Hepatoprotection by Cyclosporin A in Experimental Hepatitis. Sorting Desensitization of the Mitochondrial Permeability Transition Pore from Immunosuppression

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We studied the mitochondrial, cellular and hepatoprotective effects of Cyclosporin A (CsA) and DEBIO-025, a CsA derivative where Sar in position 3 and MeLeu in position 4 have been substituted by D-MeAla and EtVal, respectively. At variance from CsA, DEBIO-025 did not prevent nuclear translocation of a Nuclear Factor of Activated T Cells-GFP fusion protein, nor did it inhibit activation of purified mouse T cells, while it was more potent than CsA at desensitizing the mitochondrial permeability transition pore (PTP) to Ca^{2+} both *in vitro* and *ex vivo*. We have compared the effects of CsA and DEBIO-025 in fulminant hepatitis induced in the outbred CD1 mouse strain (i) by injection of lipopolysaccharide of *E. Coli* (LPS) plus D-Galactosamine (D-GalN), a treatment that sensitizes the liver to the proapoptotic effects of TNFalpha; and (ii) by injection with the Jo2 antibody, a treatment that causes hepatic damage by direct stimulation of the Fas receptor. We found comparable levels of hepatoprotection (as assessed by caspase 3 cleavage, release of aminotransferases and animal survival) with CsA and DEBIO-025 after treatment with LPS + DGalN but not with the Jo2 antibody. These results help define the hepatocyte death pathways where the PTP plays a critical role *in vivo*; allow a clear-cut separation of the effects of cyclophilin ligands from calcineurin inhibition; and suggest that DEBIO-025 may be a safe and useful tool for the treatment of TNFalpha-dependent acute hepatitis.

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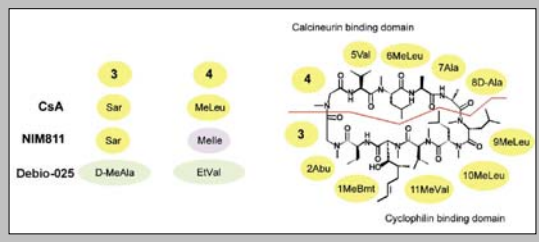
Previous work on rats demonstrated that PTP opening contributes to the hepatotoxic effects of TNF-α downstream of caspase 8 activation, and that Cyclosporin A (CsA) could be used to treat TNF-α-dependent hepatitis

We switched to mice!



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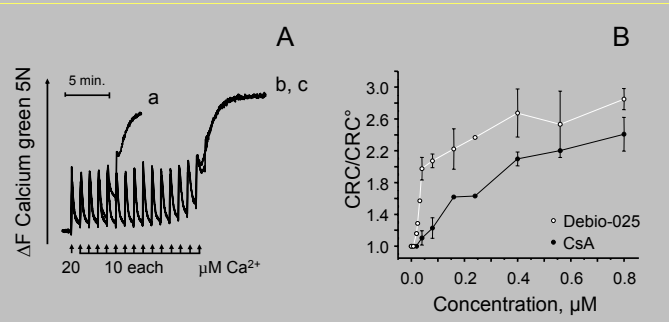
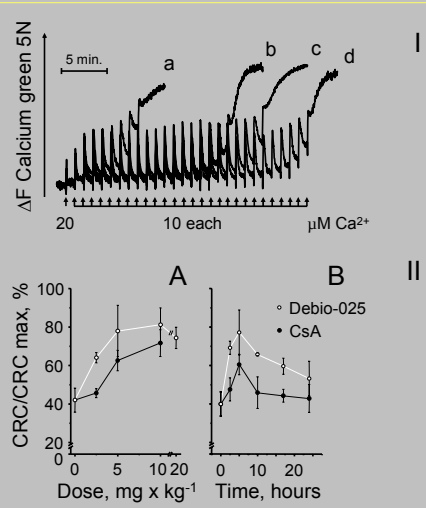
The binding site for cyclophilins to the immunosuppressive CsA molecule covers residues 1-3, 10 and 11 and the interaction with calcineurin residues 4-7. The non-immunosuppressive cyclosporin molecules NIM811 and Debio-025 are both modified at the fourth amino acid (from N-methyl-leucine to N-methyl-isoleucine and N-ethyl-valine, respectively), which prevents calcineurin binding while retaining the ability to interact with cyclophilin. Debio-025 has an additional modification at the third ammino acid (from sarcosine to N-methil-D-alanine).

