Journal of Bioenergetics and Biomembranes, Vol. 36, No. 4, August 2004 (© 2004)

The Nonimmunosuppressive Cyclosporin Analogs NIM811 and UNIL025 Display Nanomolar Potencies on Permeability Transition in Brain-Derived Mitochondria

Magnus J. Hansson,[1,2] Gustav Mattiasson,[1] Roland Månsson,[1,3] Jenny Karlsson,[4] Marcus F. Keep,[5] Peter Waldmeier,[6] Urs T. Ruegg,[7] Jean-Maurice Dumont,[8] Kamel Besseghir,[8] and Eskil Elmér[1,9]

Received April 6, 2004; accepted May 7, 2004

Cyclosporin A (CsA) is highly neuroprotective in several animal models of acute neurological damage and neurodegenerative disease with inhibition of the mitochondrial permeability transition (mPT) having emerged as a possible mechanism for the observed neuroprotection. In the present study, we have evaluated two new nonimmunosuppressive cyclosporin analogs NIM811 (Novartis) and UNIL025 (Debiopharm) for their ability to inhibit mPT in rat brain-derived mitochondria. Both NIM811 and UNIL025 were found to be powerful inhibitors of calcium-induced mitochondrial swelling under energized and deenergized 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. These compounds will be instrumental in the evaluation of mPT as a central target for neuroprotection in vivo.

KEY WORDS: Cell death; apoptosis; neuron; ischemia; neurodegeneration; traumatic brain injury; amyotrophic lateral sclerosis; cyclophilin; mitochondrial permeability transition; flow cytometry.

INTRODUCTION

Cyclosporin A (CsA) is an 11-amino-acid cyclic peptide of fungal origin that is currently used clinically as an immunosuppressive agent (Sandimmun®, Novartis). During the last decade, CsA has demonstrated potent neuroprotection in several animal models of acute neurological damage and neurodegenerative disease (Friberg and Wieloch, 2002; Keep *et al.*, 2003; Waldmeier *et al.*, 2003). Two main cellular targets have been suggested to explain the neuroprotective effect of CsA: (i) inhibition of the phosphatase calcineurin, which is generally believed to mediate its immunosuppressive properties, and (ii) the specific inhibition of the mitochondrial permeability transition (mPT) pore (Uchino *et al.*, 1995). Mitochondria are essential to the normal life of eukaryotic cells and there is now widespread agreement that mitochondria also play an active role in the biochemical signaling underlying cell death. The mechanisms behind their involvement in cell death are continually debated and remain to be fully elucidated. One pathophysiologic mechanism that has been proposed to be essential in both triggering and amplifying apoptotic and necrotic cell death is the mPT (Lemasters *et al.*, 1998; Zamzami and

[1] Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University, BMC A13, SE-221 84 Lund, Sweden.
[2] Department of Internal Medicine, Ryhov Hospital, Jönköping, Sweden.
[3] Department of Neurology, Malmö University Hospital, Sweden.
[4] Community Environmental Health Program, Department of Internal Medicine, University of New Mexico, Albuquerque, New Mexico.
[5] Division of Neurosurgery, University of New Mexico, Albuquerque, New Mexico.
[6] Department of Neuroscience, Novartis Institute for Biomedical Research, Basel, Switzerland.
[7] Pharmacology Group, School of Pharmacy, University of Lausanne, Switzerland.
[8] Debiopharm S. A., Lausanne, Switzerland.
[9] To whom correspondence should be addressed; e-mail: eskil.elmer@expbr.lu.se.

0145-479X/04/0800-0407/0 © 2004 Springer Science+Business Media, Inc.

