 h post-ischemia, pups were tested for the following neurological signs and reflexes: (i) spontaneous activity (spontaneous postural signs such as right forelimb flexion and thorax twisting and exploration of the cage); (ii) walking (after ischemia pups walk in circle rather than straight); (iii) reaction to pain (the pup escapes after the tail is pinched); (iv) paw withdrawal (pups withdraw paw from adhesive pad). Grading scale of neurological examination for each test item was: 2 for normal; 1 for intermediate; and 0 for abnormal. A neurological score (mean ± S.E.M.) was attributed for each test in each experimental condition (d.o.f. stands for degree of freedom in the $\chi^2$ statistical test), NA, not applicable.

TRP601 does not modify physiological parameters. It is established that physiological variables can influence the outcomes of ischemic injury.\(^{46}\) Importantly, when injected in rat pups, TRP601 (1 mg/kg) does not significantly alter physiological parameters, including systemic acid–base balance (arterial blood pH, partial tension of $O_2$, partial tension of $CO_2$ and plasma $[HCO_3^-]$), body weight, temperature, or heart rate (Table 3). Echography and transcranial color-coded duplex Doppler sonography showed that heart function and cerebral perfusion are unchanged in TRP601-exposed neonates (Table 3). In addition, telemetry experiments in vigil dogs indicated that...
Cardiac physiology

Vital signs, heart rate, and electrocardiogram profile including QT interval corrected for heart rate were not affected by TRP601 up to the maximal dose tested (i.e., 3 mg/kg, i.v.), suggesting low potential for fatal tachyarrhythmia. Suprapharmacological doses of TRP601 in rats had no impact on central nervous system functions, including behavior, spatial activity, coordination, and memory.

To exclude the potential risk that TRP601 might affect the physiological programmed cell death, which contributes to normal brain maturation, TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) ex vivo studies were performed, and it was found that rat pups treated with TRP601 at postnatal days 5 and 7 (after birth) showed no significant changes in developmental cell death in the brain (Supplementary Table S2). In addition, histological analysis in 7-day-old rat pups treated with high doses of TRP601 found no influence on forebrain, middle brain, and hindbrain maturation at 21 days of age (data not shown).

TRP601 safety profile in adult and neonates support further translational steps. The bench to bedside translation of any drug dedicated to the neonatal population requires specific PK/PD considerations together with combined toxicology evaluation in both adult and neonates. For these reasons, having verified that TRP601 has no hemolytic potency, no effect on bleeding time, no impact on platelet aggregation, and low toxicities on various cultured primary cells, we then investigated toxicology in adult animals, together with dedicated studies in rodent and non-rodent neonates (see Supplementary Materials and Methods). In multiple-dose regulatory studies in adult dogs, no TRP601-related cytotoxic effects were observed following i.v. administration for 14 days at doses up to 3 mg/kg per day. A good vascular and perivascular local tolerance was found in rabbit ear. TRP601 was found not to be genotoxic when evaluated in a battery of in vitro and in vivo assays, and showed no antigenic response in rat. In 7-day-old rat pups, single-dose administrations of TRP601 indicated low toxicity of TRP601 (DL50i.v. = 60 mg/kg; DL50p.o. > 200 mg/kg). Studies with multiple-dose i.v. injections (once every 3 days during a 2-week period, starting on postnatal day 1 in newborn Beagle dogs) showed no adverse effect level at 15 mg/kg per injection once every 3 days.

Table 3  Physiological variables in TRP601-treated rat pups

| Parameters                        | Vehicle   | TRP601   |
|-----------------------------------|-----------|----------|
| Blood hemodynamics and biochemistry |           |          |
| Blood flow (n = 5, ml/min)        | 4.2 ± 0.9 | 3.6 ± 0.4 |
| Resistance index (n = 8)          | 0.68 ± 0.01 | 0.64 ± 0.03 |
| Bleeding time (n = 10)            | 319 ± 34 | 395 ± 103 |
| Arterial pH (n = 8)               | 7.42 ± 0.03 | 7.42 ± 0.02 |
| PaO2 (n = 8, mm Hg)               | 70.84 ± 6.38 | 69.82 ± 2.24 |
| PaCO2 (n = 8, mm Hg)              | 37.96 ± 2.64 | 42.06 ± 2.58 |
| HCO3 (n = 8, mM)                  | 24.16 ± 0.61 | 26.30 ± 0.76 |
| Cardiac physiology               |           |          |
| Heart rate (n = 8, beat/min)      | 345 ± 13 | 344 ± 12 |
| LVEDd (n = 8, mm)                 | 0.293 ± 0.015 | 0.310 ± 0.015 |
| LVO (n = 8, ml/min)               | 0.135 ± 0.009 | 0.157 ± 0.015 |
| Fractional shortening (n = 8, %)  | 54.39 ± 0.96 | 49.97 ± 2.11 |
| VTI (n = 8)                       | 4.05 ± 0.11 | 3.92 ± 0.29 |
| Core temperature (n = 10, °C)     | 36.60 ± 0.28 | 36.40 ± 0.67 |
| Body weight (n = 10, g)           | 19.49 ± 1.43 | 19.18 ± 0.95 |
| 1 h                               | 19.35 ± 1.72 | 20.40 ± 1.67 |
| 48 h                             | 20.96 ± 2.15 | 20.93 ± 0.67 |
| 14 days                          | 69.56 ± 1.60 | 62.00 ± 2.50 |

