CARE
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CARE – Corona Accelerated R&D in Europe

WP2 – Target-based drug discovery and design

D2.4 A toolbox with at least 8 assays

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Abstract

The aim of the workpackage 2 “Target-based drug discovery and design” is to provide robust protein and enzyme assays that should benchmark the field of viral enzyme tests. They are specifically designed for coronavirus (CoV) proteins, but can be adapted to many related enzymes. They should be useful in the scientific community mainly for two purposes: discovering of validating one enzyme activity, as well as probing a compound for its capability of inhibiting an enzyme function. In that regard, the assays are often used to investigate if a given inhibitor of unknown target indeed binds and inhibit any of the enzymes listed below. The assays are also important in the hit-to-lead development process, since they are often multiplexed and/or high throughput, allowing standardized classification of inhibition power, and rapid feedback to chemical teams (WP3). Details of the assays can be found in the publications.

Assays

N7 MTase assays

Aim of the assay:
Follow the **N7-MTase activity** of nsp14 which is involved in RNA capping and is essential for mRNA translation into viral proteins and participate to RNA stability in infected cells (Ahmed-Belkacem et al., 2022; Hausdorff et al., 2023).

Description of the assay (methodology):
Briefly this assay is based on the incubation of nsp14 expressed in bacteria together with short RNA substrates in presence of ^{3}H-SAM. The ^{3}H-Met transferred on RNA substrate is next quantitated by filter binding assay performed in 96 well plates (Ahmed-Belkacem et al., 2022; Hausdorff et al., 2023).

1) nsp14 production and purification: SARS-CoV-2 nsp14 (*N7*-MTase) coding sequences were cloned in fusion with a N-terminus hexa-histidine tag in pET28 plasmids. The proteins were expressed in *E.coli* C2566 and purified in a two-step IMAC using cobalt beads. Briefly, cells were lysed by Sonication in a buffer containing 50 mM Tris pH 6.8, 300 mM NaCl, 10 mM imidazole, 5 mM $MgCl_2$, and 1 mM BME, supplemented with 0.25 mg/mL lysozyme, 10 µg/mL DNASE and 1 mM PMSF. The proteins were purified on HisPur Cobalt resin 480 (Thermo Scientific), washing with an increased concentration of salt (1 M NaCl) and imidazole (20 mM), prior to elution in buffer supplemented with 250 mM imidazole. The second step of purification was performed by size exclusion chromatography (GE Superdex S200) in a final buffer of 50 mM Tris pH 6.8, 300 mM NaCl, 5 mM $MgCl_2$, and 1 mM BME and the proteins were subsequently concentrated up to 12.5 µM and conserved at −20 °C in a buffer containing 50% of glycerol.

2) N7-MTase filter binding assay: The SARS-CoV-2 nsp14 MTase assays were carried out in reaction mixture [40 mM Tris-HCl (pH 8.0), 1 mM DTT, 2 µM SAM, and 0.1 µM ^{3}H-SAM (Perkin Elmer)] in the presence of 0.7 µM $GpppAC_4$ synthetic RNA and the N7-MTases at 50nM. Briefly, the enzymes were first mixed with the compound suspended in 50% DMSO (2.5% final DMSO) before the addition of RNA substrate and SAM and then incubated at 30 °C. Reactions mixtures were stopped after 30 min by their 10-fold dilution in ice-cold water. Samples were transferred to diethylaminoethyl (DEAE)

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filtermat (Perkin Elmer) using a Filtermat Harvester (Packard Instruments). The RNA-retaining mats were washed twice with 10 mM ammonium formate pH 8.0, twice with water and once with ethanol. They were soaked with scintillation fluid (Perkin Elmer), and ^{3}H-methyl transfer to the RNA substrates was determined using a Wallac MicroBeta TriLux liquid scintillation counter (Perkin Elmer). For IC_{50} measurements, values are normalized and fitted with Prism (GraphPad software) using the following equation: Y = 100/[1 + ((X/IC_{50})^Hillslope)].