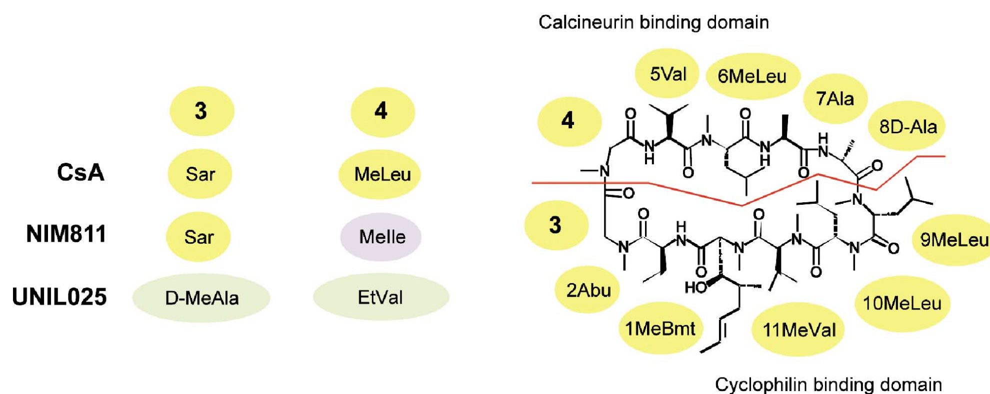

Fig. 1. Amino acid sequences for the undecapeptides cyclosporin A (CsA), NIM811, and UNIL025. The binding site for the cyclophilins to the immunosuppressive CsA molecule covers residues 1–3, 10, and 11, and the interaction with calcineurin residues 4–7. The nonimmunosuppressive cyclosporin molecules 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 cyclophilins. UNIL025 has an additional modification at the third amino acid [from sarcosine to *N*-methyl-D-alanine (D-MeAla]. (Modified from Waldmeier *et al.*, 2003)s

Kroemer, 2001). Upon various forms of cellular stress, most noteworthy calcium overload, a proteinaceous pore in the mitochondria can open, leading to inner and outer membrane permeabilization, uncoupling of oxidative phosphorylation, mitochondrial swelling, release of reactive oxygen species and liberation of apoptogenic proteins from the intermembrane space. The mitochondrial permeability transition can be blocked by CsA, supposedly by preventing the interaction of cyclophilin D (CypD) with the adenine nucleotide translocator (ANT) and/or its complex with the voltage-dependent anion channel (VDAC) (Crompton, 1999; Halestrap *et al*., 2002).

We have previously shown that CsA is a powerful inhibitor of mPT in de-energized and energized brain mitochondria (Hansson *et al*., 2003, 2004). In the energized swelling paradigm the isolated mitochondria are allowed to respire on metabolic substrates and are closer to a physiological state. Under de-energized swelling conditions, the assay suffers from less interference from possible confounding factors related to the respiratory function and active ion transport mechanisms (Halestrap *et al*., 2002). The de-energized model provides a very reliable model to study isolated pharmacological effects on mPT. Studies from our laboratory on the nonimmunosuppressive cyclosporin analog MeVal^4-cyclosporin have demonstrated that this compound besides inhibiting mPT in brain-derived mitochondria also decreased oxygen/glucose deprivation-induced neuronal damage in cultured neurons and diminished infarct size after transient focal cerebral ischemia in rat (Friberg and Wieloch, 2002). This compound is however no longer available to researchers. The objective of the present study was to characterize the effect of two new nonimmunosuppressive (non–calcineurin inhibiting) cyclosporin analogs, NIM811 (MeIle^4-cyclosporin) and UNIL025 (MeAla^{3}EtVal^4-cyclosporin) (Fig. 1) on brain-derived mitochondria. As a first step in a larger project evaluating the in vivo neuroprotection of NIM811 and UNIL025, the compounds were examined for their ability to inhibit mPT in isolated brain mitochondria. Both classical fluorometric analyses and flow cytometry were used to determine the efficiency and potency of NIM811 and UNIL025 inhibition of mPT compared to CsA.

MATERIALS AND 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) or by flow cytometric detection of side scattering (SSC) using a FACSCalibur (Becton & Dickinson, San Jose, CA). The cyclosporin analogs NIM811 (Novartis, Basel, Switzerland), UNIL-025 (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 light scattering properties under de-energized conditions and selected from background on the basis of 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 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 a percentage 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.