1



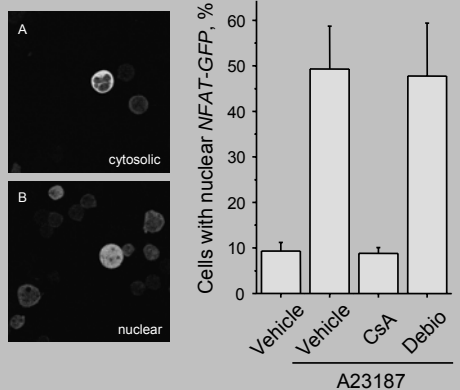
Effects of CsA and Debio-025 on the in vitro CRC of isolated mouse liver mitochondria. Liver mitochondria were isolated from CD1 male mice according to standard differential centrifugation procedures. The incubation medium contained 0.2 M sucrose, 10 mM Tris-MOPS, 5 mM Glutamate-Tris, 2.5 mM Malate-Tris, 1 mM Pi-Tris, 10 μM EGTA-Tris and 1 μM Calcium Green-5N. Final volume was 2 ml, pH 7.4, 25°C. Extramitochondrial Ca^{2+} was measured with a Perkin-Elmer LS50B spectrofluorimeter equipped with magnetic stirring and thermostatic control (excitation 505 nm-emission 535 nm). All the experiments were started with the addition of 0.5 mg x ml^{-1} of liver mitochondria. Panel A. Calcium retention capacity (CRC) of mitochondria without any further addition (trace a) is compared with CRC of mitochondria in presence of 0.8 μM of CsA or Debio-025 (trace b and c respectively). The experiments are representative of four replicates for each condition. Panel B. Values on the ordinate refer to the CRC ± S.D. relative to the CRC of the control mitochondria without any further addition in the assay cuvette (CRC°). at the indicated concentrations of CsA or Debio-025. When present, error bars refer to the S.D. of at least triplicate samples, while individual points refer to individual experiments.

2

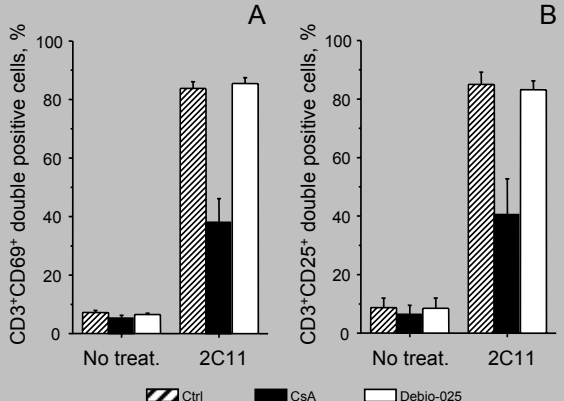


Effects of CsA and Debio-025 on the ex vivo CRC of isolated mouse liver mitochondria. Panel I. Liver mitochondria were isolated 5 hours after i.p. treatment of mice with vehicle (trace a) or with 5 mg x kg^{-1} of body weight of CsA (trace b) or Debio-025 (trace c). In trace d mitochondria from vehicle-treated animals were incubated with 0.8 μM of CsA. Panel II. Liver mitochondria were isolated from CD1 male mice according to standard differential centrifugation procedures 5 h after i.p. treatment with the indicated concentrations (A) or at the indicated times after treatment with 5 mg x kg^{-1} (B) of Debio-025 (open symbols) or CsA (closed symbols). The experimental conditions were as in Figure 1.

3



Effects of CsA and Debio-025 on NFAT-GFP distribution. Jurkat T cells expressing an NFAT-GFP fusion protein were scored for cytoplasmic (panel A) versus nuclear (panel B) localization by confocal microscopy under basal conditions or after treatment with 500 ng x ml^{-1} of A23187. Where indicated, cells had been pretreated with 0.8 μM CsA or Debio-025.



