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3 **Everolimus and Sirolimus antagonize Tacrolimus based calcineurin**
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5 **inhibition via competition for FK-binding protein 12**
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11 Johannes van Pelt¹
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34 **Journal:** Biochemical Pharmacology
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36 **Category:** inflammation and immunopharmacology
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41 **Abbreviations:** CN, calcineurin; TRL, tacrolimus; mTOR, mammalian target of
42 rapamycin; MTIs, mTOR inhibitors; FKBP, FK506-binding protein; PBMCs, peripheral
43 blood mononuclear cells; CsA, cyclosporin A; SRL, sirolimus; EVL, everolimus; PHA,
44 phytohemagglutinin; PMA, phorbol-12-myristate-13-acetate; RPMI, roswell park memorial
45 institute medium; iFCS, heat inactivated fetal calf serum; IC₅₀, half maximal inhibition
46 concentration; CI, confidence interval; E_{max}, maximal drug effect; IL, interleukin; SEM,
47 standard error of the mean
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Abstract

The calcineurin inhibitors cyclosporin A and tacrolimus and the inhibitors of the mTOR, sirolimus and everolimus bind immunophilins that are required for their immunosuppressive action. In contrast to cyclosporin A, tacrolimus and the mTOR inhibitors (MTIs) share common immunophilins, the FK506-binding proteins (FKBPs). We investigated the immunosuppressive interactions of MTIs on tacrolimus based immune suppression, since insights in immunological drug-drug interactions can be very relevant for optimization of immunosuppressive regimens in allograft transplantation medicine. Isolated peripheral blood mononuclear cells from healthy volunteers were incubated with combinations of MTIs and calcineurin inhibitors and when monitored for calcineurin activity and IL-2 excretion after mitogen stimulation, tacrolimus IC₅₀ concentrations shifted to higher concentrations in the presence of MTIs. This antagonism was absent for cyclosporin A, reproducible for 10 healthy volunteers (p<0.001) and stronger for sirolimus than for everolimus. When cell lysate was treated with and without MTI, tacrolimus and FKBP12, FKBP12 could increase calcineurin inhibition by tacrolimus and reverse the MTI antagonism for both MTIs. These results demonstrate that FKBP12 can be rate limiting for calcineurin inhibition at high tacrolimus concentrations and that the antagonism of sirolimus and everolimus on tacrolimus based immune suppression is mediated via saturation of FKBP12.

1
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3 **Keywords:** Calcineurin, tacrolimus, everolimus, sirolimus, transplantation
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1. Introduction

After organ transplantation, immune suppression to prevent allograft rejection is crucial for successful graft survival. Several immunosuppressive agents, such as the calcineurin (CN) inhibitors tacrolimus (TRL) or cyclosporin A (CsA) and the mammalian target of rapamycin (mTOR) inhibitors sirolimus (SRL) or its derivative everolimus (EVL), are used and combinational therapies are applied to achieve optimal immunosuppressive effect and reduce individual drug toxicity [1]. CsA and TRL inhibit CN, a calcium/calmodulin dependent serine/threonine protein phosphatase that plays an important role in mediating T cell receptor stimuli into cytokine (IL-2, IFN γ) transcription. SRL and EVL inhibit mTOR, a kinase with a key function in cell cycle progression [1,2]. Both classes of drugs inhibit enzyme activity only after binding to specific intracellular binding proteins, so called immunophilins [3,4]. CsA binds selectively to the cyclophilin class of immunophilins that are cytosolic peptidyl-prolyl isomerases, while TRL and the mTOR inhibitors (MTIs) bind to the FK506-binding proteins (FKBP) that also exert peptidyl-prolyl isomerase activity [3]. SRL, EVL and TRL bind similar FKBP, while distinct and selective inhibition of respectively mTOR and CN is observed [7]. Since similar FKBP are required for successful CN inhibition by TRL and mTOR inhibition by SRL or EVL, pharmacological drug-drug interactions have been investigated by several groups. Dumont et al. has shown that SRL and TRL are reciprocal antagonists [5,8], while others have demonstrated immunosuppressive synergism of these drugs [9,10].

In clinical practice, pharmacokinetic drug-drug interactions on metabolic and drug-transporter proteins are therapeutically avoided or adjusted for by therapeutic drug

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3 monitoring. When drug-drug interactions occur on the pharmacologic level and thus inhibit
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5 each others effectiveness, concentration measurement becomes less relevant since it is
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7 unable to detect this interaction [10]. In addition, combinations of immunosuppressive
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9 drugs might be associated with immunosuppressive synergism [11]. Pharmacodynamic
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11 monitoring strategies that can detect these interactions may prove to be useful to further
12
13 optimize immunosuppressive regimen [12].
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16 In this respect we studied the interaction of EVL and SRL on TRL based
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18 immunosuppression in peripheral blood mononuclear cells (PBMCs) isolated from healthy
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20 individuals. Pharmacological interaction was investigated by measurement of CN enzyme
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22 activity. For immunological monitoring, the IL-2 downstream product of CN was used.
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2. Materials and Methods

2.1 Materials

The following materials and reagents were used: Buffy coats were purchased from Sanquin (Amsterdam, NL). RPMI 1640, heat inactivated fetal calf serum (iFCS), penicillin/streptomycin, phytohemagglutinin (PHA) and IL-2 EASIA kit from Invitrogen (Carlsbad, CA), NP-40, soybean trypsin inhibitor, cyclosporin A, tacrolimus, sirolimus (rapamycin), FKBP12 and phorbol-12-myristate-13-acetate (PMA) from Sigma-Aldrich (Steinheim, Germany). Phenylmethylsulfonyl fluoride from MERCK (Darmstadt, Germany), leupeptin from Roche (Basel, Switzerland), aprotinin from Bayer (Leverkusen, Germany), phosphate standard concentrate and everolimus from Fluka (Buchs, Switzerland) and the malachite green calcineurin activity assay kit from Biomol (Plymouth Meeting, PA). All other chemicals and reagents were of the highest available grade.