RESULTS

The potencies of the nonimmunosuppressive analogs NIM811 and UNIL025 to inhibit calcium-induced mitochondrial swelling were evaluated under de-energized conditions, and compared to mPT inhibition by CsA (Fig. 2(A) and (B)). Means + S.E.M. for swelling (% maximal swelling; $n = 4$) were for vehicle (ethanol) 78.8 ± 2.2%; and decreased in a concentration-dependent manner between 65.8 ± 6.0% for 10 nM CsA and 15.3 ± 1.7% for 5 μM CsA; 39.5 ± 4.9% for 10 nM UNIL025 and 17.1 ± 2.0% for 1 μM UNIL025 and 18.5 ± 1.0% for 5 μM UNIL025; 56.6 ± 5.9% for 10 nM NIM811 to 16.8 ± 0.5% for 5 μM NIM811 (Fig. 2B). The means for swelling were not adjusted for basal (Ca^{2+}-independent) swelling (Hansson *et al.*, 2003). Both UNIL025 and NIM811 displayed significant inhibition of swelling already at 10 nM concentration ($p < 0.0001$), 10 nM CsA was near significance ($p = 0.011$) in the stringent post hoc test used. The potency of UNIL025 was significantly higher as compared to CsA, with an approximate 10-fold difference (the 10 and 100 nM values of UNIL025 were similar to the 100 nM and 1 μM CsA values, respectively). In Fig. 2A representative recorded traces are displayed for the lowest drug concentration tested (10 nM). In addition, CsA 1 μM is displayed to illustrate near-maximal inhibition of swelling.

Recently, we demonstrated that flow cytometry is a useful tool to analyze mPT at the single organelle level (Hansson *et al.*, 2004). Addition of Ca^{2+} or alamethicin resulted in a decrease in side scattering (SSC) similar to that of the fluorometric decrease in light scattering described in Fig. 2A. At the pre-Ca^{2+} measurement (Fig. 2C, top), no differences in SSC between the samples were observed. Following addition of alamethicin (bottom), all samples demonstrated a strong and similar decrease in SSC (maximal swelling = 100%). Following a 5 min Ca^{2+} insult (middle), a strong decrease in SSC (78.4 ± 4.8% of maximal swelling; $p < 0.01$, Student's t-test), indicative of mPT, is detected in the control sample, whereas no decreases compared to baseline values were detected in the samples treated with CsA, NIM811, or UNIL025 (9.0 ± 4.8, 5.9 ± 2.4, and 5.3 ± 2.6% of maximal swelling, respectively). The histograms in Fig. 2C also demonstrate that the populational spread was similar within the mitochondrial samples before and after the calcium and alamethicin insults, and that there were no subpopulations that did not respond to calcium-induced swelling (control), or that were not protected by the inhibitors of mPT (CsA, NIM811, UNIL025).

Under energized conditions, 1 μM UNIL025 and NIM811 completely blocked mPT in isolated brain mitochondria when exposed to a calcium insult, 2.0 μmol Ca^{2+}/mg mitochondrial protein, which produced extensive swelling in the absence of the cyclosporin analogs (Fig. 2D) Calculated values of the decrease in light scattering was slightly negative for UNIL025 (−0.23 ± 3.9%) and NIM811 (−4.7 ± 3.4%), but substantial for control (76.2 ± 4.1%) (Fig. 2E).

