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Supplementary material

N-heterocyclic borneol derivatives as the inhibitors of Marburg virus glycoprotein-mediated VSIV pseudotype entry

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Screening strategy

In the initial screening, all over 170 compounds of the library were tested for MarV-GP-VSV pseudotype entry inhibition (see below) at 25 mg/ml concentration, and the compounds showing

>75% inhibition were considered active. Several hit compounds have been identified among the bornyl ester containing saturated N-heterocycles, while only single active substances were found in the other classes. The group of borneols included borneol itself as well as two series of N-containing bornyl ester derivatives differing in the length of the aliphatic chain between the nitrogen atom and the ester group. For all compounds of borneol series, the half maximal cytotoxic concentrations CC_{50} were determined by MTT assay for cell metabolic activity (see below). For all borneol derivatives, rVSIV-ΔG-MarV-GP and rVSIV-ΔG-G pseudotype entry inhibition was assayed at different concentrations, the inhibition curves were constructed, and the corresponding IC50 values were determined. The experiment was replicated 3 times, and the mean IC50 was determined. The specificity of inhibition (SI) was calculated as $ICMarV_{50}/ICVSIV_{50}$. and the selectivity index (SI), a ratio of compound toxicity to inhibitory activity against rVSIV-ΔG-MarV-GP ($CC_{50}/ICMarV_{50}$) was calculated.

Materials and methods

MTT assay

We used MTT Cell Proliferation Kit I (Sigma) for colorimetric quantification of cell viability according to the Manufacturer`s instructions. The substances were added to HEK293 cells split in 96-well plates for 48 h. After the incubation period, MTT reagent was added to each well (to final concentration 0.5 mg/ml). After 4 h in a humidified atmosphere (+37°C, 5% CO2), formazan crystals were solubilized overnight by adding Solubilization solution (10% SDS in 0.01 M HCl). The spectrophotometrical absorbance of the samples was measured with Tecan Sunrise absorbance microplate reader using the destinated and reference filters of 570 nm and 670 nm wavelength, respectively.

Plasmids

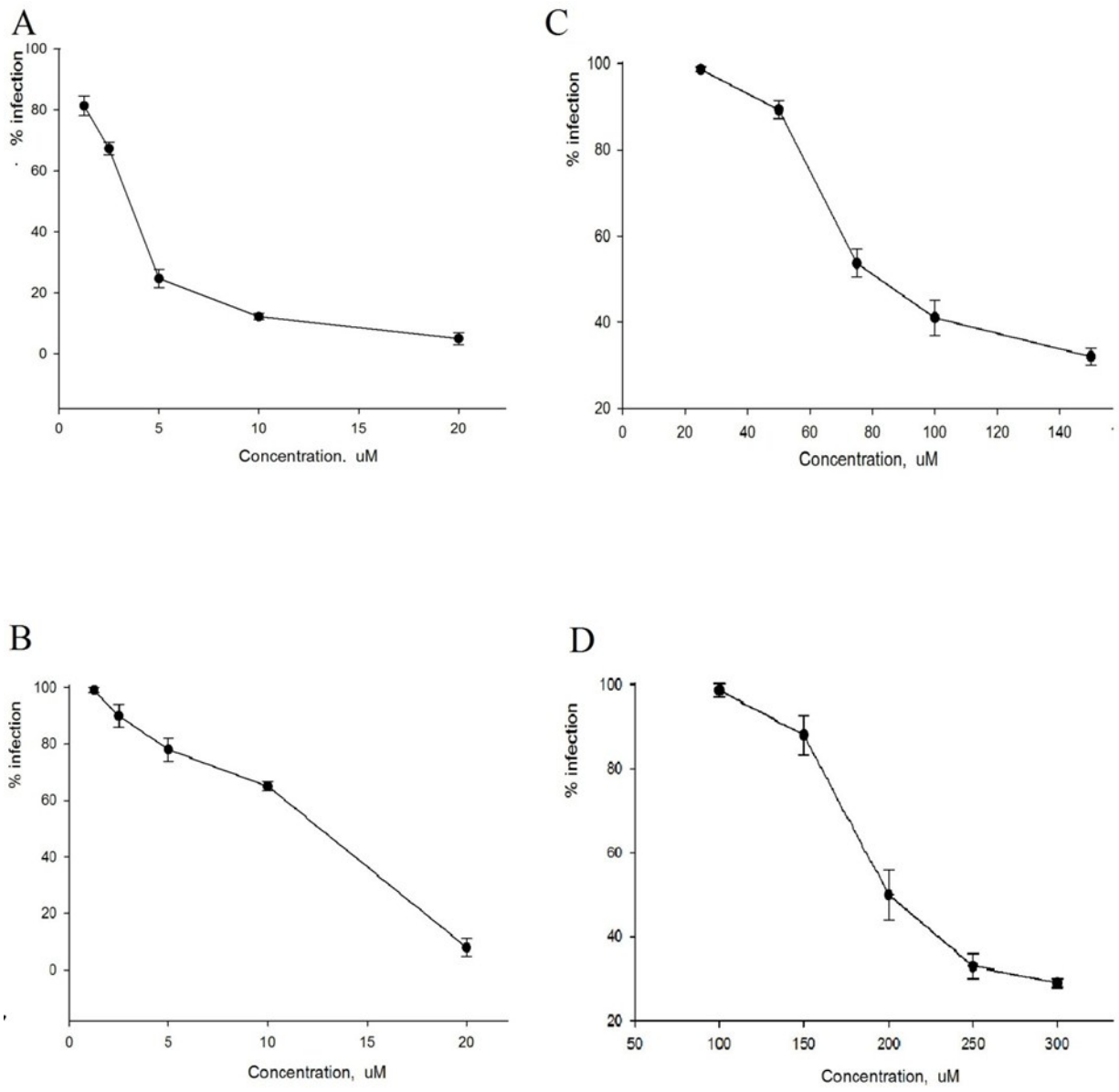
The pcDNA3-MarV-GP plasmid expressing the surface glycoprotein of Marburg virus, strain Musoke was kindly provided by Prof. R.A.Davey (Texas Biomedical Research Institute). The plasmid was constructed by cloning of the codon-optimized ORF of Marburg virus GP gene into the pcDNA3 plasmid vector. The sequence of plasmid is available upon request. The pLP-VSVG plasmid expressing G glycoprotein of VSV was also provided by Prof. R.A.Davey.

