A bioassay for screening of viral protease inhibitors in the model yeast *Saccharomyces cerevisiae*

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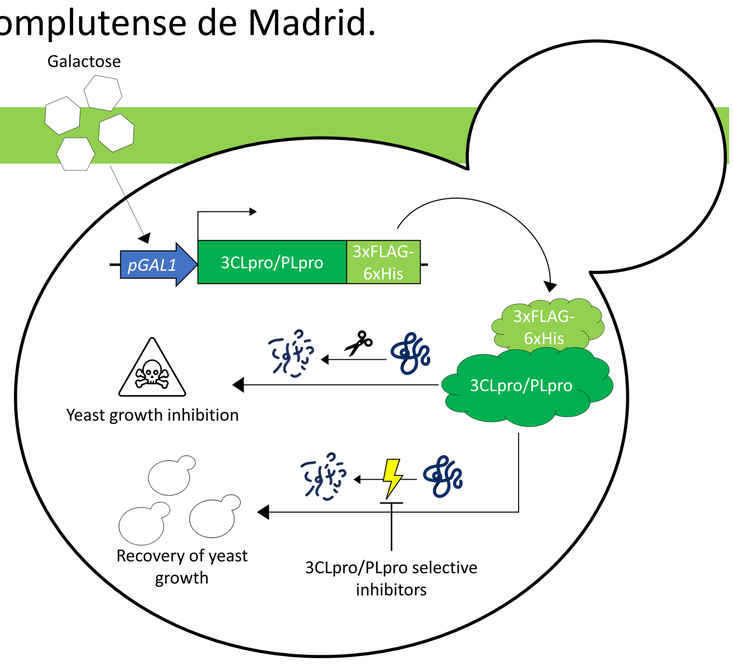
INTRODUCTION

The model yeast *Saccharomyces cerevisiae* is a **widely used system for biomedical and biotechnological studies** due to its easy manipulation, its versatility and the large number of molecular tools available. Besides, its similarity with higher eukaryotic cells provides an opportunity to extrapolate the discoveries in *S.cerevisiae* to more complex organisms.

The SARS-CoV-2 pandemic that paralyzed the world during 2020 made that all researchers´ efforts were focused on the search of treatments to fight against the virus, either preventively or during the progression of the disease. Vaccines have been essential to restore normality. However, the appearance of **new emerging variants** due to the natural viral evolutionary processes, makes it necessary to develop new strategies to get effective treatments against such variants.

One of the possible drug targets to inhibit the replication of this virus are **its proteases**, 3CLpro and PLpro, as they are essential for viral survival inside the host cell. This is because SARS-CoV-2 expresses part of its genome in 2 polyproteins, pp1a and pp1ab, which must be processed into several proteins with important functions for viral cell cycle. This processing is carried out by 3CLpro and PLpro, and drugs such as Paxlovid® can block this process by inhibiting 3CLpro.

Given the importance of these proteins for the virus, our research group decided **to use the yeast model *S.cerevisiae* as a study platform of 3CLpro and PLpro through their heterologous expression**, in order to better understand these proteases and search for compounds that are able to inhibit their activity.



OBJECTIVES

Express the SARS-CoV-2 proteases 3CLpro and PLpro in *S. cerevisiae* and study its phenotype in yeast cells.

Optimize the pharmacological screening yeast system through strategies that facilitate the entry of the compounds into cell and prevent their expulsion.

Validate our pharmacological based yeast screening system using known inhibitors of the proteases 3CLpro and PLpro.

METODOLOGY

1. Cloning and heterologous expression of 3CLpro and PLpro in *S. cerevisiae* has been carried out using a **high copy number expression vector with a galactose-inducible promoter.**
2. The study of proteases expression in *S. cerevisiae* has been performed by **Western-blotting**.
3. The phenotype produced by these proteases in cells has been analyzed through **yeast ten-fold dilution growth assays.**
4. Optimization and validation of the system has been done by incubating yeast expressing the proteases in the presence of **known inhibitors loaded onto a cellulose disc or dilutions of them in 96 well plates**. **Growth measurement** has been the reporter of 3CLpro and PLpro inhibition bioassay, determined either visually and/or by spectrophotometric measurement.