2.2 Immunosuppressants

The immunosuppressants CsA (MW 1203), TRL (MW 804), EVL (MW 958) and SRL (MW 914) were dissolved in ethanol and serial diluted in ethanol. These immunosuppressant stock solutions were diluted in the experiments resulting in final CsA, EVL and SRL concentrations of 0.025, 0.25, 2.5, 25, 250 and 2500 µg/L and final TRL concentrations of 0.00625, 0.0625, 0.625, 6.25, 62.5 and 625 µg/L. Final ethanol concentrations were 0.25% for single immunosuppressant experiments and 0.5% for the interaction experiments. For the CsA inhibition curve in figure 2, CsA ranged from 0.1 to

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3 3600 µg/L with final ethanol concentrations of 1 %. The ethanol concentrations resulted in
4
5 a 5% increase in CN activity when compared to an ethanol blank (n=4, p=0.04).
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8 9 10 **2.3 PBMC isolation and cell culture**

11
12 Peripheral blood mononuclear cell (PBMC) fraction of healthy volunteer blood was
13
14 isolated from buffy coats by ficoll density gradient centrifugation. Remaining of
15
16 erythrocytes and thrombocytes were lysed and washed out by NH₄Cl lysis buffer (8.4 g/L
17
18 NH₄Cl, 1.0 g/L KHCO₃, pH 7.3) and resuspended in phosphate buffered saline (PBS, 154
19
20 mmol/L NaCl, 1.4 mmol/L phosphate pH 7.5) for cell count on a Sysmex XE2100. Cells
21
22 were centrifuged (10 min., 350g, 4 °C) and resuspended at a concentration of 2·10⁶
23
24 cells/mL in RPMI 1640 cell culture containing 10% iFCS, 100 U/mL penicillin and 100
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26 µg/mL streptomycin.
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30 For CN activity measurement, cells were incubated for 1 hr at 37 °C with
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32 immunosuppressant(s). After incubation, samples were centrifuged (10 min., 350g, 4 °C)
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34 and cell pellets were washed once with 400 µL HEPES buffered saline (9.0 g/L NaCl, 10
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36 mmol/L HEPES pH 7.5), resuspended in 400 µL lysis buffer (50 mmol/L Tris HCl pH 7.7,
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38 1.0 mmol/L dithiothreitol, 5.0 mmol/L ascorbic acid, 0.02% (v/v) NP-40, 50 mg/L soybean
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40 trypsin inhibitor, 50 mg/L phenylmethylsulphonyl fluoride, 5.0 mg/L leupeptin and 5.0
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42 mg/L aprotinin) and lysed by 3 freeze thaw cycles (liquid N₂ / 30 °C). Cell debris was
43
44 centrifuged (10 min., 10000g, 4 °C) and supernatants were snap frozen in liquid N₂ before
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46 storage at -80 °C till CN activity measurement [13].
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50 For IL-2 excretion measurement, cells were stimulated by PHA/PMA 1 hour after
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52 incubation with immunosuppressant(s), and incubated overnight at 37 °C in a humidified
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3 environment (5%, CO₂). PMA stock solution was 4000 times diluted in cell cultures
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5 resulting in a final PMA and DMSO concentration of respectively 5.3 nM and 0.025 %,
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7 and PHA was 100 times diluted according to manufacturers' protocol. The next day, cells
8
9 were centrifuged (10 min., 350g, 4 °C) and supernatant was stored at -20 °C until IL-2
10
11 concentration measurement.
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14 15 16 17 **2.4 Cell lysate experiments**

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19 In order to investigate the role of FKBP, we isolated PBMCs from buffycoats obtained
20
21 from healthy volunteers. Untreated PBMCs were lysed as described before and aliquoted
22
23 lysate was incubated in duplicate (1 hr, 37 °C) with and without TRL, MTI and FKBP12
24
25 resulting in final lysate concentrations of 25 µg/L TRL, 50 µg/L of SRL/EVL and 2000
26
27 µg/L and 10000 µg/L of extra FKBP12. At these concentrations the molar concentrations of
28
29 the MTIs, TRL and FKBP12 were of the same order of magnitude. Directly after
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31 incubation CN activity was determined.
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38 39 **2.5 Calcineurin assay**

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41 Calcineurin phosphatase activity was measured as described before [13]. In brief, CN
42
43 phosphatase was quantified by measurement of the dephosphorylation of the
44
45 phosphorylated RII substrate in the presence of okadaic acid. 15 µL of sample lysis was
46
47 added to 40 µL of assay buffer (0.313 µmol/L calmodulin, 0.375 mmol/L RII
48
49 phosphopeptide substrate, 75 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 9 mmol/L MgCl₂,
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51 0.75 mmol/L dithiothreitol, 0.0375 % NP-40, 0.625 µmol/L okadaic acid, 0.75 mmol/L
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53 CaCl₂ and 5 mmol/L ascorbic acid) and substrate blanks were used to correct for
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3 background absorbance. The assay was run for 30 min. at 30 °C and stopped by addition of
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5 100 µL of malachite green reagent. Colour was developed the next 50 min. before
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7 absorbance measurement at 620nm, and phosphate was quantified by means of a 0-5 nmol
8
9 calibration curve.
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11 12 13 14 **2.6 Cytokine concentration measurement**

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17 IL-2 concentrations were determined with the EASIA kit (Invitrogen) and performed
18
19 according to manufacturers' protocol.
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22 23 24 **2.7 Data analysis and statistics**

