

In silico evaluation of *Candida* species enolase as a therapeutic target for routine antifungal agents against candidiasis

Evaluación in silico de la enolasa de especies de *Candida* como blanco terapéutico para antifúngicos de uso rutinario contra candidiasis

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ABSTRACT

The objective of this study was to evaluate *in silico* the enolase of various species of *Candida* and *Homo sapiens* as a potential therapeutic target for commercial antifungals, by molecular modeling. Proteins were modeled with AlphaFold3 and structurally refined using ModLoop, SAVES v6.0, and YASARA. The ligands were downloaded from PubChem, optimized in Avogadro, and coupled with AutoDock Vina in PyRx. The complexes were analyzed with ChimeraX and Discovery Studio Visualizer. Fluconazole presented the highest number of favorable interactions, especially with *C. glabrata*, *C. krusei*, and *C. parapsilosis*, with coincidences in key residues from the active site, suggesting specific affinity and possible functional inhibition. In contrast, nystatin showed fewer interactions, little species differentiation, and no match with active sites, indicating low therapeutic relevance of enolase against this drug. Caspofungin had a broad binding profile, especially in *C. parapsilosis* and human gamma enolase, although with several unfavorable interactions. Only one relevant match (Glu212) was identified in the active site of *C. parapsilosis*. These results highlight fluconazole as the compound with greater specificity towards fungal enzymes and lower risk of affecting human isoforms.

Keywords: Molecular docking; active site; protein-ligand interactions.

RESUMEN

El objetivo de este estudio fue evaluar *in silico*, mediante modelado molecular, la enolasa de diversas especies de *Candida* y de *Homo sapiens* como posible blanco terapéutico de antifúngicos de uso clínico. Las proteínas se modelaron con AlphaFold3, se refinaron con ModLoop, se evaluaron con SAVES v6.0 y se minimizaron energéticamente con YASARA. Los ligandos se obtuvieron de PubChem, se optimizaron en Avogadro y se acoplaron con AutoDock Vina en PyRx. Los complejos se analizaron con ChimeraX y Discovery Studio Visualizer. El fluconazol presentó el mayor número de interacciones favorables, especialmente con *C. glabrata*, *C. krusei* y *C. parapsilosis*, con coincidencias en residuos clave del sitio activo, lo que sugiere afinidad y posible inhibición funcional. En contraste, la nistatina mostró menos interacciones, escasa diferenciación entre especies y ausencia de coincidencias

en sitios activos. La caspofungina exhibió un perfil de unión amplio, más notable con *C. parapsilosis* y la enolasa gamma humana, aunque con varias interacciones desfavorables y solo se identificó una coincidencia relevante en el sitio activo de *C. parapsilosis*. Estos resultados muestran al fluconazol como el compuesto con mayor especificidad hacia enzimas fúngicas y menor riesgo de afectar isoformas humanas.

Palabras clave: Acoplamiento molecular; sitio activo; interacciones proteína-ligando.

INTRODUCTION

Infections caused by species of the genus *Candida* pose a significant threat to human health, especially in immunocompromised individuals. This opportunistic human pathogen can colonize both mucosal surfaces and deep tissues, causing both superficial and systemic infections (Vallabhaneni *et al.*, 2016). The pathogenicity of *Candida* is due to a combination of virulence factors that facilitate adhesion to host cells, tissue invasion, and evasion of immune mechanisms (Henriques and Silva, 2021).

Among these factors, enolase, a key enzyme in the glycolysis pathway, has gained interest as a therapeutic target. This protein not only performs essential metabolic functions in the cell cytosol, but is also located in the cell wall (CW) of *Candida*, where it interacts with host components such as vitronectin, fibronectin, and plasminogen. These interactions enhance fungal adhesion, extracellular matrix degradation, and dissemination during infection (Satala *et al.*, 2020). In addition, enolase has been shown to modulate the immune response by activating B lymphocytes and monocytes, promoting the release of inflammatory cytokines such as IL-6, and even interfering with the complement system (Langenhorst *et al.*, 2023). Several studies have pointed to the potential of enolase as a therapeutic target. Deletion of the ENO1 gene in *C. albicans* increases the fungus's susceptibility to conventional antifungals such as fluconazole, voriconazole, and amphotericin B (Arvizu-Rubio *et al.*, 2022), and vaccines based on this protein have been reported to protect against disseminated candidiasis (Shukla *et al.*, 2024). Despite this, there are currently no approved antifungal agents that specifically target enolase, which opens a window of opportunity for research into new treatments.

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In this regard, computational tools such as molecular docking offer a powerful strategy for exploring interactions between fungal enolase and antifungal compounds. This approach allows the identification of potential inhibitors that affect both its intracellular catalytic function, and its extracellular activities related to virulence. In addition, comparisons between *Candida* enolase and its human homologue are essential to evaluate selectivity and reduce toxicity risks (Pus-hpakom *et al.*, 2019). For example, compounds such as isobavachalcone (IBC) have shown selective affinity for *C. albicans* enolase, with remarkable efficacy *in vitro* and *in vivo* (Wu *et al.*, 2024). Similarly, baicalein (BE), known for its antifungal activity and synergy with fluconazole, has been proposed as a potential inhibitor of the glycolytic pathway through interaction with enolase (Li *et al.*, 2022). These findings suggest that evaluating whether current antifungals also interact with enolase could provide clues to better understand their spectrum of action, identify secondary mechanisms involved in their efficacy or toxicity, and potentially guide the rational design of more effective combination therapies.

In this study, three clinically relevant antifungal agents, fluconazole, nystatin and caspofungin were selected for evaluation due to their clinical relevance and different mechanisms of action against *Candida* species. Fluconazole, a triazole antifungal, inhibits lanosterol 14- α -demethylase, a key enzyme in the ergosterol biosynthetic pathway, leading to membrane destabilization and growth inhibition (Perfect, 2017). Nystatin, a polyene, exerts its antifungal effect by binding to ergosterol in the fungal membrane, inducing the formation of pores that cause leakage of intracellular components and cell death (Lazić *et al.*, 2024). Caspofungin, a representative echinocandin, targets the fungal cell wall by inhibit b-(1,3)-D-glucan synthase, an essential enzyme for glucan biosynthesis, resulting in osmotic instability and cell lysis (Arendrup and Patterson, 2017). These drugs were chosen not only because they represent the three major antifungal classes currently used in clinical practice, but also to explore whether their mechanisms of action may involve secondary interactions with key virulence-associated proteins such as enolase. Understanding such interactions could provide insights into additional modes of antifungal activity and guide the rational design of more effective and selective therapeutic strategies.

MATERIALS AND METHODS

Sequence selection

The protein sequences corresponding to enolase were selected from the UniProt database (<https://www.uniprot.org>), using the gene name ENO1 and the enzymatic function associated with enolase as search criteria. Complete functional isoforms were selected for the five *Candida* species, ensuring that they corresponded to complete and correctly annotated proteins. Similarly, the three human isoforms of enolase were identified: α -enolase (ENO1), β -enolase (ENO3), and γ -enolase (ENO2). The functional identity of each protein was confirmed by verifying the presence of the enolase do-

main (IPR000941) and the functional annotation in UniProt. All sequences were downloaded in FASTA format, and their respective identifiers are presented in Table 1.

Construction of three-dimensional (3D) models of the proteins

The monomeric three-dimensional (3D) structures of the proteins were predicted using AlphaFold3 (Abramson *et al.*, 2024). The corresponding sequences, in FASTA format, were uploaded to the system, and structural predictions were generated using the default parameters. This version was chosen because it offers greater structural accuracy compared to previous versions and allows the direct incorporation of metal ions (Mg^{2+}) as cofactors.

Structural quality assessment

The structural quality of the generated three-dimensional models was evaluated using the SAVES v6.0 server (<https://saves.mbi.ucla.edu/>), which integrates the ERRAT v2.0, Verify3D, and Procheck v3.5.4 tools, which allow the analysis of critical aspects such as the overall quality of the model, the compatibility between the three-dimensional structure and its primary sequence, and the stereochemical geometry using Ramachandran diagrams, respectively. Each model was reevaluated after the structural refinement stages, verifying the improvements through quantitative comparison of the values obtained by each tool and visual inspection of the Ramachandran diagrams. A model was considered to be of adequate structural quality if it met the following criteria: an ERRAT value greater than 90 %, a Verify-3D score greater than 70%, and no residues in unfavorable regions according to the Procheck analysis (Williams *et al.*, 2018).

Refinement of loops

Conformational refinement of the loops present in structurally unfavorable regions was performed using ModLoop (Fiser and Sali, 2003) (<https://modbase.compbio.ucsf.edu/mod-loop/>), with the aim of reducing local tensions and optimizing the geometry in flexible regions of the model. The selection of segments to be refined was based on the identification of residues with scores outside the limits allowed in the Ramachandran diagrams, indicating a sterically unfavorable conformation. Based on this analysis, the following residues

Table 1. Identificadores de las secuencias proteicas de enolasa obtenidas de las bases de datos UniProt y NCBI para las especies de *Candida* estudiadas.

Table 1. Identifiers of enolase protein sequences obtained from UniProt and NCBI databases for the *Candida* species.

Protein	Organism	UniProt ID	NCBI Reference
Enolase	<i>C. albicans</i>	P30575	XP_711883.1
	<i>C. glabrata</i>	Q6FTW6	XP_446328.1
	<i>C. tropicalis</i>	C5MD83	XP_002549887.1
	<i>C. krusei</i>	A0A099NYL7	OUT23677.1
	<i>C. parapsilosis</i>	A0A8X7TD55	KAF6059114.1
Gamma enolase	<i>H. sapiens</i>	P09104	CAA36215.1
Beta enolase	<i>H. sapiens</i>	P13929	CAA40163.1
Alpha enolase	<i>H. sapiens</i>	P06733	CAA34360.1

were selected for refinement: 404-410 in the enolase of *C. albicans*, 400-406 in *C. glabrata*, 400-406 in *C. krusei*, 238-242 and 402-409 in *C. parapsilosis*, 402-410 in *C. tropicalis*, 398-403 in the gamma enolase of *H. sapiens*, 398-403 in the beta enolase, and 398-402 in the alpha enolase. After refinement, an improvement in the overall quality of the models was observed, evidenced by a higher proportion of residues in allowed regions in the Ramachandran diagrams.

Protein energy minimization

The structural models with the best quality scores were subjected to an energy minimization process using the YASARA server (Land and Humble, 2018), with the aim of optimizing intramolecular interactions and reducing residual structural stresses. The minimization was carried out using the AMBER14 force field, under predetermined parameters and a maximum of 5000 steps until the system reached energy convergence. After refinement, the models were evaluated using the SCORE function integrated in YASARA, which confirmed an effective reduction in total energy, reflecting a more stable and favorable conformation.

Ligand modeling

The chemical structures of fluconazole, nystatin and caspofungin were downloaded in SDF format from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>). Subsequently, the corresponding files were imported into the Avogadro program (Hanwell *et al.*, 2023), and in this environment, the three-dimensional conformations of each ligand were automatically generated and subjected to an energy minimization process using the MMFF94 force field, chosen for its specific parameterization for organic molecules of pharmacological interest. Energy minimization was performed using the conjugate gradient algorithm, without structural restrictions, with a maximum of 500 iterations and a convergence criterion of 10^{-7} kcal/mol, which was achieved in all cases. Finally, the optimized structures were exported in mol2 format, ensuring compatibility with the molecular docking protocols used in the subsequent stages.

Molecular docking

The docking analysis was performed using AutoDock Vina 1.2.0 (Eberhardt *et al.*, 2021), through the PyRx 0.8 interface (Dallakyan and Olson, 2015). The previously optimized three-dimensional structures of proteins (enolases) and ligands (fluconazole, nystatin and caspofungin) were imported in PDB and Mol2 formats, respectively, and converted to PDBQT format within the PyRx environment. In this process, proteins were treated as rigid macromolecules, while ligands were considered flexible structures, with rotatable bonds assigned by the software.

Protein preparation

The proteins used in molecular docking were previously subjected to energy minimization in YASARA, using the AMBER14 force field. Subsequently, during their preparation in PyRx, water molecules and non-essential heteroatoms were removed, polar hydrogens were added, and Gasteiger-type partial charges were assigned. The polypeptide chains were kept rigid, in accordance with the rigid receptor model used by AutoDock Vina. As a structural control measure, the proteins were placed in an aqueous environment to verify their integrity before docking; however, the interaction analyses were performed under vacuum conditions, using the program's default parameters.

Docking configuration and parameters

A blind docking strategy was used for the ligand-receptor interaction analysis in order to allow an undirected exploration of the entire protein surface. The exhaustiveness parameter was set to 10, which represents a balance between accuracy and computational time in AutoDock Vina. This program uses an iterative local search algorithm as a stochastic global optimization method to predict the most favorable binding conformations based on the calculated free binding energy.

The search box parameters were as shown in Table 2.

The best binding conformations were selected based on the lowest free binding energy values reported by AutoDock Vina.

Tabla 2. Parámetros caja de búsqueda.

Table 2. Search box parameters.