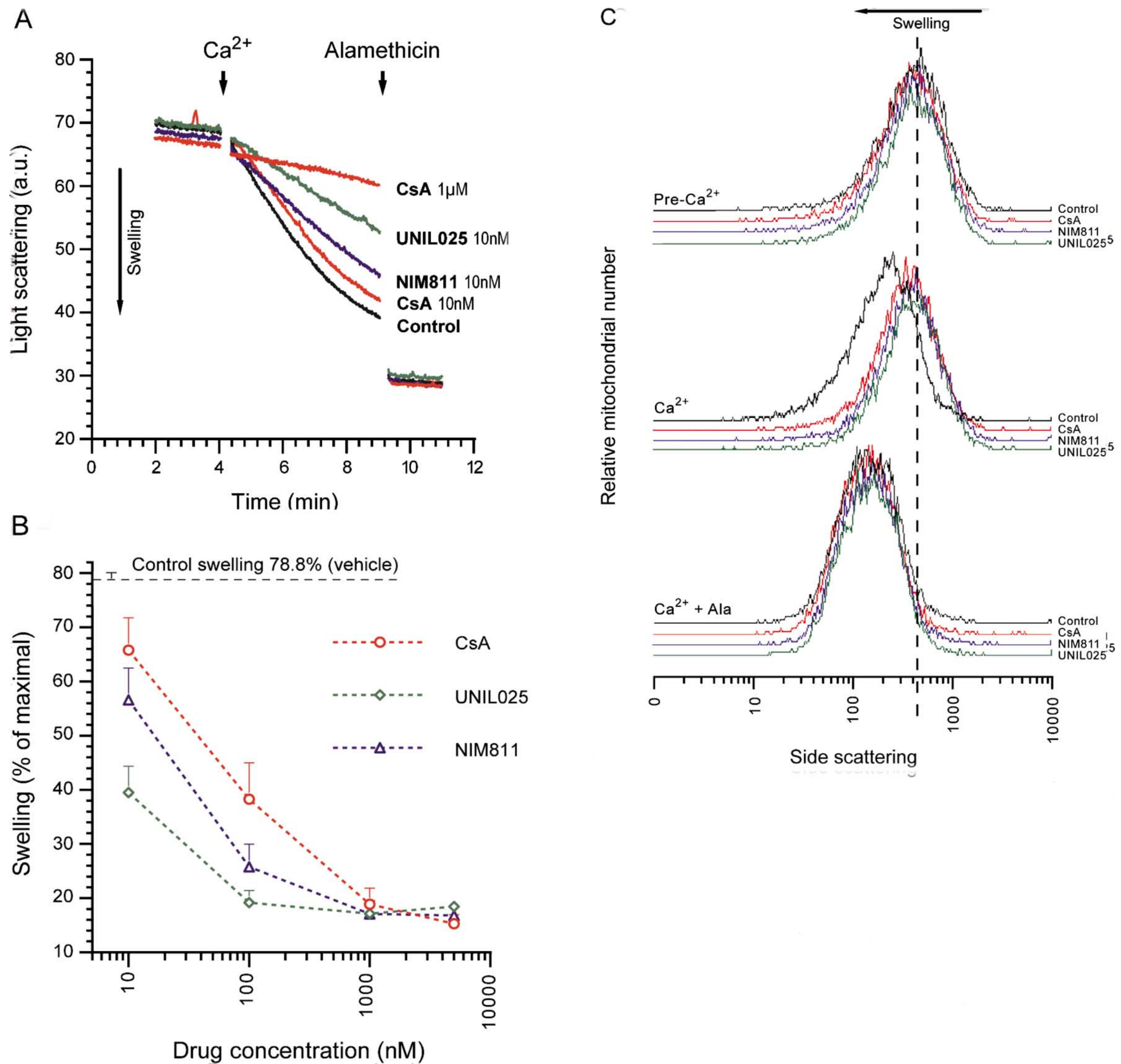

Fig. 2. The nonimmunosuppressive cyclosporin analogs NIM811 and UNIL025 inhibit permeability transition with nanomolar potencies in brain-derived mitochondria. (A) Representative traces of changes in 90° light scattering (a.u. = arbitrary units, Δ520 nm) of experiments run under de-energized conditions at 28°C in isotonic KCl-based swelling buffer, pH 7.3. UNIL025, NIM811, cyclosporin A (CsA), or vehicle (ethanol, 0.2% (v/v), control) were added to mitochondrial suspensions (25 μg protein/ml). Ca^{2+} (final concentration 100 μM) was added after 4 min and each experiment was terminated at 9 min with alamethicin (7.5 μg/ml) to induce maximal swelling. Trace disturbances due to manual mixing were deleted for clarity. (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, and NIM811. Mitochondria treated with Ca^{2+} in the presence of vehicle (ethanol, 0.2% (v/v), control) displayed 78.8% swelling. All values (except for 10 nM CsA, $p = 0.011$ in the stringent post hoc test used) were significantly different from control swelling. 