Blood hemodynamics, biochemistry, cardiac physiology, temperature, and body weight were evaluated before and 1 h after TRP601 (1 mg/kg) or vehicle injection. Anterior cerebral arteries were located by transtransducer color-coded duplex sonography to measure peak systolic (Vp) and minimum end-diastolic (Vd) velocities. Resistive index (RI) was calculated as follows: RI = (Vp - Vd)/Vp, and corresponds to an index of cerebral perfusion. For cardiac physiology, the left ventricular (LV) was imaged in short axis view, and diastolic diameter (LVEDd) was defined as the largest LV area. The fractional shortening was calculated with the following formula: FS (%) = (LVEDd - LVEDd)/LVEDd × 100. LVEDd corresponds to left ventricular end-systolic diameter. A pulsed-wave Doppler spectrum of aortic outflow was recorded from suprasternal view to evaluate left ventricular output (LVO). Velocity–time integral (VTI) of aortic flow, aortic root diameter (CSA), and heart rate (HR) were measured to calculate LVO as follows: LVO = CSA × VTI × HR. A pulsed-wave Doppler spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view as an index of diastolic function. Statistical analysis was performed by a spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view, and diastolic diameter (LVEDd) was defined as the largest LV area. A pulsed-wave Doppler spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view as an index of diastolic function. Statistical analysis was performed by a spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view, and diastolic diameter (LVEDd) was defined as the largest LV area. A pulsed-wave Doppler spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view as an index of diastolic function. Statistical analysis was performed by a spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view, and diastolic diameter (LVEDd) was defined as the largest LV area. A pulsed-wave Doppler spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view as an index of diastolic function. Statistical analysis was performed by a spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view, and diastolic diameter (LVEDd) was defined as the largest LV area. A pulsed-wave Doppler spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view as an index of diastolic function. Statistical analysis was performed by a spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view, and diastolic diameter (LVEDd) was defined as the largest LV area. A pulsed-wave Doppler spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view as an index of diastolic function. Statistical analysis was performed by a spectrum of mitral inflow (VTI) was recorded from the apical four-chamber view, and diastolic diameter (LVEDd) was defined as the largest LV area.

Discussion

Neonatal ischemic brain injury triggers multiple pathways of oxidant stress, inflammation, and excitotoxicity that lead to massive cellular death in the ischemic territory. Caspase-dependent programmed cell death importantly contributes to this fate.7,9,10,14,15,23,32,42 In contrast to previous generations of aspartyl-fluoromethylketone (D-fmk)-based inhibitors (e.g., z-VD-FMK (N-benzoyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone), boc-D-fmk (Boc-Asp (Ome)-fluoromethylketone), aspartyl-methylxoyphenylketone-based inhibitors (e.g., Q-VD-OPh) only target the caspase family, efficiently inhibit (all) caspases, have low in vivo toxicity, and present enhanced pharmacological properties.10,16,25,32 Our results show that pharmacological inhibition of a subfamily of caspases (Casp2, Casp3) with the pentapeptide-mOPh derivative, TRP601, reduces cortical and white matter damage in the neonatal brain after excitotoxicity, arterial stroke, and HI. In all of these experimental paradigms, TRP601 also reduces gliosis, an inflammatory response to brain ischemia. It has been demonstrated recently that caspase-8 and Casp3/7 are involved in regulating microglia activity, coordination, and memory. Consequently, beside its direct protective effect on neurons (as observed in primary cortical cell cultures), it might be important to investigate whether TRP601 (or the active metabolite 2Me-TRP601) could also exert cerebroprotective effects through targeting the microglia.
Our results indicate that TRP601 and its active metabolite(s) (e.g., ∆2Me-TRP601) inhibit Casp3 and Casp2 in vitro and in vivo. We also provide evidences suggesting that TRP601 interrupts the mitochondrial (intrinsic) pathway of apoptosis in the immature brain, possibly at the level of Casp-2-mediated Bid cleavage, and upstream cytochrome c release. We have recently reported that Casp2−/− mice subjected to neonatal HI presented significantly lower Casp3 activation in the thalamus and hippocampus. Lesion subjected to neonatal HI presented significantly lower previously found neuroprotective against neonatal HI, 24 one inhibitor exclude that part of the neuroprotective effect of TRP601 mediated neuroprotection is at least, in part, Casp2-dependent. In addition, we show here that combined administration of TRP601 with Casp2 silencing did not show any additive effect against ibotenate challenge. This suggests that the TRP601-mediated neuroprotection is at least, in part, Casp2-dependent. Taken together, our data suggest that the in vivo observed neuroprotection and Casp3 inhibition by TRP601 are at least, in part, Casp2-dependent. However, we could not formally exclude that part of the neuroprotective effect of TRP601 could be related to a direct Casp3 inactivation by TRP601 or ∆2Me-TRP601 (this latter being a very powerful Casp3 inhibitor in vitro). As the selective Casp3 inhibitor M826 was previously found neuroprotective against neonatal HI, one can suggest that the use of a group-II caspase inhibitor might be of therapeutic interest to cover different conditions, developmental stages, or regional mechanisms, where Casp3/7 and Casp2 are variably expressed and activated through different pathways.

