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LaBEFar Laboratório de Biologia Estrutural e Fármacos

Description of activity assays for the non-structural proteins from SARS-CoV 2 carried out at the Laboratory of Structural Biology and Drug Discovery (LaBEFar) of the Institute of Physics of São Carlos at the University of São Paulo

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RNA-dependent RNA polymerase (RdRp)

Postdoctoral researcher: Juliana Roberta Torini
Supervisor: Glaucius Oliva

- **General activity assay**

The RNA-dependent RNA polymerase (RdRp) activy of the betacoronavirus SARS-CoV-2 is mediated by a multisubunit composed of 3 viral non-structural proteins (nsp): nsp7, nsp8, and nsp12. The core component nsp12 hosts the catalytic site. The nsp12 is obtained by insect cell expression, employing pFastBac vectors (pFB) to produce recombinant baculoviruses used to transfect *Spodoptera frugiperda* cells (Sf9). The cofactor proteins (nsp7 and nsp8) are expressed in *E. coli* BL21 using pET28a vectors.

The polymerization activity is performed using a real-time polymerase elongation technique in a 50 µL mixture containing assay buffer single-stranded RNA, the RdRp complex, dUTP, BSA, SYBR Green I (SGI) and ROX reference dye. SGI is an intercalating probe that fluoresces more intensely when intercalated into two strands of DNA or RNA. Thus, elongation activity can be directly detected by the increase in fluorescence during complementary strand formation. ROX is a reference dye that improves the accuracy between technical replicates, ROX fluorescence is not affected by the amplification of the PCR product. However, it is affected by anything else that changes the overall readings such as bubbles or instrumental issues. The RdRp complex is formed by nsp12, nsp8 and nsp7 incubation, at 22°C for 30 min in a 1:2:1 ratio, respectively.

The assays are performed with 3 controls: NAC (No amplification control; contains all reaction components except the RdRp complex); NPC (No probe control; contains all reaction components except the probe) and NTC (No template control; contains all reaction components except the RNA template).

The polymerase elongation reaction is conducted by incubation at 30 °C in a real-time PCR system and monitored for 40 minutes, measuring the fluorescence every 30 s with the appropriate filter. To distinguish between fluorescence derived from specific and non-specific products included in the reaction, at the end of incubation a dissociation process is also followed. During the dissociation curve, the dsRNA product is melted into ssRNA by a stepwise increase in temperature, with fluorescence data being collected at each step. The magnitude of the reduction in fluorescence intensity provides a qualitative indicator of the proportion of dsRNA attributable to the polymerization product.

All materials used in this assay are certified as RNase, DNase and pyrogenase-free.

- **Compounds screening and inhibition assay**

For screening 100 µM of test compound diluted in DMSO is added to the reaction mixture and the polymerase elongation is monitored as described above. All compounds that showed inhibition greater than 80% in relation to native enzyme activity are submitted to IC50 assays.

To determine the IC_{50} value, different sample reactions are tested with 12 different inhibitor concentrations (serial dilution starting from 100 µM in DMSO). Reactions, without compounds, containing the same volume of DMSO are used as controls.

All assays are performed in triplicate. The inhibition graphs and the IC_{50} are obtained using GraphPad Prism 8.0 Software. In the absence of the compound, the intensity in each data set is defined as 100% of activity. In the absence of the enzyme, the intensity in each data set is defined as 0% of activity.

Main Protease (M^{pro})

PhD Student: Gabriela Dias Noske
Supervisor: Glaucius Oliva

- **Expression and Purification**

SARS-CoV-2 M^{pro} is produced as previously described [1]. Briefly, the plasmid containing M^{pro} coding sequence is used to transform *E. coli* BL21 that is cultured in ZYM-5052 [2] at 37 °C and 200 RPM to an OD_{600} of 0.8, followed by expression at 18 °C, 200 RPM for 16 h. Cells are harvested by centrifugation at 5,000 x g for 40 min at 4 °C, resuspended in lysis buffer (20 mM Tris pH 7.8, 150 mM NaCl, 1 mM DTT), and disrupted by sonication. M^{pro} is separated from the clarified extract by adding 1 M ammonium sulfate to the cell lysate followed by incubation on ice for 10 min. The precipitated protein is recovered by centrifugation at 12,000 x g for 30 min at 4 °C, resuspended in gel filtration buffer (20 mM Tris pH 7.8, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) and purified by size-exclusion chromatography using a HiLoad 26/100 Superdex 200 column (GE Healthcare). Protein fractions are exchanged to 20 mM Tris pH 8.0, 1 mM DTT, and then injected into a Mono-Q 5/50 GL column (GE Healthcare). Protein is eluted using a linear gradient of a buffer containing 20 mM Tris pH 8.0, 1 M NaCl and 1 mM DTT. Fractions containing the purified protein are buffer exchanged to gel filtration buffer.