RESULTS

Expression of 3CLpro and PLpro proteases inhibits yeast growth due to their proteolytic activity

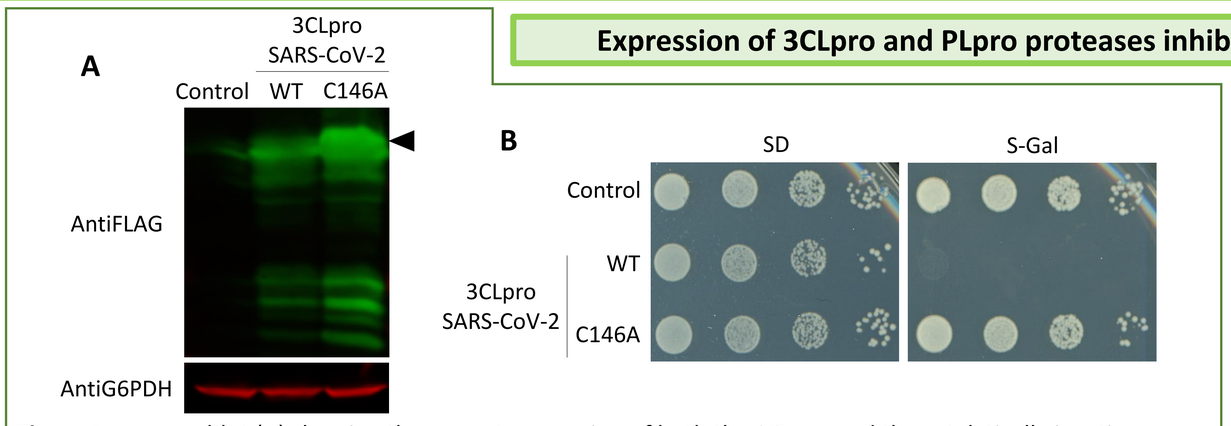


Figure 1. Immunoblot (A) showing the correct expression of both the 3CLpro and the catalytically inactive mutant C146A, C-terminal tagged with 3xFLAG-6xHis. G6PDH has been used as a loading control. The yeast growth assay (B) shows how the strain that is expressing 3CLpro WT is unable to grow, unlike the mutant C146A.

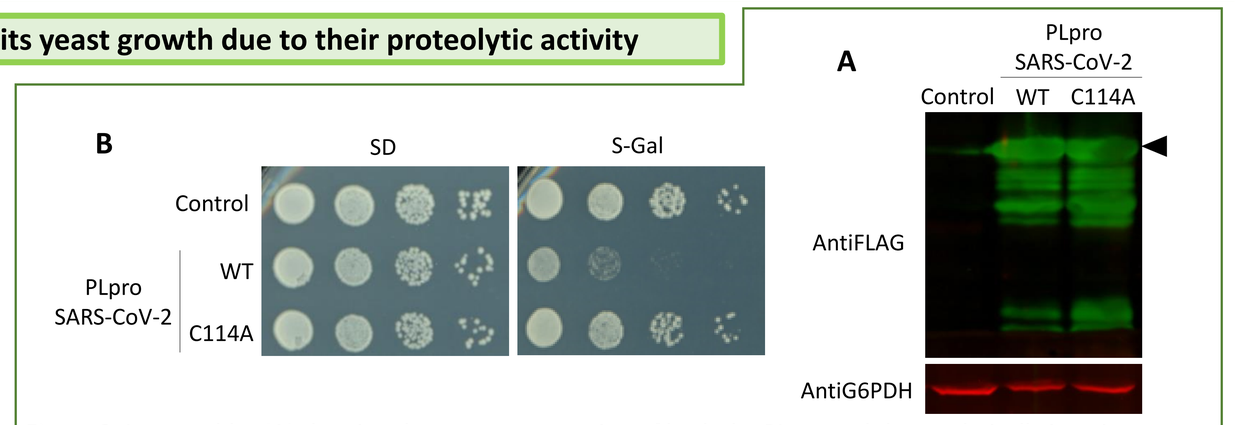


Figure 2. Immunoblot (A) showing the correct expression of both the PLpro and the catalytically inactive mutant C114A, C-terminal tagged with 3xFLAG-6xHis. G6PDH has been used as a loading control. The yeast growth assay (B) shows how the strain that is expressing PLpro WT is unable to grow, unlike the mutant C114A.

Yeast bioassay optimization with known 3CLpro and PLpro inhibitors by reverse halo assay

Strain AD1-8. Genotype:

Matα, pdr1-3, ura3, his1, yor1Δ::hisG, snq2Δ::hisG, pdr5Δ::hisG, pdr10Δ::hisG, pdr11Δ::hisG, ycf1Δ::hisG, pdr3Δ::hisG, pdr15Δ::hisG

Mutated transcription factors: Pdr1 and Pdr3.

Mutated ABC system efflux pumps (ATP-binding casette): Pdr5, Pdr10, Pdr11, Pdr15, Snq2, Yor1 and Ycf.

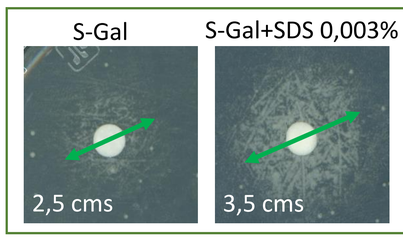


Figure 3. "Reverse halo" assay in solid medium with the inhibitor GC376 on the AD1-8 strain expressing 3CLpro in SG and SG-0,003% of SDS. The compound rescues yeast growth nearby the disc (left picture) and this effect is boosted in the presence of 0,003% of SDS, as the halo of growth around the disk is increased by 1 cm.