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26 Values are presented as mean of duplicate and blank experiments were performed in
27
28 quadruple. CN activity and IL-2 concentrations were expressed as % of blank measurement
29
30 in order to remove inter-individual variation in absolute CN activity and IL-2 excretion.
31
32 CN inhibition curves and IL-2 cytokine inhibition curves were fitted by sigmoidal dose-
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34 response curves using GraphPad software and IC₅₀ concentrations, E_{max} values and 95%
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36 confidence intervals (CI) were obtained from these regression analysis. Student t-tests were
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38 used to test significance and statistical significance was defined as p<0.05.
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3. Results

3.1 IL-2 inhibition by TRL and CsA in the presence of EVL and SRL

To gain insights whether and under which conditions the immunological interaction between the MTIs and TRL occurred, we first measured the effect of the MTIs on IL-2 inhibition by TRL. PBMCs isolated from healthy volunteers were stimulated using PHA/PMA and IL-2 excretion was measured in the presence of TRL or CsA and various concentrations of EVL or SRL. The results of these experiments are shown in figure 1 and the obtained IC₅₀ values of CsA and TRL in the presence of various concentrations of EVL and SRL are listed in table 1.

The MTIs inhibited IL-2 excretion in the PHA/PMA stimulated PBMCs themselves at the concentration range used and this inhibition increased with increasing concentrations of MTI, though no clear sigmoidal concentration response relationship was visible as well as no inhibition of total IL-2 excretion. When high TRL concentrations were used in combination with EVL or SRL, low concentration of the MTIs did not significantly effect IL-2 inhibition by TRL. However at higher concentrations (250 µg/L), significant shifts in IL-2 IC₅₀ values were observed. MTI concentrations of 25 µg/L and higher reversed IL-2 inhibition by TRL though the occurrence and magnitude of this effect is also dependent on the TRL concentration and seems to be less for EVL when compared to SRL (figure 1A and 1B). No increasing IC₅₀ values and significant differences in IC₅₀ values were observed when CsA was used in combination with EVL and SRL (figure 1C and 1D).

3.2 CN inhibition by CNIs, MTIs and combinations thereof

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3 To further investigate the origin of the observed IL-2 antagonism, we measured calcineurin
4 activity that is the pharmacological target enzyme of TRL. First, the individual effect of the
5 immunosuppressants on CN activity was investigated and therefore CN inhibition curves
6
7 were determined for CsA, TRL, EVL and SRL. The data in figure 2 shows that SRL and
8
9 EVL do not inhibit CN activity, while for TRL and CsA a clear dose-response curve was
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11 found with an IC_{50} value of 1.0 $\mu\text{g/L}$ (0.51 - 2.1 $\mu\text{g/L}$, 95% CI) and an E_{max} of 53% (48 -
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13 58%, 95% CI) for TRL and an IC_{50} value of 14 $\mu\text{g/L}$ (9.6 – 21 $\mu\text{g/L}$, 95% CI) and an E_{max}
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15 of 31% (26 - 36%, 95% CI) for CsA.
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22 Next, we incubated five concentrations of TRL (0.0625 - 625 $\mu\text{g/L}$) and a TRL blank, each
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24 in combination with five concentrations (0.25 - 2500 $\mu\text{g/L}$) and a blank of either EVL or
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26 SRL. As control experiment TRL was replaced by CsA (0.25 - 2500 $\mu\text{g/L}$). The obtained
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28 CN inhibition curves are shown in figure 3 and the calculated IC_{50} concentrations for TRL
29
30 and CsA in the presence of various concentrations of EVL and SRL are listed in table 2.
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34 A concentration dependent effect of EVL and SRL on CN inhibition by TRL was observed
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36 and significant higher TRL IC_{50} values were found when 25 $\mu\text{g/L}$ SRL and 250 $\mu\text{g/L}$ EVL
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38 or higher, were present in culture medium (figure 3A and 3B). This pattern was not
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40 detected for the CsA CN inhibition curves; here no differences in IC_{50} values were
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42 observed by increasing concentration of MTIs (figure 3C and 3D).
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46 47 **3.3 Antagonism of MTIs on TRL based immune suppression in 10 healthy volunteers**

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49 To determine whether the TRL/MTI interactions are reproducible for other individuals we
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51 investigated the interaction on PBMCs isolated from 10 healthy volunteers.
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3 For reproduction of the immunological antagonism we reproduced the experiment for 10
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5 healthy volunteers and treated the PBMCs with 0.625 $\mu\text{g/L}$ TRL and 25 $\mu\text{g/L}$ of EVL or
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7 SRL, concentrations that previously (figure 1) showed to be characteristic for the
8
9 interaction. The results are shown in figure 4.

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12 All three drugs inhibited IL-2 excretion in our cell culture system, though TRL caused a
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14 larger inhibition than SRL ($p=0.001$) and EVL ($p=0.002$) that partially inhibited the IL-2
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16 excretion in these concentrations. When combinations of TRL and MTIs were tested, SRL
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18 significantly reversed IL-2 excretion inhibition by TRL ($p=0.004$), while for EVL no
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20 difference in IL-2 levels was found ($p=0.53$). The IL-2 concentrations observed for the
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22 EVL/TRL combination was also significant lower when compared to the combination of
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24 SRL/TRL ($p=0.01$).

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29 Next, we determined CN activity in the presence/absence of TRL (6.25 $\mu\text{g/L}$) and/or
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31 EVL/SRL (25 $\mu\text{g/L}$), concentrations that were previously illustrative for the interaction
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33 (figure 3).

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36 Here no CN inhibition was found for SRL and EVL. TRL caused a mean CN inhibition of
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38 44% \pm 11% (SD) in the PBMC fractions isolated from these individuals. When MTIs were
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40 co-incubated with TRL in the culture medium, CN inhibition by TRL was partially lost and
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42 final mean inhibition in the presence of SRL and EVL was 18% \pm 14% and 26% \pm 10%,
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44 respectively. Both were significant lower ($p<0.001$) when compared to TRL alone.

