Non-immunosuppressive cyclosporin analogs inhibit mitochondrial permeability transition and neuronal death in hippocampal slice cultures

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Abstract

Cyclosporin A (CsA) has been shown to be neuroprotective in animal models of ischemia, traumatic brain injury, hypoglycemic coma as well as prolonging survival of transgenic ALS mice. Inhibition of the mitochondrial permeability transition (mPT) has emerged as a possible mechanism for the powerful in vivo neuroprotection displayed by CsA.

In the present ongoing study, we have evaluated new non-immunosuppressive cyclosporin analogs NIM811 (MeIle[4]-cyclosporin-A) and UNIL025 (MeAla[3]EtVal[4]-cyclosporin-A) for their ability to inhibit mPT in rodent brain-derived mitochondria as well as their prevention of cell death in an in vitro model of ischemic brain damage (described in Rytter A. et al. JCBF 23:23-33, 2003). Both NIM811 and UNIL025 were found to be powerful inhibitors of calcium-induced mitochondrial swelling under energized and de-energized conditions and the maximal effects were identical to those of native CsA. The potencies of mPT inhibition by NIM811 and UNIL025 were stronger, with almost one order of magnitude higher potency for UNIL025 compared to CsA, correlating to their respective inhibitory action of cyclophilin activity. In addition, both CsA and the first non-immunosuppressive cyclosporin analog tested ameliorated selective CA1 cell death in organotypic mouse hippocampal slices exposed to 15 min of oxygen and glucose deprivation.

Stringent evaluation of the efficacy displayed by CsA and its non-immunosuppressive analogs can aid in discriminating between (i) inhibition of the calcium-activated phosphatase calcineurin and (ii) counteracting the mitochondrial permeability transition as the primary neuroprotective mechanism displayed by these compounds.

Objectives

To evaluate the potency of NIM811 and UNIL025 inhibiton of mPT in rat brain-derived mitochondria

To investigate if NIM811 and UNIL025 can ameliorate selective CA1 cell death in organotypic mouse hippocampal slice cultures exposed to oxygen and glucose deprivation

Methods

Brain mitochondria were isolated from rat cerebral cortex tissue using a discontinuous Percoll gradient according to Sims, method B (Sims, 1990), with slight modifications (Hansson et al., 2003).

Activation of mitochondrial permeability transition was monitored by measuring the decrease in right angle light scattering at 520 nm (mitochondrial swelling) using a Perkin-Elmer Spectrometer LS-50B (Emeryville, CA, USA) or by flow cytometric detection of side scattering (SSC) using a FACSCalibur (Becton & Dickinson, San Jose, CA).

The cyclosporin analogs NIM811 (Novartis, Basel, Switzerland), UNIL025 (Debiopharm, Lausanne, Switzerland) and CsA (Ivax, Opava, Czech Republic) were run under de-energized conditions to evaluate potency of mPT-inhibition, as described previously (Hansson et al., 2003). The experiments were performed at 28°C in an isotonic KCl-based buffer containing the respiratory complex blockers rotenone and antimycin (both 0.5 µM) and the calcium ionophore A23187 (2 µM) to ensure free diffusion of Ca^{2+} over the mitochondrial membranes. Mitochondria were incubated with 10 nM to 5 µM of CsA, NIM811, UNIL025 or vehicle (final concentration 0.2% v/v ethanol) for 4 min and were subsequently exposed to 100 µM Ca^{2+} for 5 min before termination of experiments with the ionophore alamethicin (7.5 µg/ml).

The protocol for flow cytometric analyses of isolated brain mitochondria was described earlier (Mattiasson et al., 2003). Mitochondria were analyzed for distribution properties under de-energized conditions and selected from background based on specific staining with nonyl acridine orange. Experiments were performed as described above for the fluorometric analyses but run at room temperature. The cyclosporin analogs were tested at 1 µM and mPT was detected as a decrease in side scattering (SSC).

Inhibition of mPT by NIM811 and UNIL025 was also evaluated under energized conditions. Experiments were run as described previously in a sucrose-based buffer with 5 mM malate and glutamate as respiratory substrates, in the presence of 20 µM ADP and 1 µg/ml oligomycin (Hansson et al., 2004). Mitochondria were incubated with the analogs at 1 µM concentration for 3 min and then exposed to 2 µmol Ca^{2+}/mg mitochondrial protein for 10 min before termination of experiments with alamethicin.