- **General activity assay**

M^{pro} enzymatic assays are carried out using FRET-based substrate DABCYL-KTSAVLQ↓SGFRKM-E(EDANS)-NH_2 in assay buffer (20 mM Tris pH 7.3, 1 mM EDTA, 1 mM DTT), purchased from GenScript, at a protein final concentration of 0.14 µM. Fluorescence measures are performed in SpectraMax Gemini EM Microplate Reader with $\lambda_{exc}/\lambda_{emi}$ of 360/460 nm, every 30 s over 60 min at 37 °C. All assays are performed in triplicates.

References

[1] G.D. Noske, A.M. Nakamura, V.O. Gawriljuk, R.S. Fernandes, G. M. A. Lima, H. V. D. Rosa, H.D. Pereira, A. C. M. Zeri, A. A. F. Z. Nascimento, M. C. L. C. Freire, D. Fearon, A. Douangamath, F. von Delft, G. Oliva, A.S. Godoy, A Crystallographic Snapshot of SARS-CoV-2 Main Protease Maturation Process, J. Mol. Biol. (2021) 167118. https://doi.org/10.1016/j.jmb.2021.167118.

[2] F.W. Studier, Protein production by auto-induction in high density shaking cultures., Protein Expr. Purif. 41 (2005) 207–234. https://doi.org/10.1016/j.pep.2005.01.016.

Papain-like Protease (PL^{pro})

PhD Student: Mariana Ortiz de Godoy
Supervisor: Rafael V. C. Guido

- **Expression and purification**

The plasmids containing PL^{pro} coding sequence are used to transform Rosetta 2 (DE3) *E. coli* cells (Novagen, Madison, WI, USA), which are grown in Lysogen Broth (LB) medium, supplemented with 50 µg/mL kanamycin, and 34 µg/mL chloramphenicol at 37 °C until the OD_{600} reached 0.6. The protein expression is induced by the addition of 0.5 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) and 1 mM zinc chloride ($ZnCl_2$), for 16 h at 18 °C. Cells are harvested by centrifugation, and cell pellets are resuspended in lysis buffer (50 mM Tris-HCl pH 8.5, 150 mM NaCl, 10 mM imidazole, and 1 mM DTT). Cells are then lysed by sonication and centrifuged at 15,000× g to clarify the supernatant.

The SARS-CoV-2 PL^{pro} is purified using an AKTA Purifier System (GE Healthcare, Boston, MA, USA). The first purification step is affinity chromatography using a HisTrap HP 5.0 mL column (GE Healthcare, Boston, MA, USA). The protein is eluted with an elution buffer (50 mM Tris-HCl pH 8.5, 150 mM NaCl, 250 mM imidazole, and 1 mM DTT) and after that, the second purification step is done through size-exclusion chromatography on a Superdex 200 10/300 column (GE Healthcare, Boston, MA, USA) pre-equilibrated with 20 mM Tris-HCl pH 7.4, 100 mM NaCl, and 1 mM TCEP. The protein is concentrated to 1.0 mg/mL followed by the addition of 5% glycerol, and samples are then flash-frozen and stored at −80 °C for activity assays.

- **General activity assay**

The SARS-CoV-2 PL^{pro} inhibition assay is performed using the FRET-peptide Abz-TLKGG↓APIKEDDPS-EDDnp (↓ cleavage site). The assay is standardized with an enzyme concentration

of 70 nM. The fluorescent substrate is used at 27 µM in an assay buffer containing 50 mM HEPES pH 7.5, 0.01% Triton X-100 and 5 mM DTT. The negative control is made with only 1% DMSO, while blank reactions were made using assay buffer without enzyme.