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47 Antagonism of SRL on TRL mediated CN inhibition was significantly larger than that of
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49 EVL for these concentrations ($p=0.01$).

50 51 52 53 54 55 **3.4 Role of FKBP**

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3 To investigate whether the mechanism of antagonism between both mTOR inhibitors and
4
5 TRL was mediated via competition for saturated FKBP binding sites, we prepared PBMC
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7 lysate from untreated cells of 4 healthy volunteers in duplicate and incubated (1 hr, 37 °C)
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9 these with and without mTOR inhibitor, TRL and FKBP12. CN activity was determined
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11 directly after incubation. The results of these experiments are shown in figure 5.
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15 In this test system no inhibition of CN was found by the MTIs, FKBP12 and combinations
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17 thereof (data not shown). Inhibition by TRL in cell lysate was observed though it was much
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19 smaller ($\pm 10\%$) compared to the cell systems. Since the addition of 2000 $\mu\text{g/L}$ FKBP12
20
21 increased TRL inhibition ($\pm 35\%$, $p < 0.001$) it seemed that the FKBP12 pool from the
22
23 intracellular compartment was diluted in the cell lysate, limiting CN inhibition by TRL.
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26 Addition of SRL to TRL reduced TRL inhibition of CN ($p = 0.005$), while for EVL
27
28 antagonism under these conditions could not be detected ($p = 0.7$). In the presence of 2000
29
30 $\mu\text{g/L}$ FKBP12 antagonism for both SRL and EVL was found (for both $p < 0.001$) and when
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32 10000 $\mu\text{g/L}$ FKBP12 was used, antagonism of both MTIs was no longer observed.
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4. Discussion

In transplant recipients, multiple immunosuppressive agents are normally used in order to optimally prevent acute rejection episodes and to reduce the side effects of the individual drugs [1]. The CNI and MTI class of drugs require intracellular binding proteins (immunophilins) to exert their immunosuppressive action. TRL binds to its intracellular binding proteins (FKBPs) and especially the 12 kDa protein FKBP12 is considered critical for the immunosuppressive action of TRL [14]. The MTIs SRL and its analog EVL require binding to the same protein though an other immunosuppressive mechanism is observed for these drugs [6,15]. Where TRL inhibits CN and block T-cell receptor induced activation/proliferation of T cells, the mTOR inhibitors inhibit IL-2 induced T-cell proliferation [7,15]. In this study, using a CN assay, we confirmed that CN enzyme activity is not inhibited by EVL and SRL but selectively by TRL and CsA (figure 2), which is in agreement with previous observations.

In studies by Dumont et al., reciprocal antagonism was observed for SRL and TRL which was explained by the use of similar intracellular binding sites of the drugs [5,8]. Here we show that both SRL and EVL antagonize TRL mediated CN inhibition and TRL inhibition of IL-2 excretion in PHA/PMA stimulated PBMCs, an effect which is not observed for the CNI CsA. In addition, we show that the antagonism between MTIs and TRL in cell lysate is reversed by the addition of excess of FKBP12 (10000 µg/L), indicating that the antagonism is mediated via this protein.

The *in vitro* antagonism observed in our cellular system is significant starting at SRL concentrations of 25 µg/L for CN activity and 250 µg/L for IL-2 excretion and at EVL

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3 concentrations of 250 $\mu\text{g/L}$ for both CN inhibition and IL-2 excretion inhibition by TRL.

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5 These relative high concentrations of MTIs in combination with TRL seem to saturate
6
7 FKBP binding sites that are required for CN inhibition by TRL. Competition between the
8
9 MTIs and TRL for these binding sites is clearly illustrated by the IL-2 interaction
10
11 experiments in figure 1. Here it is shown that 0.625 $\mu\text{g/L}$ TRL itself inhibits IL-2 excretion,
12
13 but when increasing concentrations of SRL are introduced, above a certain threshold (25
14
15 $\mu\text{g/L}$ SRL), IL-2 inhibition by TRL is lost and the IL-2 response is more comparable to the
16
17 25 $\mu\text{g/L}$ SRL response itself.
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21 The MTIs themselves reduce IL-2 excretion in our cell culture system, without showing a
22
23 clear sigmoidal concentration-response relationship. This effect is probably best explained
24
25 to be indirect and mediated through inhibition of proliferation/activation of IL-2 producing
26
27 cells. In our system the lowest MTIs concentrations used already significantly decreased
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29 IL-2 excretion. Under these conditions of low MTI concentration, immunosuppressive
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31 additivity or even synergism could be very well achieved in patients in combination
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33 protocols with CsA, but also TRL; although MTIs are mechanistically antagonists of TRL,
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35 antagonism only occurs at saturating conditions for FKBP. Immunosuppressive synergy
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37 has been demonstrated for SRL/TRL and SRL/CsA combinations on lymphocyte
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39 proliferation and lymphocyte activation markers [10].
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46 25 $\mu\text{g/L}$ of both EVL and SRL partially reversed CN inhibition by 6.25 $\mu\text{g/L}$ TRL though
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48 the interference by SRL was significant larger when compared to EVL in 10 healthy
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50 volunteers. In addition, only SRL could significantly reverse IL-2 inhibition by 0.625 $\mu\text{g/L}$
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52 TRL. These results indicate relevant differences between both MTIs that could be
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54 explained by the weaker binding of EVL to FKBP12 [6].
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5 Kung and Halloran showed that at high concentrations of CNIs, immunophilins limit CN
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7 inhibition for both CsA and TRL [16]. It is however not known whether this is relevant for
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9 CNI treated patients. When using combination immunosuppressant therapy of both MTI
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11 and TRL, saturation of FKBP and thus pharmacological drug-drug interaction is even
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13 more likely and this interaction could therefore be clinically relevant. The MTI
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15 concentrations for which TRL antagonism was observed (25 µg/L) are of the same
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17 magnitude of MTI blood concentrations in allograft recipients treated with these drugs [17].
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19 In addition, also the TRL concentrations at which the interference was observed are found
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21 in allograft recipients treated with this drug [17]. The drug concentrations that are used in
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23 our *in vitro* model can however not directly be translated to full blood drug concentrations,
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25 since drug concentrations from cell culture experiments do not necessarily show similar
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27 cellular responses compared to drug concentrations in blood due to the presence of
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29 irrelevant binding sites as has been demonstrated for CsA [18]. The true clinical relevance
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31 remains therefore unclear.
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38 If CN inhibition is limited by the immunophilin content in patients receiving CNI, this
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40 could be of great importance; higher CNI concentrations are ineffective. Especially early
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42 after graft implantation, when the blood concentrations are highest, this might become
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44 relevant. Consequently, under condition of immunophilin saturation, inter-individual
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46 variation in the content of immunophilins could be of great importance for allowing
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48 different maximal CN inhibitions.
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53 Finally, blood drug concentrations and also intracellular drug concentrations are not able to
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55 detect the limitation of CN inhibition by immunophilins. Only pharmacodynamic strategies
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3 are theoretically capable of detecting these. This also holds for detecting the interaction
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5 between the MTI and TRL in individuals receiving both drugs.
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8 In conclusion, it is shown that mTOR inhibitors can antagonize TRL and not CsA mediated
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10 immunosuppression. FKBP12 that is bound by EVL and SRL, and required for TRL based
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12 CN inhibition is a key mediator; saturation of this protein by high concentrations of MTIs
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14 and TRL results in competition for FKBP12.
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Legends for figures