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). Samples were stained with the mitochondrial marker nonyl acridine orange, and 10 000 mitochondria were used for each measurement. 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 (Ca^{2+}, middle) and after induction of maximal swelling by the addition of alamethicin (Ca^{2+}+ Ala, 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. All histograms are representative of three individual experiments. (D) Complete inhibition by UNIL025 and NIM811 of the induction of permeability transition under energized conditions. Experiments were run at 37°C in a sucrose-based buffer containing 5 mM malate/glutamate, 1 μg/ml oligomycin, 20 μM ADP, and 1 μM UNIL025, NIM811, or vehicle (ethanol, 0.2% (v/v), control). 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 (% 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.

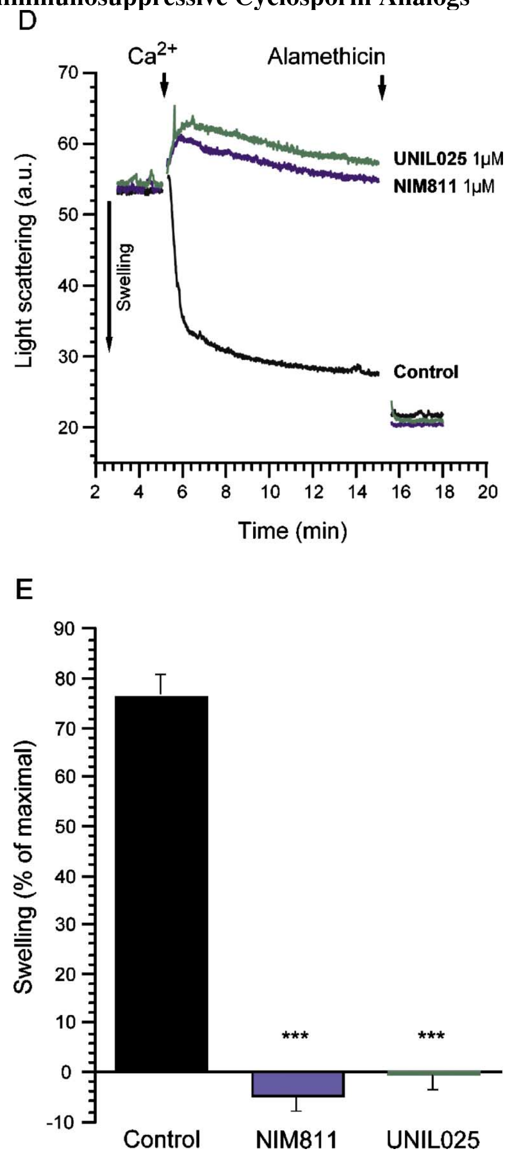

Fig. 2. Continued.