Effects of CsA and Debio-025 on activation of mouse T lymphocytes. T lymphocytes were prepared by a negative selection procedure. $CD3^+$ cells were treated with a stimulatory antibody (2C11) and scored for expression of the activation markers CD69 (panel A) and CD25 (panel B) by FACS analysis. Cells had been pretreated with vehicle (hatched bars), 0.8 μM CsA (closed bars) or Debio-025 (open bars).

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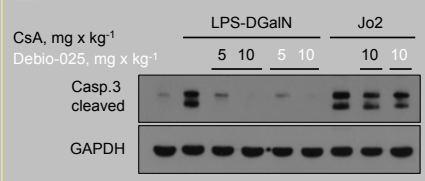

Cleavage of caspase 3 following treatment of CD1 mice with LPS-DGalN or with Jo2 antibody. Effects of Debio-025 and CsA. CD1 mice were treated with vehicle, with 5 or 10 mg x kg^{-1} CsA, with 5 or 10 mg x kg^{-1} Debio-025, followed 1 h later by 20 μg LPS plus 700 mg x kg^{-1} of DGalN or by 200 μg x kg^{-1} of Jo2 antibody. After further 5 h liver extracts were prepared and analyzed for caspase 3 cleavage and for GAPDH by SDS-PAGE and Western blot.

Treatment with	Animals positive for cleaved Caspase 3	% animals positive for cleaved Caspase 3
LPS-DGalN	15/18	83.3
CsA + LPS-DGalN	6/19	31.6
Debio-025 + LPS-DGalN	12/24	50.0

Treatment with	Animals positive for cleaved Caspase 3	% animals positive for cleaved Caspase 3
Jo2	13/13	100
CsA + Jo2	15/18	83.3
Debio-025 + Jo2	6/6	100

Summary of the effects of treatment with CsA and Debio-025 on caspase 3 cleavage induced by LPS-DGalN or Jo2 antibody in CD1 mice. CD1 mice were treated with vehicle, 20 μg LPS plus 700 mg D-GalN x kg^{-1} , 0.2 mg x kg^{-1} Jo2 antibody and CsA or Debio-025 as denoted in the first column, and scored for cleaved caspase 3. The dose of CsA and Debio-025 was between 5 and 10 mg x kg^{-1}. The results are presented together for clarity because there were no significant difference in the protection or lack thereof in this dose range.

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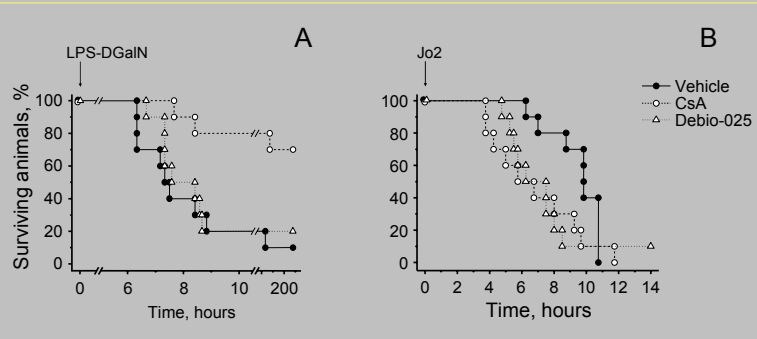

Survival curves of CD1 mice treated with LPS-DGalN or Jo2 antibody. Effects of Debio-025 and CsA. CD1 mice were treated with vehicle (closed symbols) or with 10 mg x kg^{-1} of CsA (open circles) or Debio-025 (open triangles), followed 1 hour later by 20 μg x kg^{-1} of LPS plus 700 mg x kg^{-1} of DGalN (panel A) or by 200 μg x kg^{-1} of Jo2 antibody (panel B).

These results help define the hepatocyte death pathways where the PTP plays a critical role *in vivo*; allow a clear-cut separation of the effects of cyclophilin ligands from calcineurin inhibition; and suggest that despite its short-term protective effects on activation of the PTP-dependent mitochondrial cell death pathway Debio-025 may not be as effective as CsA for the treatment of TNFα-dependent acute hepatitis.