The protein is diluted in an assay buffer and incubated with 10 µM of each compound (1% DMSO), at 37 °C for 30 min. Then, the diluted substrate is added to the solution and the enzymatic activity is monitored in the spectrofluorometer system Spectramax Gemini EM (Molecular Devices, San Jose, CA, USA), with λex = 320 nm and λem = 420 nm, at 37 °C every 30 s for 15 min. Data are expressed as fraction velocity using DMSO controls with and without enzyme as the base.

Compounds that inhibited the SARS-CoV-2 PL^{pro} activity in more than 80% at 10 µM are assayed in a concentration-dependent manner to determine their half-inhibitory concentrations (IC_{50}). Enzyme and substrate concentrations are maintained at 70 nM and 27 µM concentrations, respectively, and compounds are 2-fold serially diluted in DMSO in 10 points (from 10 µM to 0.019 µM). Reactions containing compounds are incubated at 37 °C for 30 min prior to the addition of the substrate. The negative control is made with only 1% DMSO, while blank reactions were made using assay buffer without enzyme. Then, the diluted substrate is added to the solution and the enzymatic activity was monitored in the spectrofluorometer system Spectramax Gemini EM (Molecular Devices, San Jose, CA, USA), with λex = 320 nm and λem = 420 nm, at 37 °C every 30 s for 15 min. Data are expressed as fraction velocity using DMSO controls with and without enzyme as the base.

The IC_{50} values from two independent experiments are averaged for final values. The results are analyzed using OriginPro 9.0 Software (Origin Lab, Northampton, MA, USA) and the IC_{50} values for each compound are determined using the Hill1 non-linear function.

Post Doctor: Luana Galvão Morão

Supervisor: Glaucius Oliva

ATPase activity assay for Nsp13-SARS-CoV-2

The process of RNA duplex unwinding by Nsp13 is linked to the amount of ATP required for initial duplex binding, translocation, and substrate unwinding. It is known that Nsp13 – SARS-CoV-2 can hydrolyze the four types of NTPs as an energy source, however, to perform its unidirectional translocation activity during the duplex unfolding process, preference is given to ATP hydrolysis cycles. The ATP hydrolytic activity exerted by Nsp13 still requires the presence of divalent metal ions and exhibits optimal ATPase activity when using Mg2+. Assays for evaluating the ATPase activity of Nsp13 were carried out using the commercial kit BIOMOL® Green (Enzo Life Sciences). This assay quantifies the activity through the colorimetric quantification of phosphate (abs. 600-680 nm) remaining in the solution after a reaction that is measured through the addition of a reagent which interrupts the reaction and starts the development of the color, which is read in 20-30 min later. The absorbance value is correlated with the amount of PO4 present and is directly correlated with the amount of protein activity. The negative control was considered only reaction buffer containing 2% DMSO with ATP (without protein), while the positive control (CP) was the reaction buffer containing 2% DMSO with ATP + protein. In addition, we performed a third control, called standard control (CS), which consisted of a commercially available drug with an Nsp13 inhibitory action. After incubation, the reagent was added to each well and incubated for 20 min and the absorbance reading was performed at 620 nm. For inhibition studies, Nsp13 was initially incubated with different concentrations of compounds evaluated for 10 min before the addition of ATP in order to evaluate its inhibitory potential or not.

Assay of duplex unwinding activity for Nsp13-SARS-CoV-2

Basically, the reaction starts with Nsp13 binding to a 5' single-stranded tail in the presence of ATP, yet without effecting its hydrolysis. Hydrolysis only occurs with the addition of magnesium ions which allows unwinding along the duplex to occur through unidirectional translocation (5'-3') by the unfolded ssRNA. To monitor the Nsp13 unwinding assay, we requested the synthesis of two single RNA strands, one of which was labeled with Hexachlorofluorescein at the 5' end and the other unlabeled. Helix oligonucleotides were generated by hybridizing the strands which were mixed in an annealing reaction. The reaction occurred with incubation at 37 °C and was monitored by a fluorimeter for about 50 minutes. Readings were recorded every 10 seconds and comparative analyzes between controls and reaction were evaluated. During the reaction, aliquots of each condition (controls and reaction) were removed and the samples were subjected to electrophoresis on a 15% Native-PAGE gel, followed by scanning using a Gel Doc EZ Imager (Bio Rad). In this way, it was possible to assess whether there was Nsp13 helicase activity happening and whether this activity was partial or total, by viewing the height of the RNA bands compared to the controls as well as the fluorimeter scan.