Figure 1. IL-2 inhibition by TRL and CsA in the presence of MTIs. IL-2 inhibition by TRL was determined in the presence of various concentrations of SRL (A) and EVL (B). C and D show the CN inhibition by CsA in the presence of the same concentrations of SRL and EVL respectively. Data show the mean of duplicate measurement for one healthy individual.

Figure 2. CN inhibition by CsA, TRL, SRL and EVL. Data are normalized and expressed as % by dividing through CNI blank measurement (performed in quadruple) and maximum effect was fixed at 100% for the sigmoidal dose-response analysis in Graphpad. Data are presented as mean of duplicate measurement.

Figure 3. CN inhibition by CsA and TRL in the presence of MTIs. CN inhibition by TRL was determined in the presence of SRL (A) and EVL (B). C and D show the CN inhibition by CsA in the presences of the same concentrations of SRL and EVL respectively. Data show the mean of duplicate of one healthy individual and the lines are the CN inhibition curves of TRL and CsA in the presence of various concentrations of EVL and SRL fitted for sigmoidal dose-response curves by Graphpad software. The IC₅₀ values for CsA and TRL obtained from these curves are listed in table 2. Data was normalized by dividing through blank measurement in the absence of CNI and MTI. CNI 0 values were set at 10⁻⁴ µg/L for the regression analysis.

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5 **Figure 4. MTI antagonism in 10 healthy volunteers.** Left: IL-2 excretion after
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7 PHA/PMA stimulation for combinations of TRL (0.625 µg/L TRL) and MTIs (25 µg/L).
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9 Right: CN activity in the presence/absence of 6.25 µg/L TRL, and 25 µg/L EVL or SRL.
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11 Both were tested on PBMCs isolated from 10 healthy volunteers and data was normalized
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13 to remove inter-individual variation in absolute IL-2 excretion and CN activity. Data are
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15 presented as mean ± SEM and statistical significance was tested using paired student t-tests
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17 and presented as *** for p<0.001 and ** for p<0.01.
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24 **Figure 5. CN activity after FKBP12, TRL and MTI addition to cell lysate.** CN activity
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26 was determined in PBMC lysate in the presence/absence of TRL, FKBP12 and either SRL
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28 or EVL and expressed as % of blank measurement. Final concentrations were 25 µg/L
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30 TRL, 50 µg/L of SRL/EVL and 0, 2000 and 10000 µg/L of FKBP12 was added to cell
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32 lysate. Results were obtained from duplicate measurement of 4 healthy volunteers and
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34 presented as mean ± SEM. Statistical analysis were performed between TRL and TRL +
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36 SRL or EVL conditions, except when lines indicate otherwise and presented as *** for
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38 p<0.001 and NS for not significant.
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Table 1.