Irrespective of these mechanistic considerations, TRP601 is a lead candidate for neuroprotective strategy in a variety of perinatal brain injury conditions. The lack of detectable side effects following in vivo administration of TRP601 to newborn rodents and dogs support its further evaluation, possibly in combination with hypothermia or other candidate treatments.

Materials and Methods

Animal models. Three different animal models were used: neonatal stroke, excitotoxicity, and HI. Neonatal stroke (focal brain ischemia with reperfusion in the 7-day-old rat) was carried out according to Renolleau et al. For HI, 8-day-old rats or alternatively 9-day-old mice were subjected to unilateral ligation of the left carotid artery, followed by hypoxia (7.8% O2 for rats and 10% O2 for mice, 36°C) for 50 min, as described. For drug-induced excitotoxicity, ibotenate was administered i.c.v. to 5-day-old mice, as described. Details for each animal models are given in Supplementary Material and Methods. SiRNAs were prepared as a solution in JetSI (Polyplus-transfection, Illkirch, France). Final solution contained RNAse free water, 0.08 mM DOPE, 0.24% 7% EtOH, glucose 12.5%, and 0.04 mM JetSI. For intracerebral siRNA administration, 20 ng of human active recombinant caspase-3 (Sigma; no. C5974), and 100 ng of human active recombinant caspase-2 (Biomol, Plymouth, PA, USA) or 117 ng of human active recombinant caspase-2 (Biomol, Plymouth, PA, USA) or 20 ng of human active recombinant caspase-3 (Sigma; no. C5974), and 100 μM of the purine portion of the progress curve. The formation of pNA was followed every 30 s for 150 min at 37°C with a microtiter plate reader (Paramet Multi-Mode Microplate Detection Platform, Softmax Pro 5.2 software; Molecular Devices, St. Grégoire Cedex, France). V0, relative velocities, Ks, and KC0 were determined from experimental data using the GraphPad Prism 5.01 software program. Standard deviations of the reported values were below 10%. Inhibitor binding affinity (disaccharide constant, K0 and first-order rate constant (k0) parameters were estimated using R (http://www.R-project.org) to fit the following equation from Wu and Fritz:

\[
P_{ij} = [E]_0 \left( \frac{[S]_i [K_i]}{[K_i] + [S]} \right) \left( 1 - e^{-k_1 t} \right) \left( 1 + [S]/K_s \right)
\]

Other in vitro assays for calpains, cathepsins, and granzyme activities were performed as described.33

Ex vivo biochemistry. Brains of rat or mice pups were removed just after decapitation. Contralateral and ipsilateral hemispheres were frozen immediately and kept at −80°C. Alternatively, the ipsilateral hemisphere was rapidly micro-dissected (at 4°C) to mainly select the lesioned area (i.e., the estimated penumbra zone for the neonatal stroke model). Brain samples processing and related biochemical analyses are described in Supplementary Material and Methods.

Physiological parameters. Blood gas analyses and hemodynamic evaluations conducted in newborn rats are described in Supplementary Materials and Methods.
Safety pharmacology and toxicology. Safety experiments conducted in newborn dogs and rats are described in Supplementary Materials and Methods.

Statistics. Statistical analyses were conducted with Prism 4.0. Data are presented as mean ± S.E.M. and were first analyzed with a KS normality test. When the data passed the normality test, the appropriate parametric test has been applied (Student’s t-test or ANOVA), followed by the suitable post hoc test if necessary (Bonferroni test). When the data did not pass the normality test, the appropriate non-parametric test has been applied (Mann-Whitney or Kruskal-Wallis tests), followed by the suitable post hoc test if necessary (Dunn’s test). Neurological scores were statistically analyzed with a \( \chi^2 \). Statistical significance is indicated by a single asterisk (\( P < 0.05 \)), and two or three asterisks (\( P < 0.01 \) or \( < 0.001 \), respectively).

Conflict of Interest

Some of the authors (DC, SA, MB, RC, MS, A-PT, EB, and EJ) were former employees of a biopharmaceutical company (Therapostis SA, Paris, France) that closed down in late 2008. Other authors (SR, SH, LS, CR, JH, GD, YC, XM, WH, J-MR, SM, AL, PR, JM, HH, PG, CC-M) declare no conflict of interest. First public disclosure of TRPE01-related data occurred in June 2008 (6th Hershsey Conference, Ecqueville, France). TRPE01 rights were acquired in 2009 by Chiesi Farmaceutici SpA, Parma, Italy (http://www.chiesigroup.com/web/guest/chi-siamo/key-figures/report-finanziari; Annual Report 2009, p 50).

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