2’OMTase assays

Aim of the assay:
Follow the **2’O-MTase activity** of nsp16 which is involved in RNA capping and is essential to avoid mRNA detection by RIG-I and favours RNA escape to IFITs mediated sequestration.

Description of the assay (methodology):
Briefly this assay is based on the incubation of nsp10/16 complexes expressed in bacteria together with short RNA substrates in presence of ^{3}H-SAM. The ^{3}H-Met transferred on RNA substrate is next quantitated by filter binding assay performed in 96 well plates.

1) nsp10 and nsp16 production and purification: SARS-CoV-2 nsp16 (*2’O*-MTase) and nsp10 coding sequences were cloned in fusion with a N-terminus hexa-histidine tag in pET28 plasmids. The proteins were expressed in *E.coli* C2566 and purified in a two-step IMAC using cobalt beads. Briefly, cells were lysed by Sonication in a buffer containing 50 mM Tris pH 6.8, 300 mM NaCl, 10 mM imidazole, 5 mM $MgCl_2$, and 1 mM BME, supplemented with 0.25 mg/mL lysozyme, 10 µg/mL DNASE and 1 mM PMSF. The proteins were purified on HisPur Cobalt resin 480 (Thermo Scientific), washing with an increased concentration of salt (1 M NaCl) and imidazole (20 mM), prior to elution in buffer supplemented with 250 mM imidazole. The second step of purification was performed by size exclusion chromatography (GE Superdex S200) in a final buffer of 50 mM Tris pH 6.8, 300 mM NaCl, 5 mM $MgCl_2$, and 1 mM BME and the proteins were subsequently concentrated up to 12.5 µM and conserved at −20 °C in a buffer containing 50% of glycerol.

2) 2’O-MTase filter binding assay: The SARS-CoV-2 nsp16 2’O-MTase assays were carried out in reaction mixture [40 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM $MgCl_2$, 2 µM SAM, and 0.1 µM ^{3}H-SAM (Perkin Elmer)] in the presence of 0.7 µM ^{7M}GpppAC_4 synthetic RNA and the nsp16 and nsp10 at 200 nM and 1 mM respectively. The enzymes were mixed with the compound suspended in 50% DMSO (2.5% final DMSO) before the addition of RNA substrate and SAM and then incubated at 30 °C. Reactions mixtures were stopped after 30 min by their 10-fold dilution in ice-cold water. Samples were transferred to diethylaminoethyl (DEAE) filtermat (Perkin Elmer) using a Filtermat Harvester (Packard Instruments). The RNA-retaining mats were washed twice with 10 mM ammonium formate pH 8.0, twice with water and once with ethanol. They were soaked with scintillation fluid (Perkin Elmer), and ^{3}H-methyl transfer to the RNA substrates was determined using a Wallac MicroBeta TriLux liquid scintillation counter (Perkin Elmer). For IC_{50} measurements, values are normalized and fitted with Prism (GraphPad software) using the following equation: Y = 100/[1 + ((X/IC_{50})^Hillslope)].

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Cellular Protease/convertase assays

Aim of the assay:
Follow the protease activity of furin which is involved in spike protein endoproteolytical maturation downstream of PRRAR sequence and increase the exposition of the RDBD domain which recognize the ACE2 receptor and therefore favour the viral entry (PMID 35343766).

Description of the assay (methodology):
Briefly this assay is based on the incubation commercially available furin together with fluorogenic peptides encompassing the furin cleavage site of SARS-CoV-2 spike protein. The protease activity is quantitated by determining the increase fluorescence in 384 well plates (Essalmani et al., 2022).