Pseudotype production

Pseudotypes expressing Marburg virus or Vesicular stomatitis Indiana virus surface glycoproteins were produced by the transfection of 293FT producer cells (Invitrogen) with the plasmids expressing the corresponding viral glycoproteins, either pcDNA3-MarV-GP or pLP-VSVG, with the subsequent infection of glycoprotein-expressing cells by the **rVSV-ΔG-G** pseudotype (Whitt, 2010) carrying the firefly luciferase reporter gene in its genome. The profucer cells were transfected with 5 μg of pcDNA3-MarV-GP or 0.5 μg pLP-VSVG plasmids per 60 mm culture dish (~70-80% confluent) by calcium phosphate method using BES buffered system (pH~6.95). Growth medium of the producer cells was replaced for the fresh culture medium without geneticin (G-418) 1-2 h before the transfection. About 12 h post-transfection, the medium was replaced again, and the producer cells were infected with 20 μl (corresponding to - 2 x 10^8 RLU) of **rVSV-ΔG-G**. The virus-containing medium was discarded 4 h later, the cells were washed twice, and fresh growth medium was added. The **rVSV-ΔG-MarV** and **rVSV-ΔG**-G pseudotypes were collected at 24 h post-infection, pooled, cleared by low-speed centrifugation, filtered through a 0.45-μm syringe filter, and frozen in aliquots until required. Viral titers were determined using plaque-forming assay (Whitt, 2009) and were in 10^6-10^7 PFU/ml range in different preparations.

Pseudotype entry inhibition assay

Target HEK293 cells were split at 10^5 cells per well in 96-well plates the day before infection and were infected by 10^5 PFU (10 μl of 10^7 PFU/ml of the pseudotype stock) in the presence and absence of the inhibitors, making the effective MOI ~ 0.5 – 1 PFU/cell. The transduced luciferase signal was assayed 24 h post-infection by adding 50 μl of Bright-Glo reagent (Promega) and incubating the plate for 5` at room temperature before being read by Luminoscan Ascent microplate luminometer (Thermo Electron Corporation). All tested compounds were prepared and stored as 100 mg/mL DMSO stock solutions and were added to the target cells at 1 to 250 μg/mL at the moment of infection. The lysosomotropic agent, chloroquin, which is a pan-pH-dependent virus inhibitor, and the Ca channel blocker, verapamil, which was shown to be a specific filovirus entry inhibitor, when used at therapeutic concentrations, were used as the reference compounds for the positive controls of pseudotype infection inhibition. All assays were performed in triplicates, and the obtained data are presented as mean values and standard deviations.



Dose response curves of rVSIV-ΔG-MarV-GP and rVSIV-ΔG-G pseudotype inhibition by compound **6** and reference compound, verapamil. A and B - rVSIV-ΔG-MarV-GP entry inhibition by compound **6** and verapamil, respectively. C and D - rVSIV-ΔG-G entry inhibition by compound **6** and verapamil, respectively.