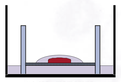
The decrease in light scattering from the pre-Ca^{2+} incubation value to that after alamethicin administration was defined as maximal swelling (100%). The decreases in light scattering following 5 and 10 min Ca^{2+} exposure for de-energized and energized experiments, respectively, were calculated and displayed as % of maximal swelling. Swelling responses in the flow cytometric analyses were calculated in a similar fashion using the geometrical mean values (CellQuest, Becton & Dickinson, San Jose, CA).

Data were analyzed with ANOVA and the Bonferroni post hoc test unless otherwise stated and presented as means + S.E.M.

Hippocampal organotypic cultures

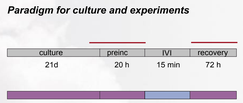
Hippocampi from 6-day-old Balb/c mice were dissected out, cut into 250 µm thick transverse sections using a McIlwain Tissue Chopper and plated onto 0.4 µm Millicell culture inserts. The culture medium consisted of 50% MEM (Eagles with Earl's balanced salt solution), 25% horse serum, 18% HBSS (Hank's balanced salt solution), supplemented with 4 mM L-glutamine, 50 units penicillin-streptomycin/ml, 20 mM D-glucose and pH was adjusted to 7.2 with $NaHCO_3$. During the first week of culture 2% B27 was added to the medium.

McIlwain Tissue Chopper



Millicell membrane

Paradigm for culture and experiments



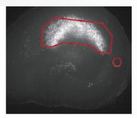
Induction of in vitro ischemia (IVI)

Prior to IVI, cultures were washed from beneath in glucose-free medium and transferred to the anaerobic incubator.

Inside the incubator cultures were transferred to wells with anoxic IVI-medium containing (in mM): 0.3 $CaCl_2$, 70 NaCl, 5.25 $NaHCO_3$, 70 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 40 sucrose and pH 6.5.

Quantification of cell death

Cell death was evaluated with the the fluorescent cell death marker propidium iodide (PI). Fluorescence intensity was measured in a standardized area in the CA1 region and in a small hexagon placed in an undamaged area outside the CA2/3 cell layer (background). Values of cell death were obtained by subtracting mean fluorescence intensity (MFI) in the background area from the MFI levels in the standardized CA1-area.

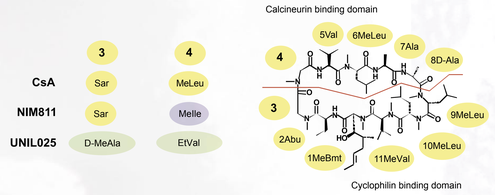


Statistics

Data are expressed as mean ± S.E.M. Differences between groups were analysed using two-way analysis of variance (ANOVA) with Scheffé´s post-hoc test. Differences over time were compared with repeated measures ANOVA, Scheffé´s post hoc test. Variability between experimental dates was compensated for by including date as a factor.

Results

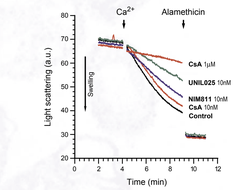
1 Amino acid sequences for the cyclic undecapeptides Cyclosporin A (CsA), NIM811 and UNIL025



The binding site for the cyclophilins to the immunosuppressive CsA molecule covers residues 1-3, 10 and 11 and the interaction with calcineurin residues 4-7. The non-immunosuppressive cyclosporin molecules NIM811 and UNIL025 are both modified (as compared to CsA) at the fourth amino acid (from N-methyl-leucine to N-methyl-isoleucine, MeIle and N-ethyl-valine, EtVal, respectively) preventing calcineurin binding while retaining the ability to interact with cyclophilin. Modified from (Waldmeier et al., 2003). UNIL025 has an additional modification at the third amino acid (from sarcosine to N-methyl-D-alanine, D-MeAla).

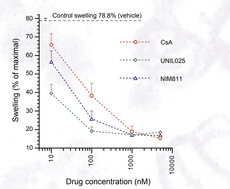
2 The non-immunosuppressive cyclosporin analogs NIM811 and UNIL025 inhibit permeability transition with nanomolar potencies in brain-derived mitochondria

A



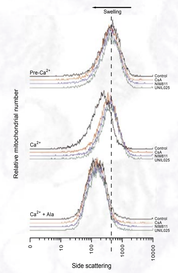
Representative traces of changes in 90° light scattering (arbitrary units, Δ520 nm) of experiments run under de-energized conditions at 28°C in isotonic KCl-based swelling buffer, pH 7.3. UNIL025, NIM811, cyclosporin-A (CsA) or vehicle (EtOH) was added to mitochondrial suspensions (25 µg protein/ml). Ca^{2+} (100 µM) was added after four minutes and each experiment was terminated at 9 min with alamethicin (7.5 µg/ml) to induce maximal swelling.