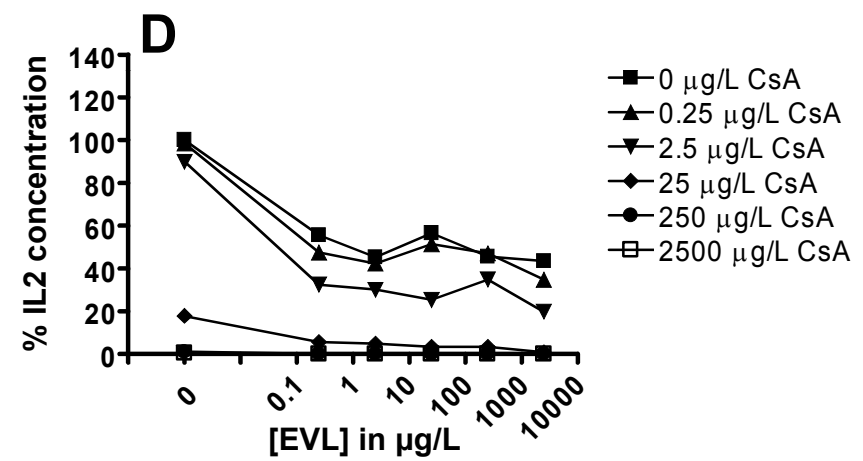
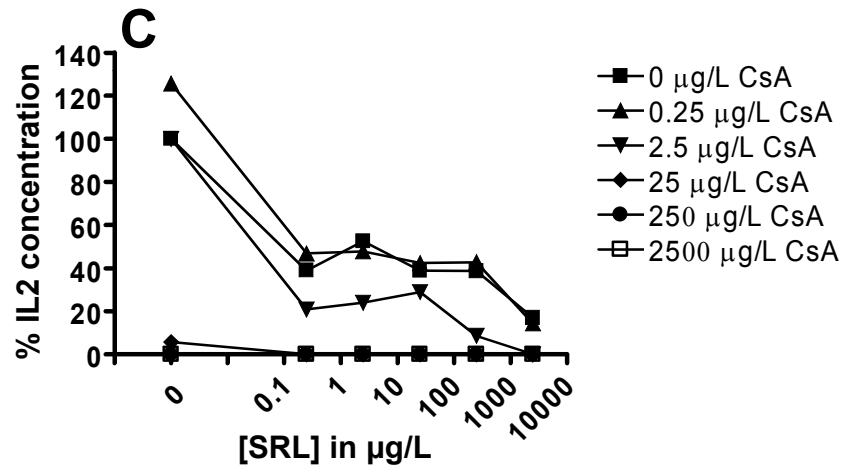
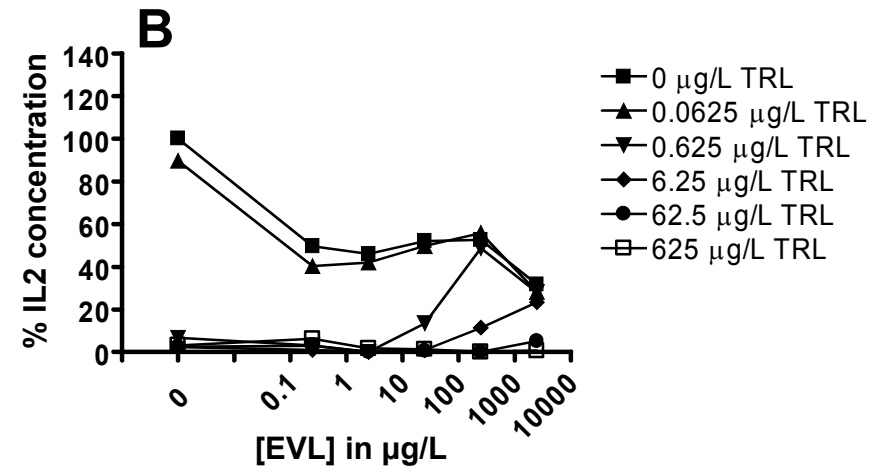
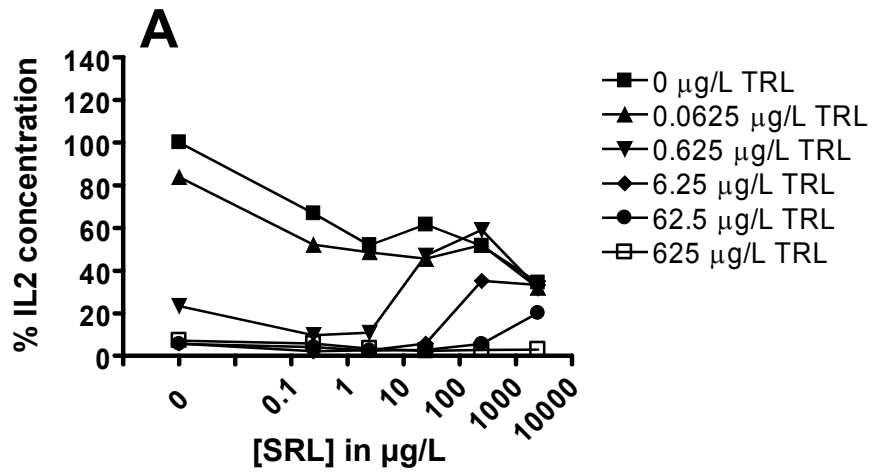
	[mTOR inhibitor] in µg/L	Tacrolimus IC ₅₀ in µg/L (95% CI)	Cyclosporin A IC ₅₀ in µg/L (95% CI)
Sirolimus	0	0.20 (0.12 - 0.33)	8.5 (2.6 - 27)
	0.25	0.14 (0.09 - 0.23)	2.7 (1.1 - 6.7)
	2.5	0.22 (0.11 - 0.44)	2.1 (1.1 - 4.1)
	25	1.6 (0.57 - 4.6)	4.7 (2.4 - 9.2)
	250	11 (4.8 - 26)	1.2 (0.35 - 4.1)
	2500	114 (56 - 234)	0.59 (0.05 - 6.8)
Everolimus	0	0.15 (0.06 - 0.36)	9.5 (4.7 - 19)
	0.25	0.13 (0.05 - 0.31)	3.6 (2.1 - 6.3)
	2.5	0.13 (0.05 - 0.38)	4.7 (2.9 - 7.5)
	25	0.28 (0.14 - 0.55)	2.1 (0.84 - 5.0)
	250	2.6 (1.4 - 4.6)	5.6 (3.1 - 10)
	2500	19 (11 - 32)	2.0 (0.84 - 4.9)