Recombinant furin is purchased from BioLegend (no. 719406) and the DABCYL/Glu-EDANS labeled peptides encompassing the spike protein furin cleavage sites $TNSPRRARSVAS_{689}$ from Genscript. Reactions are performed at room temperature in black 384-well polystyrene low-volume plates (CELLSTAR-Greiner Bio-One; 784476) at a final volume of 15 µL. The fluorescent peptide is used at 5 µM and the reactions are performed in 50 mM Tris buffer (pH 6.5 or 7.5), 0.2% Triton X-100, 1 mM $CaCl_2$; furin is added at a final concentration of 1 nM. The inhibitors must be dissolved in DMSO (1 mM) to provide a final DMSO concentration of 5% in the enzymatic assay. Cleavage of the synthetic peptides is quantitated by determining the increase of EDANS (493 nm) fluorescence following release of the DABCYL quencher, which was excited at 335 nm using a Safire 2 Tecan fluorimeter. The fluorescence was followed for 90 min, and the enzymatic activity was deduced by measurement of the increase of fluorescence during the linear phase of the reaction.

MPro assay

Aim of the assay:
Follow the protease activity of **the MPro (nsp5)** which is involved in the maturation of the viral polyprotein PP1A and PP1AB (proteolysis of the peptide bond between nsp4 and nsp16) into 16 proteins forming the viral replication transcription complex. Its inhibition was demonstrated to strongly impair the viral replication.

Description of the assay (methodology):
Briefly this assay is based on the incubation of purified nsp5 expressed in bacteria together with fluorogenic peptides mimicking the MPro cleavage site and the enzymatic activity is followed by measuring the increase fluorescence in 384-well plates (Chen et al., 2022; Hassan et al., 2023). The assays were optimized with two kind of fluorescent substrates due to autofluorescence issues of some compounds.

MPro production and purification: The nsp5 protein was produced in E. coli as previously described (Zhang et al., 2020). Briefly, the pGEX-6P-1 plasmid was transformed in E coli BL21 DE3 gold, and the nsp5 protein was produced overnight at 17 °C upon IPTG induction (250 µM). Bacterial pellets were resuspended in lysis buffer (50 mM Tris pH 8, 300 mM NaCl, imidazole 15 mM, 5 mM $MgSO_4$, 1mM BME, 10% Glycerol, 0.1%Triton X-100, supplemented with 0.25 mg/mL Lysosyme, 1 mM PMSF, and 10 µg/mL DNAse I). After 3 cycles of sonication and clarification, the 6xHis-tagged

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protein was purified by NTA affinity on cobalt beads, and the nsp5 protein was eluted in lysis buffer supplemented with 250 mM imidazole. The nsp5 protein was then concentrated on Vivaspin 20 centrifugal concentrators 10 kDa MWCO (GE Healthcare #VS2001), dialysed against the elution buffer in the absence of imidazole, and stored at −80 °C upon addition of 50% glycerol.

MPro protease assay with peptides linked to Dabcy/Edans: The fluorescence resonance energy transfer (FRET)-based *in vitro* activity assays are performed in black 384-well HiBase non-binding plates (Greiner Bio One #784900). Briefly, the inhibitors are incubated with purified nsp5 protein (55 nM) and 5 µM of a florescent synthetic peptide (Dabcyl-KTSAVLQ↓SGFRKM-Edans-NH2, purchased from Genscript) in HEPES buffer (20 mM, pH 6.5) containing 120 mM NaCl, 0.4 mM EDTA, 4 mM DTT and 10% glycerol. The DMSO final concentration must be adjusted at 0.5%. Cleavage of the synthetic peptide by nsp5 separates the Edans/Dabcyl fluorophore-quencher pair. The time courses of the enzymatic reaction is followed during 60 min by monitoring the increase of fluorescence emission at 493 nM (excitation 335 nM) using CLARIOstar Plus (BMG Labtech) fluorimeter. The enzymatic activities is estimated by taking the slope of the linear part of the reaction curve after normalization regarding the activity measured in the absence of inhibitor. The IC_{50} values can be determined by plotting the % of activity as a function of the inhibitor concentration and by fitting the curves with Prism (GraphPad) using the following equation: $Y = 100/(1+((X/IC_{50})$^Hill slope).