DISCUSSION

Cyclosporins have several biological activities, and their major receptors are a group of intracellular proteins known as cyclophilins. The immunosuppressive activity of CsA is generally attributed to the inhibition of the protein phosphatase calcineurin, mediated by the complex of cyclophilin A (CypA) and CsA (Borel *et al.*, 1996). The binding domains for CypA and calcineurin are located at different sites on the cyclosporin molecule (Fig. 1). Small structural alterations on the latter can dramatically reduce the binding to calcineurin while preserving high affinity to cyclophilins (Waldmeier *et al.*, 2003). A few such cyclosporins have been identified, including the two evaluated in this study, NIM811 and UNIL025. Both are modified on the fourth amino acid, and UNIL025 has an additional modification at the third amino acid (Fig. 1). The previously available nonimmunosuppressive analog MeVal^4-cyclosporin (PKF/SDZ 220-384, Novartis) was also modified at the fourth amino acid. Changes at residue 4 are within the critical binding site to calcineurin, and even subtle changes in the binding can largely abolish the immunosuppressive activity without reducing the cyclophilin affinity (Waldmeier *et al.*, 2003). The present study demonstrates that NIM811 and UNIL025 are equally effective at inhibiting mPT in brain mitochondria using both the de-energized and energized assays, with maximal effects similar to those of CsA (Hansson *et al.*, 2003, 2004). The dose–response studies in the de-energized model display a tendency of increased potency of NIM811 and a significant (almost 10-fold) increased potency of UNIL025 inhibiting the mPT relative to CsA. These results correlate well with the CypA binding activites of these cyclosporins. Using a competitive solid phase binding assay, NIM811 was reported to have a 1.7 times higher binding potency to CypA as compared to that of CsA (Billich *et al.*, 1995). In a cyclophilin cis–trans isomerization assay, the inhibitory potency for UNIL025 was 3.4 times that of CsA (Ruegg *et al.*, unpublished observations). The cyclosporin binding pocket of all cyclophilins is formed by a highly conserved region. Of the known cyclophilins, the mitochondrial CypD has the highest homology to the cytosolic CypA. Further, in the actual cyclosporin binding region the sequence identity is 100% between CypA and CypD (Waldmeier *et al.*, 2003). Therefore, CypA affinity is likely a very good predictor of mPT inhibition, which is dependent on the interaction between CypD and the ANT.

Mitochondria from the brain are derived from different cellular sources, glial as well as neuronal. The characteristics of mPT vary depending on the tissue and cell type from which they are derived (Bambrick *et al.*, 2004). Our previous results indicate that brain-derived mitochondria in general display CsA-sensitive permeability transition, even though they display regional differences in the sensitivity to calcium and other inducers of mPT (Friberg *et al.*, 1991; Hansson *et al.*, 2003, 2004; Mattiasson *et al.*, 2003). Here, using flow cytometry under de-energized conditions, NIM811 and UNIL025 as well as CsA displayed powerful inhibition of mPT in the whole mitochondrial population when exposed to a calcium concentration that rapidly induces mPT and large amplitude swelling when the cyclosporins were not present.

The potent inhibition of mPT by NIM811 and UNIL025 opens the opportunity to further and more stringently assess the hypothesis that mPT is involved in

neuronal cell death. Animal studies strongly implicate mPT as an important mechanism for the neuroprotection displayed by CsA. FK506, a calcineurin-inhibiting immunosuppressant that does not affect the mPT pore has generally been used as a control for calcineurin-mediated neuroprotection, and the more pronounced protective effect of CsA in certain in vivo and in vitro models has been attributed to the inhibition of mPT (for review see Hansson *et al.*, 2004; Keep *et al.*, 2003; Waldmeier *et al.*, 2003). Early results on MeVal^4-cyclosporin has demonstrated that mPT is not dependent on calcineurin activity (Nicolli *et al.*, 1996; Petronilli *et al.*, 1994), which is further confirmed by the present data. MeVal^4-cyclosporin has displayed neuroprotection in an animal model of focal ischemia and in a cell culture model of oxygen/glucose deprivation (Friberg and Wieloch, 2002). NIM811 has previously been demonstrated to inhibit mPT in rat liver mitochondria and to block TNF-induced apoptosis in cultured rat hepatocytes. In contrast to CsA, NIM811 was not as cytotoxic at high concentrations, implying a wider therapeutic window when inhibition of the calcineurin enzyme is absent (Waldmeier *et al.*, 2002). There are two important obstacles in translating the promising preclinical neuroprotective effects of CsA to human use. Unless the blood–brain barrier is disrupted, the penetration of CsA to the central nervous system by regular intravenous or oral administration is poor (Begley *et al.*, 1990), and immunosuppression is an undesirable side effect for many neurological indications (especially chronic diseases). The reported immunosuppressive activity of NIM811 is 1700 times less than that for CsA (Billich *et al.*, 1995) and the toxicity data also seem more favorable, with no significant change in kidney-specific parameters following 10 days of 50 mg/kg of oral NIM811 (Rosenwirth *et al.*, 1994). The same dose regimen of CsA increased serum creatinine and urea concentrations as signs of renal dysfunction. UNIL025 has a similar profile with 7000 times less immunosuppression (as compared to CsA) measured using a nuclear factor of activated T cell dependent reporter assay in Jurkat T cells (Ruegg *et al.*, unpublished observations). In addition, single dose intravenous toxicity for UNIL025 measured as LD50 in Sprague Dawley rats is between 160 and 190 mg/kg (Debiopharm, unpublished observations) which is considerably higher than that reported for CsA (104 mg/kg) (data from the SPC for Sandimmun®). The lesser degree of toxicity may facilitate administration of the analogs for neurological indications. CsA is actively transported over the cerebral capillary membranes back to the blood by the *p*-glycoprotein (P-gp), but the transport can be saturated by high concentrations of CsA and may also be blocked sufficiently by other P-gp substrates (Tsuji *et al.*, 1993). Local administration in the carotid artery also saturates the transport (Yoshimoto and Siesjö, 1999) and intra-thecal delivery has been successful in the treatment of transgenic SOD1 G93A mice (a model of amyotrophic lateral sclerosis) (Keep *et al.*, 2001). In animal models of traumatic brain injury, where the blood-brain barrier is disrupted due to the injury, CsA has been administered via simple intravenous injection or infusion and has displayed potent neuroprotective properties (Okonkwo *et al.*, 2003; Sullivan *et al.*, 2000). In addition, human clinical trials are underway using intravenous CsA treatment of patients with severe closed head injury (Alves *et al.*, 2003; Brophy *et al.*, 2003). If CsA proves to be clinically effective, nonimmunosuppressive cyclosporins may have a therapeutic potential in these patients by retaining the mitochondrial protection while avoiding immunosuppressive and calcineurin-related side effects.

