Supporting Information to:

Antimicrobial, antiproliferative, cytotoxic and tau inhibitory activity of rubellins and caeruleoramularin produced by the phytopathogenic fungus Ramularia collo-cygni

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Table 1S: Antimicrobial activity of compounds 1–5 determined by agar diffusion test (200 µM; zone of inhibition: diameter in mm).

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td><strong>Bacillus subtilis</strong> ATCC 6633</td>
<td>13</td>
<td>16</td>
<td>16.5</td>
<td>12c</td>
<td>12.5c</td>
<td>29a</td>
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<td>37a</td>
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<td><strong>Mycobacterium vaccae</strong> IMET 10670</td>
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<td>19</td>
<td>18</td>
<td>17.5</td>
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<td>0</td>
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<td>0</td>
<td>14b</td>
</tr>
</tbody>
</table>

ª Ciprofloxacin (15 µM).
ª Amphotericin B (11 µM; Sigma, No. A-4888).
ª Partial inhibition, some colonies in the inhibition zone.

Antimicrobial assays

Agar diffusion test

Antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria, yeasts and fungi was determined qualitatively by agar diffusion tests according to European Pharmacopoeia [1] as described [2]. Shortly, 100 µL suspension of the test organism with a density of McFarland standard 0.5 were inoculated into 32 mL of sterile melted agar medium and poured into petri dishes. Holes of 9 mm in diameter were cut in the agar and filled with 50 µL of a 0.2 mM (or as stated otherwise) solution of the compound. Inhibition zones were read after overnight incubation.

Determination of MICs

Active compounds were studied quantitatively by determination of minimal inhibitory concentrations (MIC) according to the NCCLS guidelines [3] using the broth microdilution method [4]. The bacteria were grown overnight at 37 °C in Mueller-Hinton broth (MHB) (Difco). 50 µL of a compound solution of 2 mM were serially diluted by factor two with
MHB. Then the wells were inoculated with 50 µL of test organisms to give a final concentration of $5 \times 10^5$ CFU/mL. Microtiter plates were incubated at 37 °C for 24 h, the MIC values were read with a Nepheloscan Ascent 1.4 automatic plate reader (Labsystems, Vantaa, Finland) as the lowest dilution of antibiotic allowing no visible growth. *Candida albicans* was grown in yeast nitrogen base (Difco) supplemented with 1 % glucose and incubated at 30 °C.

**CPE inhibitory test for antiviral activity and cytotoxicity test in GMK cells**

50 µL of drug solution and 50 µL of a constant amount of virus (0.1, 0.001, and 0.0005 MOI for CVB3, poliovirus 2 and 3, respectively) were added to confluent HeLa cell monolayers grown in 96-well plates. The inhibition of the virus-induced CPE was scored after crystal violet staining spectrophotometrically 24 h (CVB3) or 48 h (poliovirus 2 and 3) post infection when untreated infected control cells showed maximum cytopathic effect. To determine the 50 % cytotoxic concentration ($CC_{50}$) for GMK (green monkey kidney) cells, confluent cell monolayer grown in 96-well plates were incubated with serial dilutions (factor 2, concentration range 0.30 – 90 µM) of the respective compounds for 72 h (37 °C, 5 % CO₂). Then, the cells were fixed and stained with a crystal violet formalin solution. Cytotoxicity was quantified spectrophotometrically with a plate reader. $CC_{50}$ values were calculated by the sigmoidal fitting method using Microcal™ Origin® 6.0.

**Cytotoxicity test in SK cells**

The use of the MTT-cell culture bioassay for the evaluation of the cytotoxic properties of samples is reported. The principle of this bioassay is based on the transformation of the yellow salt MTT by viable, living cells (via mitochondrial dehydrogenase) to purple formazan [5-6]. The MTT-assay was carried out with swine kidney cells (SK) as target cells, which are known to react sensitive towards a wide range of mycotoxins and have been already used in
assaying crude extracts from various samples. Aliquotes of the isolates (extracts) and defined quantities of mycotoxin standards were dissolved in special cell culture medium (MEM with 1.7 % ethanol and 0.3 % DMSO) and log 2 solution series tested in duplicates together with the cells in 96-well tissue culture plates. Final concentration of the crude extract of the samples tested was 1 mg/mL, of the mycotoxin standards 50 µg/mL (Patulin and Deoynivalenol) and 25 µg/mL (Gliotoxin). All plates were incubated for 44 h at 37 °C and 5 % CO₂ in a humidified atmosphere and parallel under the same conditions but with or without illumination (fluorescent lamp: Osram Dulux FL longlife). A volume of 20 µL each of the MTT stock solution was then added to the wells and the plates incubated for another 4 h. Supernatants were then removed and 100 µL DMSO added to each well in order to dissolve the dark formazan crystals. The optical density of each well was measured spectrophotometrically with an ELISA reader at a wavelength of 510 nm. Mean extinction values and standard deviations of each sample concentration were calculated and toxicity expressed as % cleavage activity (IC₅₀ value in µM cell culture medium) in comparison to cell controls.

**Cytotoxicity and proliferation assays in HeLa, Huvec and K562 cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>cell culture medium</th>
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<tbody>
<tr>
<td>HUVEC (ATCC CRL-1730)</td>
<td>DMEM (CAMBREX 12-614F)</td>
</tr>
<tr>
<td>K-562 (DSM ACC 10)</td>
<td>RPMI 1640 (CAMBREX 12-167F)</td>
</tr>
<tr>
<td>HeLa (DSM ACC 57)</td>
<td>RPMI 1640 (CAMBREX 12-167F)</td>
</tr>
</tbody>
</table>

Cells were grown in the appropriate cell culture medium supplemented with 10 mL/L ultraglutamine 1 (Cambrex 17-605E/U1), 500 µL/L gentamicin sulfate (CAMBREX 17-
518Z), and 10 % heat inactivated fetal bovine serum (PAA A15-144) at 37 °C in high density polyethylene flasks (NUNC 156340).