MPro protease assay with peptides linked to 5-FAM/QXL520: The assay must be performed in similar conditions except that the fluorescent synthetic peptide (5-FAM-KTSAVLQ↓SGFRKMK-QXL520 (7 µM), purchased from Eurogentech) is incubated with purified nsp5 protein at 55 nM and the enzymatic reaction is followed during 60 min by monitoring the increase of fluorescence emission at 518 nM (excitation 492 nM).

PLpro assay

Aim of the assay:
Follow the protease activity of **the PLpro (domain of nsp3)** which is involved in the maturation of the viral polyprotein PP1A and PP1AB (proteolysis of the peptide bond between nsp1 and nsp4) into 16 proteins forming the viral replication transcription complex. Its inhibition is proposed to impair the viral replication.

Description of the assay (methodology):
Briefly this assay is based on the incubation of purified nsp3 PLpro expressed in bacteria together with fluorogenic peptides mimicking the PLpro cleavage site and the enzymatic activity is followed by measuring the increase fluorescence in 384 well plates. The assays were optimized with two kind of fluorescent substrates due to autofluorescence issues of some compounds.

PLpro protease assay with peptides linked to Dabcy/Edans: The fluorescence resonance energy transfer (FRET)-based *in vitro* activity assays are performed in black 384-well HiBase non-binding plates (Greiner Bio One #784900). Briefly, the inhibitors are incubated with purified nsp3 protein (150 nM) and 5 µM of a fluorescent synthetic peptide (Dabcyl- FTLKGG↓APTK-Edans-NH2, purchased from Genscript) in Tris HCl buffer (20 mM, pH 6.5) containing 5 mM NaCl, 1 mM EDTA, 5 mM DTT and 10% glycerol. The DMSO final concentration must be adjusted at 0.5%. Cleavage of the synthetic peptide

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by PLpro separates the Edans/Dabcyl fluorophore-quencher pair. The time courses of the enzymatic reaction is followed during 60 min by monitoring the increase of fluorescence emission at 493 nM (excitation 335 nM) using CLARIOstar Plus (BMG Labtech) fluorimeter. The enzymatic activities is estimated by taking the slope of the linear part of the reaction curve after normalization regarding the activity measured in the absence of inhibitor. The IC_{50} values can be determined by plotting the % of activity as a function of the inhibitor concentration and by fitting the curves with Prism (GraphPad) using the following equation: $Y = 100/(1+((X/IC_{50})$^Hill slope).

PLpro protease assay with peptide linked to coumarin fluorophore: The assay must be performed in similar conditions except that the fluorescent synthetic peptide is FTLKGG-MCA (5 µM), purchased from Eurogentec) is incubated with purified nsp5 protein at 100 nM and the enzymatic reaction is followed during 60 min by monitoring the increase of fluorescence emission at 518 nM (excitation 492 nM).

RTC assays: 384 and 1536 format

Aim of the assay:
The assay, initially developed in 96 wells (Eydoux et al., 2021) was miniaturized into 384 wells (15 µl final volume) and recently into 1536 wells (7 µL). The Fr-PPIChem, a 10 314 compounds library dedicated to the protein-protein interactions inhibitors was tested, as well as more then 8 700 non-NUC compounds identified from several industrial virtual screens. This high throughput assay, in several "sizes", allows us to respond to screening requests from a few compounds to several hundred thousand compounds.

Description of the assay (methodology):
The activity of the compounds against RTC was evaluated in a fluorescent assay which utilizes a biochemically active complex consisting of purified nsp12 (RdRP) and co-factors nsp8L7 and nsp8. In this assay, a homo-polymeric polyA template is used which currently limits the screening of NUC triphosphates to the UTP analogues and non-NUCS. The RTC assay is a fluorescent assay based on the addition of an intercalant fluorescent agent (Picogreen) into the double RNA strand formed by the RTC. In the presence of inhibitor compounds, the single strand RNA (Poly A) is not detected by the Picogreen and the fluorescence decreases allowing the determination of percentage of inhibition and IC_{50}. The compounds were tested in two doses (5 µM and 20 µM concentrations). After identification of the hits, best compounds were further characterized in dose-response (IC_{50}). Interesting hits were expanded by analogue expansion (IC_{50} determination).