In summary, we have shown that NIM811 and UNIL025 are potent inhibitors of mPT in brain mitochondria. This study is part of a larger project evaluating their neuroprotective potentials and we are currently exploring the neuroprotective effect of the compounds in an in vitro mouse hippocampal slice model of oxygen/glucose deprivation. Further in vivo evaluation of these interesting cyclosporin analogs will reveal if mPT is a central target for neuroprotection and if calcineurin blockade affords additional neuroprotection or counteracts the benefits by dose-dependent cytotoxicity. We envisage that nonimmunosuppressive cyclosporin analogs may be neuroprotective and may prove valuable in the treatment of severe diseases of the central nervous system.

ACKNOWLEDGMENTS

The present study was funded by the Swedish Research Council (Project No. 08644), the Swedish Society of Medicine, the Swedish Brain Foundation, the Swedish Association of Neurologically Disabled, and the foundations of T&E Segerfalk, Bergvall, the Royal Physiographic Society and Åhlén. Authors M. F. K. and E. E. are founders of MaasBiol AB, LLC and NeuroPharma AB, which hold intellectual property rights and develop the use cyclosporins for neurological treatment. The authors are grateful to the Section for Tumour Immunology at Lund University for generous access to their FACSCalibur.

REFERENCES

Alves, O., Tolias, C. M., Lewis, C., Hayes, R., Choi, S., Gilman, C., Enriquez, P., Povlishock, J., and Bullock, M. R. (2003). *J. Neurotrauma* **20**, 1125. (NNS Symposium abstract).

Bambrick, L., Kristian, T., and Fiskum, G. (2004). *Neurochem. Res.* **29**, 601–608.

Begley, D. J., Squires, L. K., Zlokovic, B. V., Mitrovic, D. M., Hughes, C. C., Revest, P. A., and Greenwood, J. (1990). *J. Neurochem.* **55**, 1222–1230.

Billich, A., Hammerschmid, F., Peichl, P., Wenger, R., Zenke, G., Quesniaux, V., and Rosenwirth, B. (1995). *J. Virol.* **69**, 2451–2461.

Borel, J. F., Baumann, G., Chapman, I., Donatsch, P., Fahr, A., Mueller, E. A., and Vigouret, J. M. (1996). *Adv. Pharmacol.* **35**, 115–246.