Validation of the yeast bioassay with known inhibitors of 3CLpro and Plpro in multiwell plate format in liquid media

3CLpro is inhibited in yeast by the compound GC376

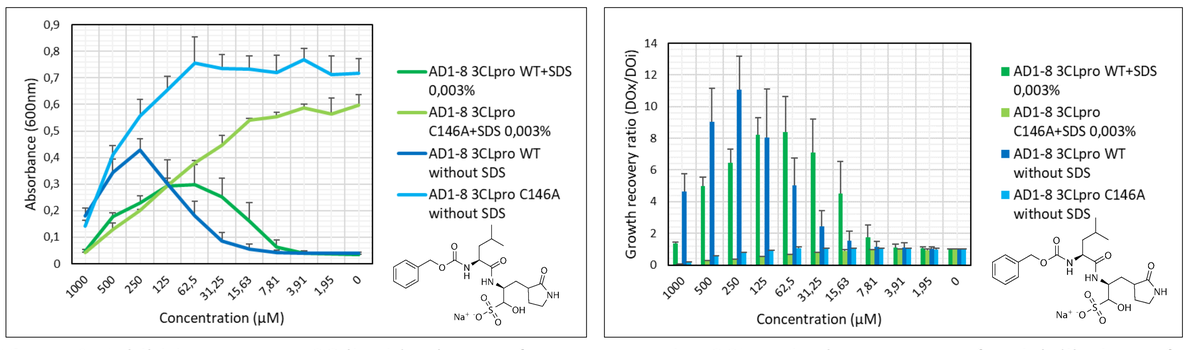


Figure 4. Inhibition assay on SG liquid cultures of yeast expressing 3CLpro in the presence of serial dilutions of GC376. Graphs represent the yeast growth (Absorbance at 600nm) at the concentrations of compound indicated. GC376 can inhibit 3CLpro in AD1-8 strain. Up to 10-fold recovery of growth is achieved with this compound (Graph on the right).

PLpro is inhibited in yeast by the compound GRL-0617

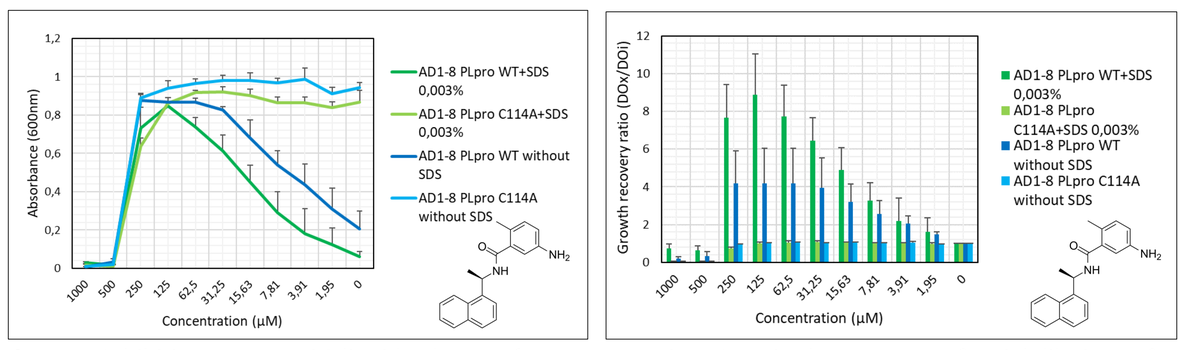


Figure 5. Inhibition assay on SG liquid cultures of yeast expressing PLpro in the presence of serial dilutions of GRL-0617. Graphs represent the yeast growth (Absorbance at 600nm) at the concentrations of compound indicated. GRL-0617 can inhibit PLpro in AD1-8 strain. Up to 8-fold recovery of growth is achieved with this compound (Graph on the right).

CONCLUSIONS

The expression of the proteases 3CLpro and PLpro in *S.cerevisiae* leads to **a yeast growth inhibition phenotype**. However, this phenotype was slightly different depending on the protease expressed, resulting in a drastic growth inhibition when expressing 3CLpro and a milder, partial growth inhibition when expressing PLpro.

The use of SDS as a cell membrane permeabilising agent, as well as the use of AD1-8 strain, has allowed the **optimization of the pharmacological screening** system for detecting 3CLpro/PLpro selective inhibitors.

Validation of this pharmacological screening platform with 2 known inhibitors of 3CLpro and PLpro (GC376 and GRL-0617 respectively), has probed that our **yeast bioassay is effective for the screening of molecules that inhibit these proteases**, which could be potential therapeutic agents.

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