Individual nsp12 and (nsp12+nsp8L7) TSA assay

Aim of the assay:
After the screening campaign on the RTC assay, the best hits needed to be validated by orthogonal assays. As the RTC assay was based on the activity of the RTC, we have developed in addition a biophysical technique by using thermashift (TSA) assays. By determining the apparent constant of dissociation (Kd), we were able to confirm an interaction between the selected hit and nsp12 and/or the (nsp12 + nsp8L7) complex. Best compounds identified at this step were send to crystallography and/or cryo-microscopy programs.

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Description of the assay (methodology):
The principle of TSA assays is based on the determination of the protein of interest Tm (melting temperature, °C) by using a fluorescent probe (Sypro-orange). Conditions of the assays (concentrations of protein and probe, buffer composition) were determined for nsp12 alone and for the (nsp12 + nsp8L7) complex. This technique was not appropriate to nsp8, nsp7 or nsp8L7 alone; certainly due to exhibition of hydrophobic parts at the protein surfaces (dye titration).

Tm measurement in the presence of the compound and determination of the variation (ΔTm) gave the confirmation of binding on the protein and if the compound was stabilizing (ΔTm>2°C) or destabilizing the protein (ΔTm<2°C). By using increasing concentration of compounds, we were able to determine an apparent Kd.

Order-of-addition RTC assay

Aim of the assay:
The order-of-addition assay has a higher informational content than standard RTC assays in that it gives hints on the target of an inhibitor compound into the RTC complex. As described in Eydoux et al. (2021), the initial RTC assay was developed on a preformed (nsp12 + nsp8 + nsp8L7) complex, in the presence of the poly A template, which was added with the compound. The assay was started by the addition of UTP. Regarding the low hit rate on certain screens dedicated to protein/protein interactions, we have decided to improve the RTC assay by changing the order of addition of the test components. This strategy optimized the detection of inhibitory compounds and is now used by default.

Description of the assay (methodology):
Compounds were dispended on the experiment plate, then a mix of (nsp8+nsp8L7) was added and incubated for 8 min. nsp12 was then added and incubated for an additional 8 min. The assay was started by adding the RNA template (Poly A) and nucleotides (UTP). The following steps were the same as described. Use of detergent is mandatory in the assay (NP40 0.01%) to prevent proteins from sticking to plastic.

Macro Domain assay

Aim of the assay:
The assay allows screening for *Macro* Domain ligands that prevent Poly-ADP ribose (PAR) binding, thus restoring innate immunity of the infected host cell.

Description of the assay (methodology):
Purified recombinant SARS-CoV and SARS-CoV-2 *Macro* domains are coated on 96-well microtiter ELISA plates overnight at 4°C. Non-specific sites on the plates are blocked 1h at 37°C with coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6), supplemented with 2% of BSA. The inhibition of PAR binding is assessed by adding increased concentrations (50-1000 μM) of the tested inhibitors to the coated *Macro* domains for 2h at RT. At the end of the incubation period, purified free PAR is added to the plate for 2h at room temperature (RT) with shaking. After this step, anti-poly-ADP-ribose binding reagent diluted 1:2000 in blocking buffer is added to the wells. Following 1h incubation at RT, the second antibody anti-rabbit IgG is dispensed to each well and incubated 1h at 37°C. The plates are washed three times with PBS-Tween after each step. The PAR–antibody complex is revealed by the addition of o-phenylenediamine

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solution. The reaction is stopped with 3 M sulfuric acid. Optical densities of formed complexes are measured at 492 nm.

Conclusion

The assays have been used throughout the project, and they are important assets in the communication between WP1, 2 and 3. They should be extended to the maximum number of possible targets, since the target identification is essential, and deserves to be determined as early as possible during the project.
Most of the assays have been used outside of their origin labs, as well as published, indicating that they are reliable and helpful to the drug-discovery and design in the coronavirus field.

Publications

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