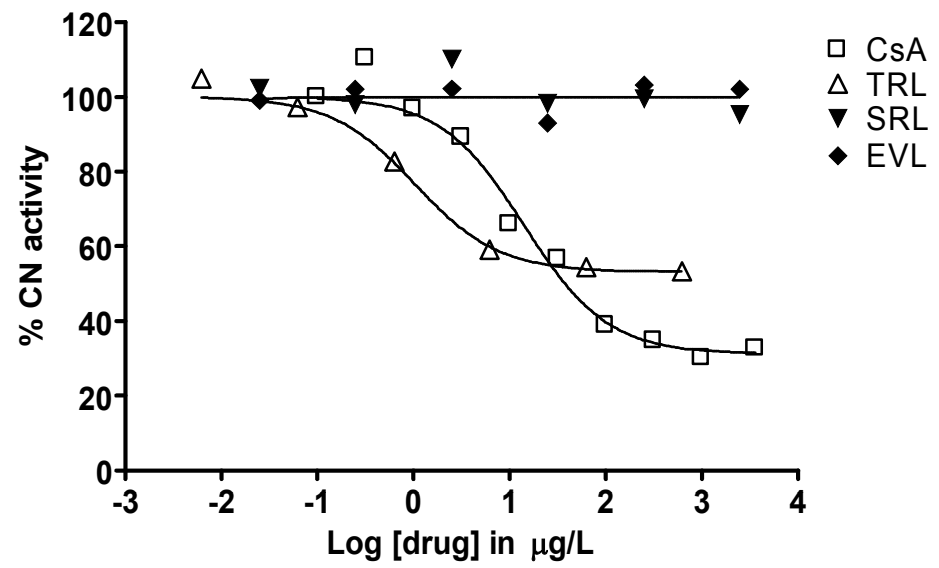
Table 1. IL-2 IC₅₀ values of TRL and CsA in the presence of various concentrations of SRL and EVL. IC₅₀ values and 95% Confidence Intervals (CI) were obtained from the sigmoidal concentration-response regressions when the data in figure 1 is presented with the CNI inhibitor on the x-axis and the individual curves are variations in EVL or SRL concentrations.

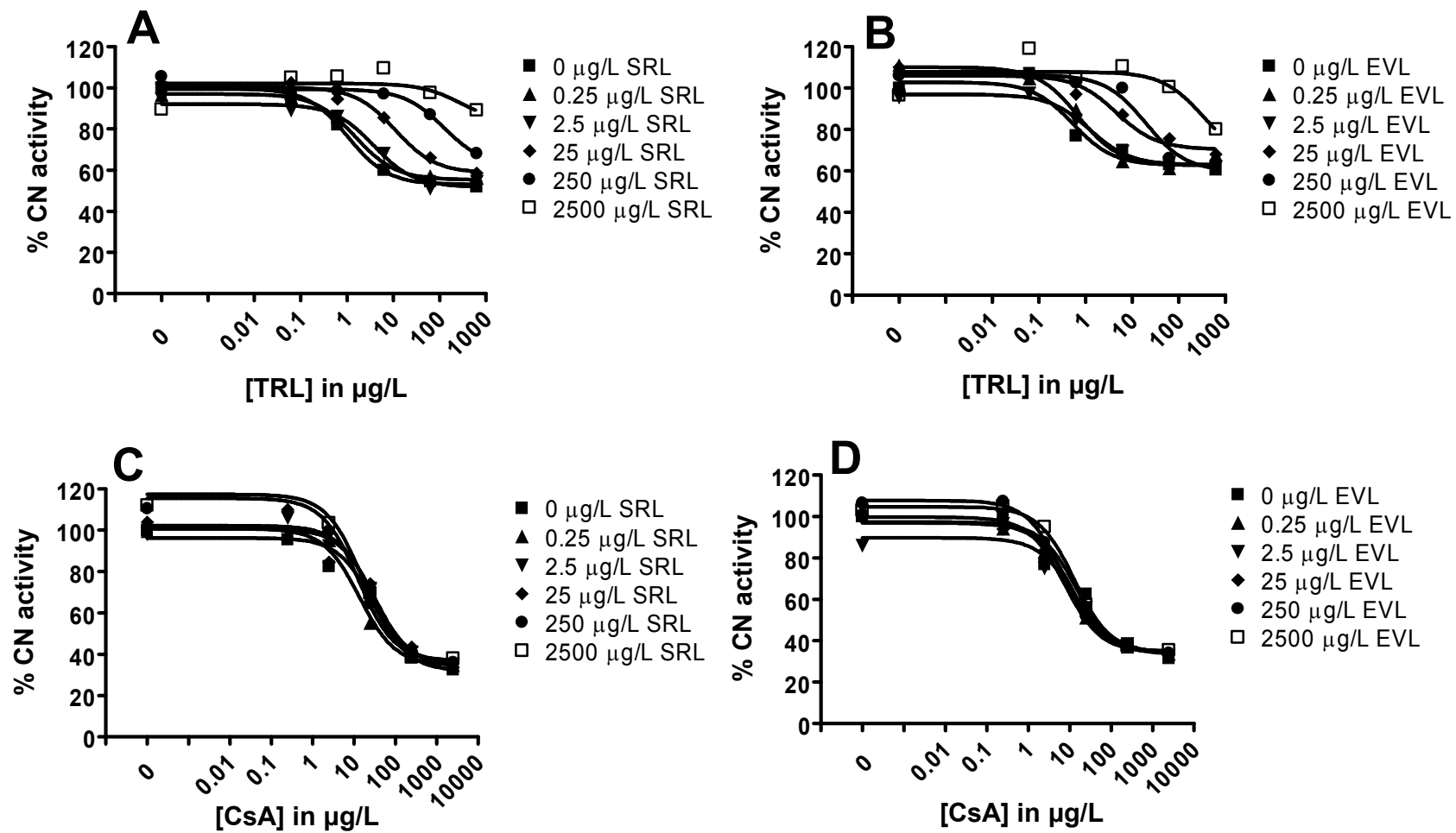
Table 2.