**Proliferation assay**

The test substances were dissolved in DMSO before being diluted in DMEM. The adherent cells were harvested at the logarithmic growth phase after soft trypsinization, using 0.25 % trypsin in PBS containing 0.02 % EDTA (Biochrom KG L2163). For each experiment approximately 10000 cells were seeded with 0.1 mL culture medium per well of the 96-well microplates (NUNC 167008).

**Cytotoxicity assay**

HeLa cells were 48 h preincubated without the test substances. The dilutions of the compounds were carried out carefully on the subconfluent monolayers of HeLa cells after the preincubation time.

**Condition of incubation**

The cells were incubated with dilutions of the test substances for 72 h at 37 °C in a humidified atmosphere and 5 % CO₂.

**Method of evaluation**

For estimating the influence of chemical compounds on cell proliferation of K-562 we determinate the numbers of viable cells present in multiwell plates via CellTiter-Blue® assay. It uses the indicator dye resazurin to measure the metabolic capacity of cells as indicator of cell viability. Viable cells of untreated control retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. Under our
experimental conditions, the signal from the CellTiter-Blue® reagent is proportional to the number of viable cells.

The adherent HUVEC and HeLa cells were fixed by glutaraldehyde and stained with a 0.05 % solution of methylene blue for 15 min. After gently washing the stain was eluted by 0.2 mL of 0.33 N HCl in the wells. The optical densities were measured at 660 nm in SUNRISE microplate reader (TECAN).

The GI\textsubscript{50} and CC\textsubscript{50} values were defined as being where the dose response curve intersected the 50 % line, compared to untreated control. These comparisons of the different values were performed with software Magellan (TECAN).

**Proliferation assay MCF-7 – E-Screen-Assay**

Human breast adenocarcinoma MCF-7 cells (ATCC) were seeded in 96-well microtitre plates (Greiner Bio-One, Frickenhausen, Germany) with a density of 3500 cells per well and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10 % fetal bovine serum (FBS) and penicillin/streptomycin (100 U / 100 µg/mL) (Biochrom, Berlin, Germany) in a humidified incubator at 37 °C and 5 % CO\textsubscript{2}. After 24 h medium was removed, cells were washed with phosphate-buffered saline (PBS), and phenol red-free DMEM (Gibco\textsuperscript{TM}, Invitrogen, Karlsruhe, Germany) supplemented with 10 % charcoal-stripped, hormone-free serum (CSS *) and test substances (concentration range 0.1 – 100 µM) were added. Medium was exchanged after 72 h incubation time. After 120 h cells were quantified by MTT assay [5-6] as follows. Medium was removed and 100 µL MTT (Fluka, Buchs, Switzerland), dissolved in phenol red-free DMEM at 0.5 mg/mL, were added to each well. The plates were incubated at 37 °C and 5 % CO\textsubscript{2} for 4 h. Formazan crystals were solubilized by addition of 100 µL of 20 % sodium dodecylsulfate (SDS) in H\textsubscript{2}O followed by incubation overnight at 37 °C. Optical density was measured at 544 nm using a Galaxy FluoStar microplate reader (BMG Labtechnologies, Offenburg, Germany) with background substraction. Relative proliferation
values were calculated as percentage of negative control (0.1 % DMSO value = 100 %).
Experiments were repeated at least three times with 4 to 6 replicates per test concentration. 

$GI_{50}$ values were calculated by the sigmoidal fitting method using Microcal™ Origin® 6.0.
The acute cytotoxic potential of compounds 1–5 was determined with the Cytotoxicity 
Detection Kit (LDH) (Roche Diagnostics, Mannheim, Germany). Optical density was measured at 492 nm using the HTS 7000 Bioassay Reader (Perkim-Elmer, Überlingen, 
Germany) with background subtraction. After incubation of 12500 cells per well with test 
compounds for 24 h, no acute cytotoxicity could be noted at concentrations up to 30 µM.

* Preparation of CSS
Hormone-free charcoal-dextran stripped serum (CSS) was prepared from FBS by agitating 
with 0.5 % charcoal (Norit A) (Serva Feinbiochemica, Heidelberg, Germany) and 0.05 % Dextran-T70 (Pharmacia, Uppsala, Sweden) at 37 ºC for 60 min. After centrifugation at 3500 rpm, CSS was filter-sterilized (0.22 µm) twice and stored at -20 ºC.

Thioflavine S fluorescence assay – tau aggregation
A 10 µM concentration of tau protein K19 was incubated with the compounds (concentration 
range 0.1 nM – 200 µM) in the presence of 2.5 µM heparin in 50 mM NH₄Ac buffer 
overnight at 37 ºC following the protocol of Friedhoff et al. (1998) [7]. As a control the 
protein was replaced with H₂O to measure the fluorescence of the compounds. After addition 
of 20 µM thioflavin S, the signal was measured at 521 nm (emission) and an excitation 
wavelength of 440 nm in a spectrofluorimeter (Ascent; Labsystems, Frankfurt, Germany). 
$IC_{50}$ and $DC_{50}$ values were calculated by the sigmoidal fitting method using Microcal™ 
Origin® 6.0.
References:


