Development of a yeast bioassay to screen for Nirmatrelvir drug resistance mutations in the SARS-CoV-2 protease 3CLpro.

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SPANISH Drug Discovery Network

INTRODUCTION

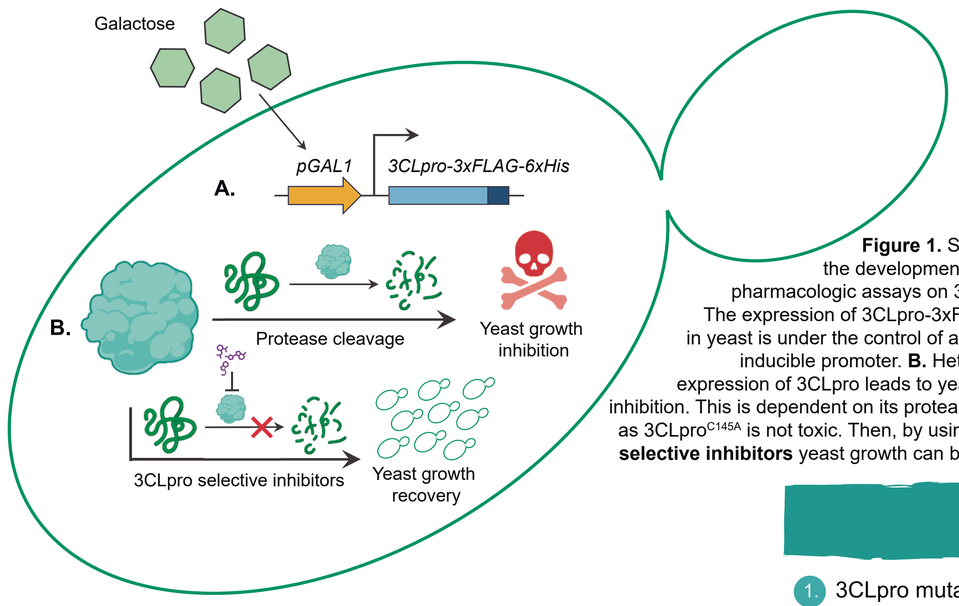

Figure 1. Strategy for the development of in vivo pharmacologic assays on 3CLpro. **A.** The expression of 3CLpro-3xFLAG-6His in yeast is under the control of a galactose inducible promoter. **B.** Heterologous expression of 3CLpro leads to yeast growth inhibition. This is dependent on its protease activity, as 3CLpro^{C145A} is not toxic. Then, by using **3CLpro selective inhibitors** yeast growth can be restored.

BACKGROUND

Model yeast *Saccharomyces cerevisiae* is a widely used system for the study of different kinds of diseases (infectious, neurodegenerative, autoimmune, etc.) due to its versatility and the great number of molecular tools available. In fact, its malleability allows us to develop unique strategies that could be quite complicated to design in more complex eukaryotic cells.

Using **yeast as a screening platform** brings us many advantages, such as its fast growth and its low cost. Besides, its similarity with human cells allows us not only to screen for new potential molecules with pharmacological activity but also to analyze their toxicity in a *in vivo* system.

That´s why we have developed a screening platform in yeast for the discovery of new drugs against the main protease of SARS-CoV-2 (the etiological agent of the 2020 pandemic), 3CLpro. This protease, along with PLpro, is **essential for viral survival** into the host cell. SARS-CoV-2 expresses a part of its genome in 2 polyproteins, pp1a and pp1ab that must be cleaved by 3CLpro and PLpro into 16 functional non structural proteins. This is the reason why 3CLpro inhibitors such as Nirmatrelvir, which is already commercialised and used in therapy for COVID-19 disease, have been designed. Nevertheless, the genetic evolution of the virus during infection and the selection pressure exerted by 3CLpro inhibitors may give rise to the appearance of mutations conferring viral resistance to the existing therapies, such as Nirmatrelvir[1].

Thus, we have decided to adapt our screening system to search for 3CLpro Nirmatrelvir resistance mutations. As seen in **Figure 1**, our system is based on the toxic effect of 3CLpro when expressed in yeast. Therefore, resistance mutations in 3CLpro would impair the growth rescue exerted by Nirmatrelvir.

OBJECTIVES

1. Randomly mutate the protease 3CLpro by **mutagenic PCR** and transform the product in our yeast model.
2. **Classify the mutants** based on their activity (which depends on 3CLpro ability to inhibit yeast growth).
3. Assay with Nirmatrelvir in those mutants that maintain their activity in order to look for **resistance mutations.**

MATERIALS AND METHODS

1. 3CLpro mutant variants were obtained by **PCR-based random mutagenesis** with the error-prone polymerase Mutazyme II® over the coding sequence of the protease.
2. Transformation of the PCR products in **AD1-8 yeast strain**[2] has been carried out by **gap-repair**, thus taking advantage of the recombination capabilities of yeast. This strain is mutated in efflux pumps so that assayed compound cannot leave the cell.
3. Classification of mutants by their activity has been carried out by **yeast growth assay** in solid media.
4. **Drug resistance assays** have been done in 96 multi-well plates in liquid media supplemented with the appropiate carbon source and 0.003% SDS to facilitate the drug entry into the cell.

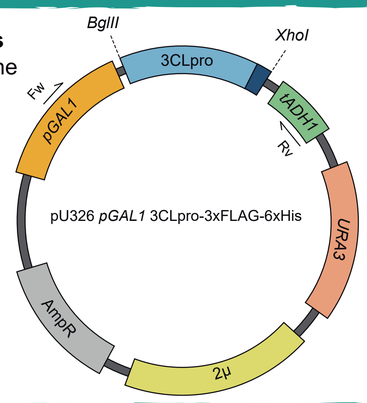

Figure 2. Expression vector employed for random mutagenesis. It is a high copy number expression vector with a galactose inducible promoter. *BglII* and *XhoI* are the restriction enzymes used to eliminate the coding sequence of 3CLpro WT and to **open the vector for subsequent recombination**. The half arrows represent the forward (Fw) and reverse (Rv) **primers used for the mutagenic PCR** and their hibridation site (*pGAL1* and *tADH1* respectively). These assays have been done on uracil-free media to maintain plasmid auxotrophy.

RESULTS

GENERATION OF A LIBRARY OF 3CLPRO MUTANTS IN YEAST BY ERROR-PRONE PCR AND GAP-REPAIR

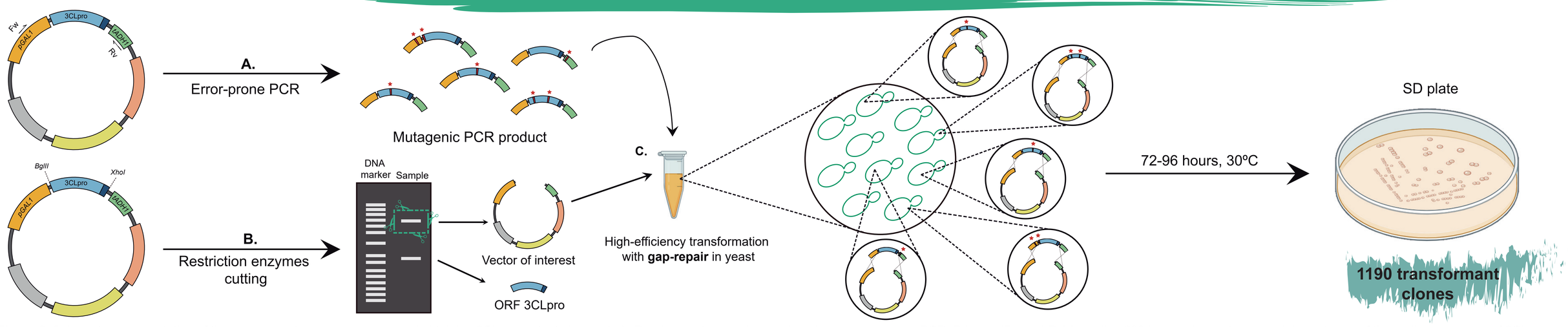

Figure 3. Schematic representation of the random mutagenesis process and obtaining 3CLpro mutants in yeast. **A.** Random mutagenesis was done by an **error-prone PCR**. Primers (Fw and Rv) hybridize 200 base pairs upstream and downstream of the coding sequence of 3CLpro in order to generate extremes capable of recombining with the processed vector during the gap repair. This PCR has been designed to incorporate 0-4,5 mutations/kb. **B.** Restriction enzyme cuts **right at the beginning and at the end of the coding sequence of 3CLpro**, using the restriction enzymes *BglII* and *XhoI*. Fragments obtained have been **separated** by electrophoresis in an agarose gel, and the **vector of interest** has been collected for the gap-repair. **C.** High-efficiency transformation with gap repair in yeast. AD1-8 competent yeast cells have been collected in the exponencial phase. The addition of the DNA has been done in a 2:1 ratio (Mutagenic PCR product: vector digested) and yeast cells have been cultivated in SD plates. During this time, yeast has been recombining our processed vector with the PCR product (a process known as gap- repair), and they will only be able to grow those yeast cells that have reconstitute the whole plasmid. **1190 transformant** have been obtained by this method and, due to the error-prone PCR, each one theoretically carries a different mutation in the gene of interest.

SELECTION OF 3CLPRO POTENTIAL MUTANTS WITH UNALTERED PROTEASE ACTIVITY

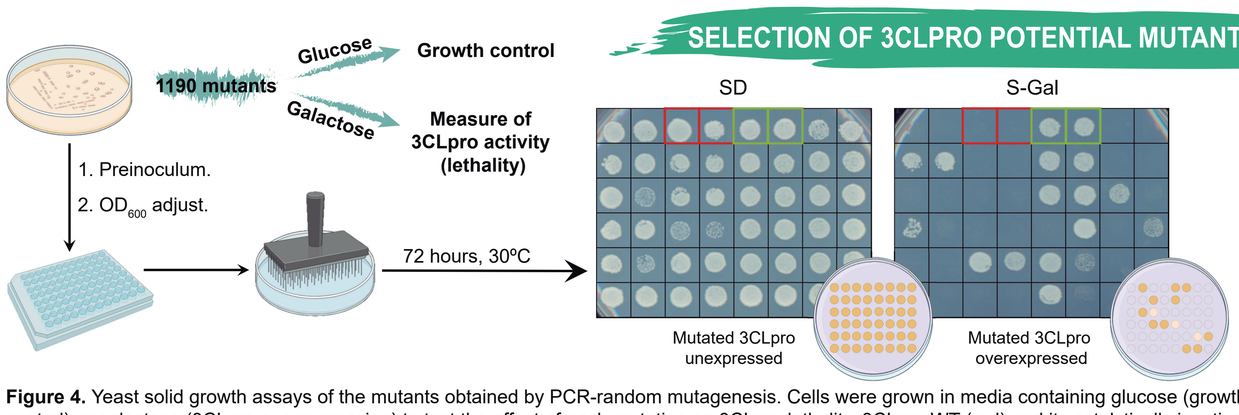

Figure 4. Yeast solid growth assays of the mutants obtained by PCR-random mutagenesis. Cells were grown in media containing glucose (growth control) or galactose (3CLpro overexpression) to test the effect of each mutation on 3CLpro lethality. 3CLpro WT (red) and its catalytically inactive version 3CLpro^{C145A} (green) were employed as growth controls.

	Fully inactive mutants of 3CLpro	Partially inactive mutants of 3CLpro	Active mutants of 3CLpro	Failed clones	Amount
Selection criteria (qualitative)	Growth in glucose Growth in galactose	Growth in glucose Partial growth in galactose	Growth in glucose No growth in galactose	No growth in glucose	
Number of mutants	245	76	**825**	44	1190
%	20.59%	6.39%	69.33%	3.70%	100%

Table 1. Classification of clones according to their activity in yeast. Those that inhibit yeast growth in galactose media are considered as **active mutants of 3CLpro**, meanwhile those that have lost their activity completely or partially are considered **catalytically inactive mutants of 3CLpro**. **Failed clones** are those that haven´t grown in glucose during our assays, which means they are not suitable for further screening.

