WP1.11. Development of compounds disrupting protein-protein interaction in influenza A polymerase (Konvalinka)

Research aims

- To develop compounds inhibiting PB1-PA interaction and characterize them in detail thermodynamically and structurally with the polymerase subunit PA.
- To perform the thermodynamic analysis of the PB1-derived peptides binding to PA to determine the minimal peptide sequence responsible for efficient binding.

Research plan and methodology

Recently, the AlphaScreen technology (Perkin Elmer) has been used in our laboratory to develop a high-throughput assay for screening of compounds disrupting protein-protein interactions between two subunits of influenza polymerase. The illustration of the developed method is shown in Figure 2. Briefly, upon illumination by laser at 680 nm, streptavidin coated donor beads containing the photosensitizer phthalocyanine convert triplet oxygen to an excited state of singlet oxygen $^1O_2$. Within its half-life of ~ 4 μs the singlet oxygen could diffuse approximately 200 nm in solution. If glutathione coated acceptor bead is within that proximity upon PB1 peptide-CPA interaction, energy is transferred from the singlet oxygen to thioxene derivative within the acceptor bead and a signal at 520 - 620 nm is emitted. In the absence of a nearby acceptor bead since the screened compound inhibits PB1-CPA interaction, the singlet oxygen falls to the ground state and does not produce any signal.

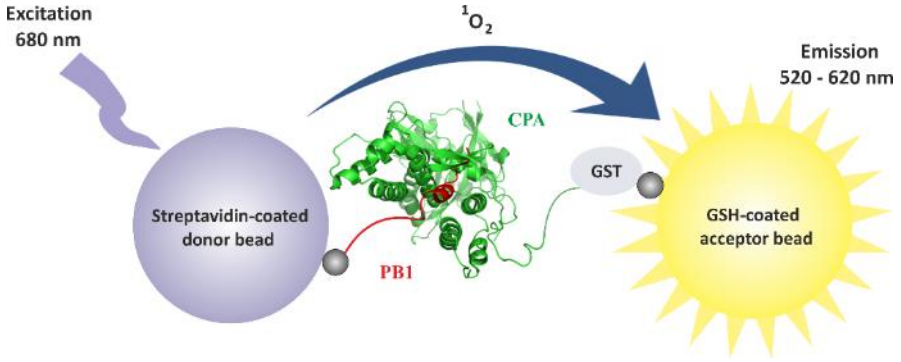

Fig.2 Illustration of the AlphaScreen protein-protein interaction assay, using streptavidin coated donor beads, GSH-coated acceptor beads, biotinylated PB1 peptide and GST-tagged C-terminal part of the PA subunit.

Preliminary screening experiments of our focused library[1] initially made for disruption of different protein-protein interaction (essential for HIV-1 capsid assembly) identified two classes of compounds capable to interfere with PA-PB1 complex (Figure 3). The first class of hits represents isomeric nitrophenylenediamines which exhibited moderate micromolar activity in AlphaScreen-based assay. Their structures resemble already reviewed derivatives of nitrobenzofurazanes and cyanopyridine derivatives shown in Figure 3. The second described class of inhibitors (the 2*H*-benzotriazoyl urea-derivative) possesses central core exhibiting an orthoquinonoid pattern and this derivative is therefore isosteric with benzofurazanes.

In this project, we propose to use the PA-PB1 protein-protein interaction in the influenza virus as a target for the development of low molecular weight molecules capable of competitively binding the PA protein and thereby disintegrate the viral RNA polymerase. Hit optimization process will be used to obtain better understanding of the structure-activity relationship of our promising hits of classes I and II. The initial hits shown in Figure 3 will serve as structural scaffolds and will be used in the further development of more potent inhibitors.

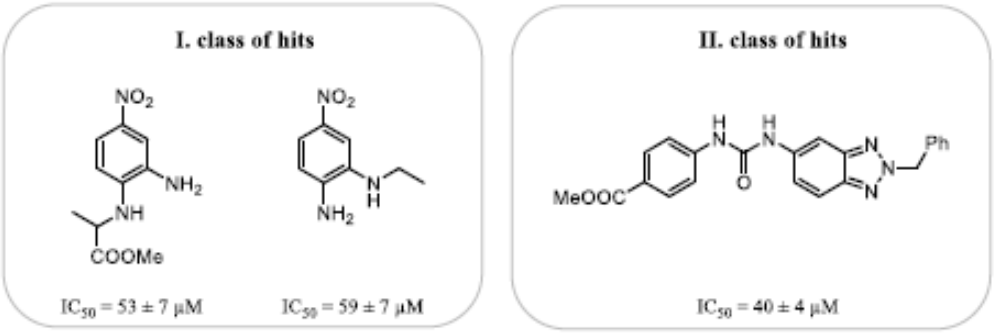

Fig.3. Structures of preliminary identified inhibitors.