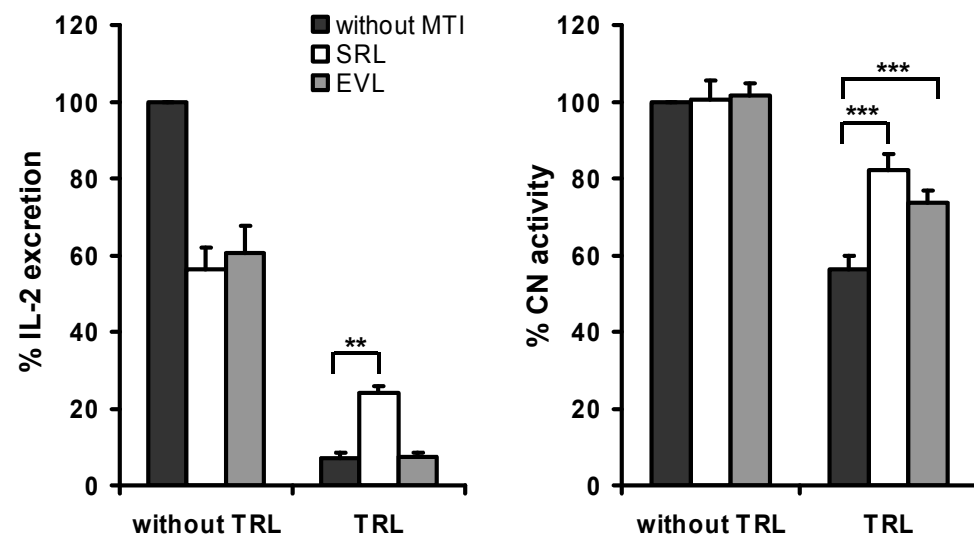
	[mTOR inhibitor] in µg/L	Tacrolimus IC ₅₀ in µg/L (95% CI)	Cyclosporin A IC ₅₀ in µg/L (95% CI)
Sirolimus	0	1.0 (0.32 - 3.4)	30 (12 - 74)
	0.25	1.5 (0.69 - 3.4)	13 (7.8 - 23)
	2.5	3.8 (1.6 - 8.8)	31 (15 - 63)
	25	11 (6.7 - 18)	29 (11 - 79)
	250	125 (25 - 620)	13 (5.3 - 31)
	2500	290 (0.08 - 1.1·10 ⁶)	16 (8.5 - 31)
Everolimus	0	0.54 (0.15 - 1.9)	14 (5.1 - 41)
	0.25	0.70 (0.34 - 1.4)	7.6 (3.7 - 16)
	2.5	1.2 (0.39 - 3.9)	13 (4.0 - 43)
	25	4.6 (0.91 - 23)	11 (5.2 - 23)
	250	20 (3.7 - 104)	7.1 (4.0 - 13)
	2500	320 (0.21 - 5.0·10 ⁵)	13 (9.8 - 17)

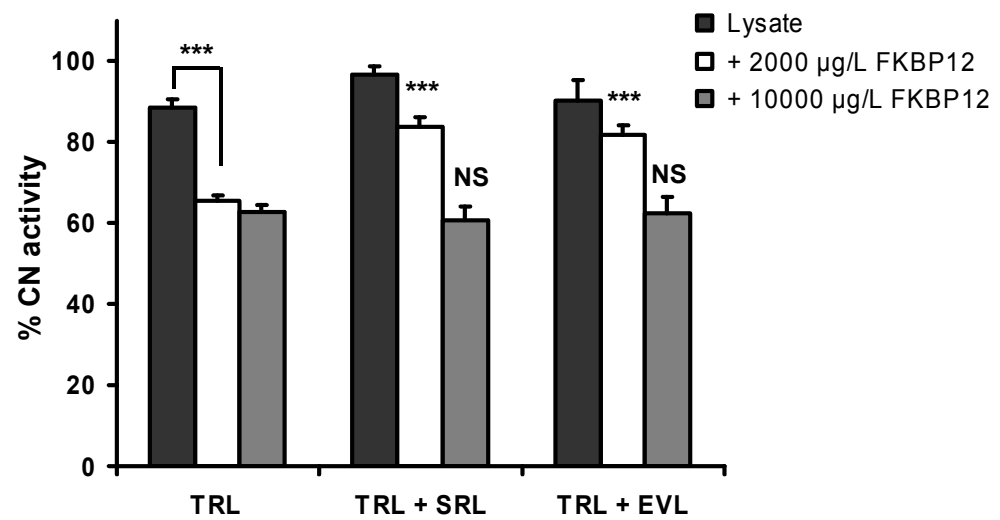
Table 2. CN IC₅₀ values of TRL and CsA in the presence of various concentrations of SRL and EVL. IC₅₀ values and 95% Confidence Intervals (CI) were obtained from the sigmoidal concentration-response regressions presented in figure 3 by Graphpad software.



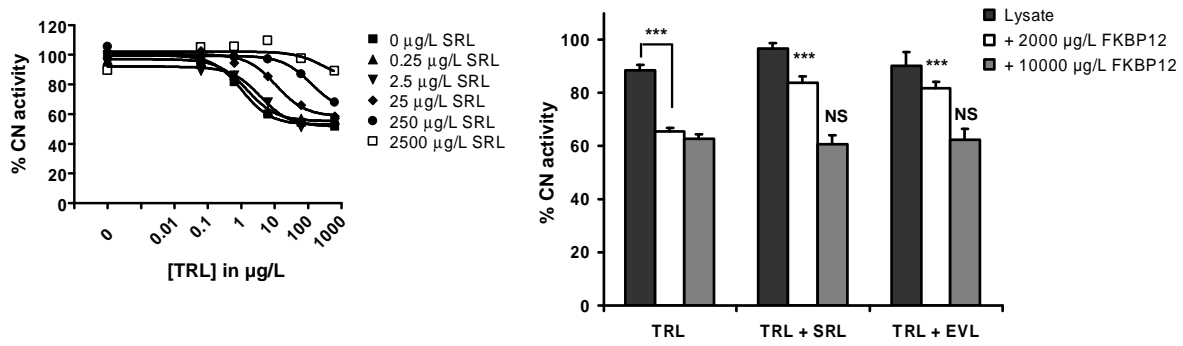








Graphical abstract



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