SEARCH FOR RESISTANCE MUTANTS FROM ACTIVE 3CLPRO CLONES

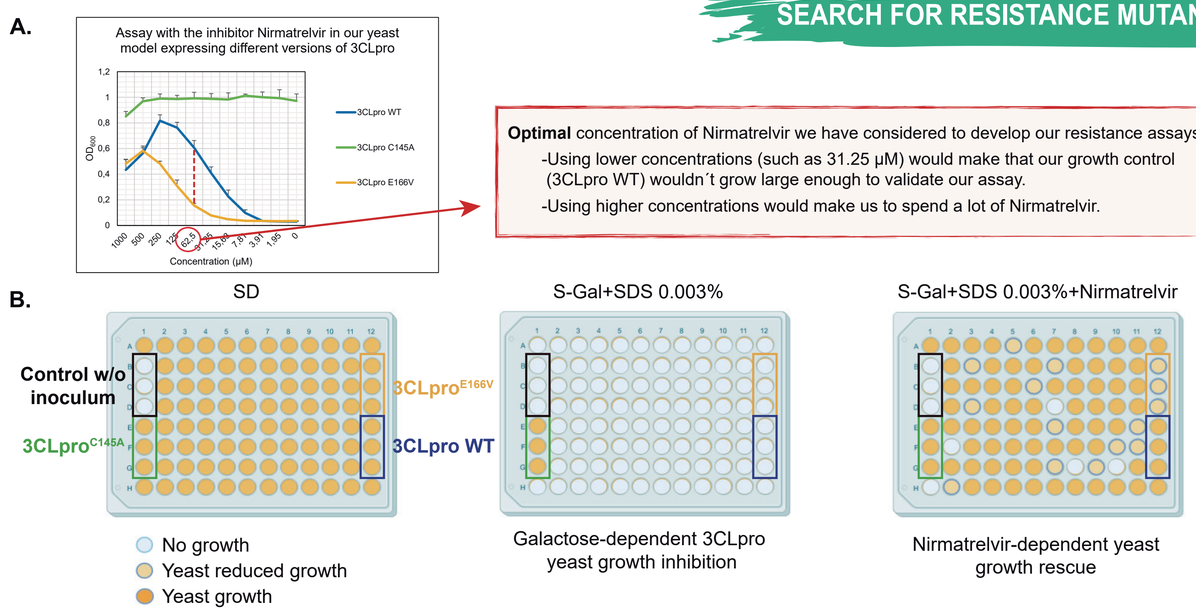

Figure 5. Nirmatrelvir-resistance screening of 3CLpro catallytically active mutants. **A.** Dose-response assay to select the **optimal working concentration of Nirmatrelvir.** 3CLpro^{C145A} version (catallytically inactive, green) was used as positive growth control. **3CLpro^{E166V} version[3] (yellow) was used as Nirmatrelvir-resistant control**. 3CLpro WT (blue) growth was compared to 3CLpro^{E166V} and 62.5 µM was selected as the lowest Nirmatrelvir concentration that allow a proper discrimination of the growth achieved by yeast mutants. **B.** Nirmatrelvir-resistance assays in liquid media. 3CLpro mutants were cultured in parallel in medium with glucose (positive growth control), galactose (lethal activity control of 3CLpro), and galactose containing 62.5 µM of Nirmatrelvir. 3CLpro^{C145A}, 3CLpro WT and 3CLpro^{E166V} were included as controls in every assay (the same distribution of samples and controls were applied for the three replicates).

	Nirmatrelvir-sensitive mutants	Nirmatrelvir-resistance mutants	Failed clones	Amount
Selection criteria (quantitative)	Growth recovery ratio >30%	Growth recovery ratio <30%	No growth in glucose or growth in galactose	
Number of mutants	517	**267**	41	825
%	62.66%	32.36%	4.97%	100%

Table 2. Preliminary results from our first screening for resistance mutations. Nirmatrelvir-sensitive mutants are those that are not able to grow in galactose media but restore their growth when they are exposed to nirmatrelvir obtaining a **growth recovery ratio >30%**. Nirmatrelvir-resistant mutants are those that are not able to grow in galactose media and don´t restore their grow when they are exposed to nirmatrelvir, obtaining a **growth recovery ratio <30%**. All of these mutants should be able to grow in glucose media, if not, they are considered failed clones, which means they are not suitable for further screening.

CONCLUSIONS AND FUTURE PERSPECTIVES

1. The yeast bioassay described in this work has reproduced the Nirmatrelvir resistance of the E166V version of 3CLpro[3], constituting a proof-of-principle approach for the identification of unknown 3CLpro mutations that confer resistance to this drug.
2. Error-prone PCR has been carried out successfully. 1190 mutants have been obtained and assayed in our model yeast, obtaining **825 3CLpro mutants that maintain their activity.**
3. Nirmatrelvir-resistance assays with 825 active 3CLpro clones have resulted in **267 nirmatrelvir-resistant mutants**. Nevertheless, those mutants must be **re-tested** in triplicate.
4. Preliminary results have shown that around **30 mutants** will pass the recomprobation assays. These mutants will be **sequenced** and re-tested with nirmatrelvir in order to carry out a **dose-response** curve that, in the last instance, will confirm and validate the resistance mutations obtained.

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