In parallel to the structure-activity relationship study of the first hits, we aim to perform a high-throughput screening of the "IOCB library", a proprietary library of approx. 5000 compounds containing various classes of organic compounds and derivatives prepared at the IOCB CAS, v.v.i. by different research groups with the aim to identify other hits disrupting the interaction of polymerase subunits. For this task, we shall make extensive use of the microplate handling robot which is a part of the proposed capital investment of this project.

The prospective rational drug design will be based on the results of initial screening and information obtained from the structural and biochemical studies, including microcalorimetry or X-ray crystallography.

The anti-influenza activity of the most active derivatives will be analyzed by determining the extent to which the test compounds inhibit replication of influenza A H1N1 strain in Madin-Darby canine kidney (MDCK) cells. The *in vitro* infectivity will be assessed either by viral immuno-stained plaque assay or by cell protection assay evaluated by XTT method.[2] The cytotoxicity of the compounds will be evaluated by the XTT method as well. To monitor the effect of identified hits and their derivatives on polymerase activity *in vivo*, the minireplicon assay will be established and optimized as described.[3,4]

The binding mode of the most active compounds into CPA protein will be characterized by X-ray structural analysis in the laboratory of Dr. Pavlína Řezáčová, IOCB. The understanding of the structure of complex will be crucial for further rational drug design. In addition, the direct interaction between CPA and the most active compounds will be characterized by isothermal titration calorimetry using VP-ITC (Malvern, MicroCal division) at 25°C. Monitoring the nature of inhibitor-CPA binding with ITC will help us with the discovery of agents with optimal thermodynamic profiles. Moreover, the detailed thermodynamic analysis of the PB1-derived peptides binding to CPA and determination of the minimal peptide sequence responsible for binding will be performed.

Finally, the most active identified inhibitor of the protein-protein interaction will be tested for development of viral resistance. The *in vitro* selection of resistant influenza virus variants will be performed by serial passage of virus grown in MDCK cells in the presence of increasing concentrations of tested compound in the laboratory of Jan Weber at the IOCB. The specific mutations in the PA or PB1 region will be identified by DNA sequencing and the anti-influenza activity of the most active inhibitor will be determined toward this selected mutated virus.

The project will be realized in a close collaboration with Pavel Majer medicinal chemistry team, J. Cvačka mass spectrometry department and P. Řezáčová and J. Weber Groups.

[1] Machara, A.; Konvalinka, J.; Kotora, M. *Chemistry Select.* **2016**, 1, 101.
[2] Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, 48, 4827.
[3] Muratore, G.; Goracci, L.; Mercorelli, B.; Foeglein, A.; Digard, P.; Cruciani, G.; Palu, G.; Loregian, A. *Proc. Natl. Acad. Sci. USA* **2012**, 109, 6247.
[4] Pleschka, S.; Jaskunas, R.; Engelhardt, O. G.; Zurcher, T.; Palese, P.; Garcia-Sastre, A. *J. Virol.* **1996**, 70, 4188.

Research schedule

2017 – 2019

- Final optimization of the AlphaScreen assay for high-throughput screening using microplate handling robot.
- Optimization of hits identified in preliminary screening in order to get improved competitors.
- High-throughput screening (using microplate handling robot) of the "IOCB library" containing various classes of organic compounds and derivatives prepared at the IOCB CAS, v.v.i. in order to identify other hits disrupting the interaction between polymerase subunits.

2020 – 2022

- X-ray structural and thermodynamic analyses of the most active compounds determined by the AlphaScreen assay.
- Validation of the most active compounds by *in vitro* infectivity testing and cell based assay for polymerase activity testing.
- Detailed thermodynamic analysis of the PB1-derived peptides binding to CPA and determination of the minimal peptide sequence responsible for efficient binding.
- Analysis of the resistance development against the most active inhibitor by the *in vitro* selection of virus performed by serial passage in the presence of increasing concentrations of tested compound.

Publications and patents

Publications (Jimp)

	Jimp
2017	0
2018	1
2019	1
2020	1
2021	1
2022	1
Total	5

Journal of Biological Chemistry
Journal of Virology
PNAS
Journal of Molecular Biology
Protein Science
Plos one
FEBS Journal
Antimicrobial Agents and Chemotherapies
Journal of Medicinal Chemistry
Biochemistry
European Journal of Medicinal Chemistry

Patents and patent applications

	Patents (granted)	International patent applications (filed)
2017	0	0
2018	0	0
2019	0	0
2020	0	0
2021	0	1
2022	1	0
Total	1	1

We expect IP protection in the following areas: The most active compounds disrupting influenza polymerase subunits.

Cooperation with foreign institutions

- Prof. Hans-Georg Krausslich, University of Heidelberg and Prof. Barbara S. Slusher, Brain Research Institute, Johns Hopkins University, Baltimore USA.
- Dr. Tomáš Cihlář, VP for Virology, Gilead Sciences Inc., Foster City, USA.