B



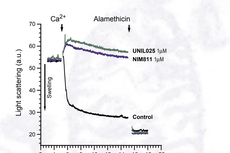
Calculations of concentration-dependent inhibition of de-energized swelling by CsA, UNIL025 or NIM811. Mitochondria were exposed to Ca^{2+} in the presence of 10 nM to 5 µM CsA, UNIL025 or NIM811. All values (except CsA 10 nM, p=0.011 in the stringent post hoc test used) were significantly different from control swelling (78.8%). Means+S.E.M., n=4.

C



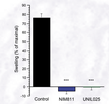
Flow cytometric analyses of isolated brain mitochondria exposed to Ca^{2+} under de-energized conditions. The histograms show side scattering (SSC) properties of mitochondria (x-axis) and relative mitochondrial number at the respective intensities (y-axis). SSC values were determined before Ca^{2+} (final concentration 100 µM) was added to the samples (Pre-Ca^{2+}, top), following 5 min of Ca^{2+} incubation (middle) and after induction of maximal swelling by the addition of alamethicin (7.5 µg/ml) (bottom). Taken together, the histograms show that Ca^{2+} -induced swelling is prevented by 1 µM CsA, NIM811 or UNIL025, and that there was no subpopulation in the samples that was not protected by these inhibitors of mPT.

D



Complete inhibition by UNIL025 and NIM811 of the induction of permeability transition under energized conditions. Mitochondria (25 µg/ml) were exposed to 2 µmol Ca^{2+}/mg protein for 10 min before maximal swelling was induced by application of alamethicin. Representative traces of changes in 90° light scattering are displayed. a.u. = arbitrary units.

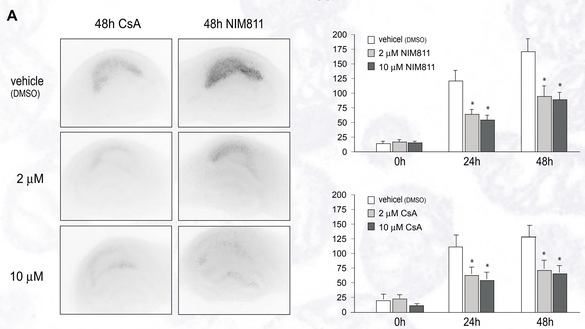
E



Calculations of the extent of inhibition displayed in (D). The extent of swelling (% of maximal) was calculated as described in Methods. Means+S.E.M., n=4. *** p<0.0001, one-way ANOVA followed by the Bonferroni post hoc test.

3 Cyclosporin A and non-immunosuppressive analog prevent cell death in hippocampal slice culture

A



The effects of CsA and NIM811 in slices exposed to in vitro ischemia. Photographs of representative hippocampal slices after 48 hours of recovery (A). Quantification of cell death in the CA1 region, 0-48 h following ischemia (B). CsA, NIM811 or vehicle (DMSO) was present in the medium from 20 h before experiment, during ischemia and in the recovery period. n=12 for CsA and n=14 for NIM811 experiments. *P<0.05 compared to vehicle group.

Conclusions

Both NIM811 and UNIL025 were found to be powerful inhibitors of calcium-induced mitochondrial swelling under energized and de-energized conditions and the maximal effects were identical to those of native CsA.

The potencies of mPT inhibition by NIM811 and UNIL025 were stronger, with almost one order of magnitude higher potency for UNIL025 compared to CsA, correlating to their respective inhibitory action of cyclophilin activity.

Both CsA and the first non-immunosuppressive cyclosporin analog tested ameliorated selective CA1 cell death in organotypic mouse hippocampal slices exposed to 15 min of oxygen and glucose deprivation. The neuroprotective effects were very similar which strongly implicates mPT as a central pathophysiologic mechanism in this model.

Further in vivo evaluation of NIM811 and UNIL025 in relation to native CsA will reveal if mPT or calcineurin blockade is the main target for the displayed neuroprotection.

Immunosuppression is an undesirable side effect for many neurological indications (especially chronic diseases). The reported immunosuppressive activity of NIM811 and UNIL025 is 1700 and 7000 times less than that for CsA, respectively. In addition both drugs seem to have more favorable toxicity profiles.

We envisage that non-immunosuppressive cyclosporin analogs may be neuroprotective and may prove valuable in the treatment of severe diseases of the central nervous system.

Acknowledgements

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