Brophy, G. M., Bullock, M. R., Tolias, C. M., Alves, O., Enriquez, P., and Gilman, C. (2003). *J. Neurotrauma* **20**, 1125. (NNS Symposium abstract).

Crompton, M. (1999). *Biochem. J.* **341**, 233–249.

Friberg, H., and Wieloch, T. (2002). *Biochimie* **84**, 241–250.

Friberg, H., Connern, C., Halestrap, A. P., and Wieloch, T. (1999). *J. Neurochem.* **72**, 2488–2497.

Halestrap, A. P., McStay, G. P., and Clarke, S. J. (2002). *Biochimie* **84**, 153–166.

Hansson, M. J., Persson, T., Friberg, H., Keep, M. F., Rees, A., Wieloch, T., and Elmér, E. (2003). *Brain Res.* **960**, 99–111.

Hansson, M. J., Månsson, R., Mattiasson, G., Ohlsson, J., Karlsson, J., Keep, M. F., and Elmér E. (2004). *J. Neurochem.* **89**, 715–729.

Keep, M., Elmér, E., Fong, K. S., and Csiszar, K. (2001). *Brain Res.* **894**, 327–331.

Keep, M. F., Uchino, H., and Elmér, E. (2003). In *Immunosuppressant Analogs in Neuroprotection* (Borlongan, C. V., Isacson, O., and Sanberg, P. R., eds.), Humana Press, Totowa, pp. 3–32.

Lemasters, J. J., Nieminen, A. L., Qian, T., Trost, L. C., Elmore, S. P., Nishimura, Y., Crowe, R. A., Cascio, W. E., Bradham, C. A., Brenner, D. A., and Herman, B. (1998). *Biochim. Biophys. Acta* **1366**, 177–196.

Mattiasson, G., Friberg, H., Hansson, M., Elmér, E., and Wieloch, T. (2003). *J. Neurochem.* **87**, 532–544.

Nicolli, A., Basso, E., Petronilli, V., Wenger, R. M., and Bernardi, P. (1996). *J. Biol. Chem.* **271**, 2185–2192.

Okonkwo, D. O., Melon, D. E., Pellicane, A. J., Mutlu, L. K., Rubin, D. G., Stone, J. R., and Helm, G. A. (2003). *Neuroreport* **14**, 463–466.

Petronilli, V., Nicolli, A., Costantini, P., Colonna, R., and Bernardi, P. (1994). *Biochim. Biophys. Acta* **1187**, 255–259.

Rosenwirth, B., Billich, A., Datema, R., Donatsch, P., Hammerschmid, F., Harrison, R., Hiestand, P., Jaksche, H., Mayer, P., Peichl, P., *et al.* (1994). *Antimicrob. Agents Chemother.* **38**, 1763–1772.

Sims, N. (1990). *J. Neurochem.* **55**, 698–707.

Sullivan, P. G., Rabchevsky, A. G., Hicks, R. R., Gibson, T. R., Fletcher-Turner, A., and Scheff, S. W. (2000). *Neuroscience* **101**, 289–295.

Tsuji, A., Tamai, I., Sakata, A., Tenda, Y., and Terasaki, T. (1993). *Biochem. Pharmacol.* **46**, 1096–1099.

Uchino, H., Elmér, E., Uchino, K., Lindvall, O., and Siesjö, B. K. (1995). *Acta Physiol. Scand.* **155**, 469–471.

Waldmeier, P. C., Feldtrauer, J. J., Qian, T., and Lemasters, J. J. (2002). *Mol. Pharmacol.* **62**, 22–29.

Waldmeier, P. C., Zimmermann, K., Qian, T., Tintelnot-Blomley, M., and Lemasters, J. J. (2003). *Curr. Med. Chem.* **10**, 1485–1506.

Yoshimoto, T., and Siesjö, B. K. (1999). *Brain Res.* **839**, 283–291.

Zamzami, N., and Kroemer, G. (2001). *Nat. Rev. Mol. Cell. Biol.* **2**, 67–71.