Species	Center			Dimensions		
	X	Y	Z	X	Y	Z
<i>C. albicans</i>	-0.787834	Y: 1.0422	-0.4696	57.834649 Å	52.5611 Å	65.0045 Å
<i>C. glabrata</i>	0.0062	Y: 0.8441	-0.3686	61.6272 Å	58.5696 Å	56.0320 Å
<i>C. krusei</i>	0.2398	-0.1795	0.3155	63.4118 Å	56.5162 Å	53.6784 Å
<i>C. parapsilosis</i>	0.3831	-0.7423	0.8080	69.6486 Å	48.7150 Å	53.3010 Å
<i>C. tropicalis</i>	-1.3749	0.1341	0.154	58.9396 Å	69.7462 Å	53.8525 Å
Alpha enolase <i>H. sapiens</i>	1.3362	1.2713	0.9960	57.446 Å	53.5090 Å	59.7429 Å
Beta enolase <i>H. sapiens</i>	-0.6446	0.208	-0.4299	59.6769 Å	57.3480 Å	63.0099 Å
Gamma enolase <i>H. sapiens</i>	-0.134	-1.3112	0.507	65.5136 Å	61.057 Å	49.6810 Å

Post-docking analysis

The protein-ligand complexes generated after docking were visualized and analyzed in three dimensions using UCSF ChimeraX (Meng *et al.*, 2023). For detailed characterization of the molecular interactions, BIOVIA Discovery Studio Visualize 2024 was used, which allowed the interactions to be represented in two dimensions. Different types of interactions were identified and evaluated, including hydrogen bonds, hydrophobic (alkyl-type) interactions, p-p interactions, and electrostatic interactions, with the aim of evaluating the quality of the ligand-protein docking.

RESULTS AND DISCUSSION

To investigate whether commonly used antifungal agents could interact with *Candida* enolase, molecular docking analyses were performed for fluconazole, nystatin, and caspofungin. These compounds represent the major classes of antifungals currently employed in clinical practice, and were selected to provide a comparative framework for assessing potential cross-mechanistic interactions with this multifunctional enzyme. Previous studies have shown that certain conventional antifungals can interact with unexpected protein targets, contributing to secondary effects or synergistic activities (Pushpakom *et al.*, 2019). Therefore, exploring their affinity toward enolase offers an opportunity to identify noncanonical binding events that may influence antifungal efficacy or virulence modulation. The following results describe the predicted binding affinities, interaction residues, and structural implications of these drugs when docked with enolases from *Candida* species and human enzymes, providing a comparative perspective on selectivity and potential therapeutic relevance.

Interaction between fluconazole and enolases

Fluconazole showed the highest number of protein-ligand interactions among the antifungals evaluated. Twenty interactions were identified in *C. glabrata*, 17 in *C. tropicalis*, 14 in *C. krusei*, and 13 in *C. parapsilosis*. *C. albicans*, on the other hand, is the species whose enolase showed the lowest number of interactions (7), suggesting a lower relative affinity. Similarly, it can be observed that most of these interactions correspond to hydrogen bonds or attractive charge bonds (salt bridges) (Table 3, Fig. 1). Furthermore, it is important to mention that the enolase of *C. albicans*, *C. krusei*, and *C. parapsilosis* did not show unfavorable interactions, which could correlate with greater stability of the complex formed, favoring possible enzyme inhibition. This interaction profile suggests a more specific, and possibly functional coupling, particularly for *C. krusei* and *C. parapsilosis*.

In human enolases, interactions were more limited: alpha isoform with 5, beta with 9, and gamma with 10, with hydrogen bond interactions predominating (Table 3, Fig. 2). However, in these complexes there are unfavorable interactions (Fig. 2), except for the alpha isoform, where all interactions are favorable, suggesting a more stable complex.

These data suggest a significant binding affinity of fluconazole with *Candida* spp. enolases, especially *C. krusei* and *C. parapsilosis*, which show several favorable interactions that stabilize the complex. Furthermore, the data shown in Table 4 suggest that the interaction between enolase of these *Candida* species and human enzymes, is favorable because a significant affinity energy was determined in the molecular coupling, and based on the values obtained, the possibility of interaction is suggested. Additionally, we evaluated whether this interaction occurs at the active site of the enzyme. Based on the data described in UniProt (Table 5) and the results obtained from the molecular docking showing the amino acid residues involved in the enolase-fluconazole complex, a comparison was made to determine the existence of interactions in key residues or nearby residues that could structurally or functionally influence the catalytic activity of the enzyme. The results showed that, in all the enzymes analyzed except for the enolase from *C. albicans*, there are residues involved in the enolase-fluconazole coupling and that they are found in the active site or substrate binding site (Table 5), a finding that suggests a possible more significant interaction between this drug and the enzymes analyzed. The fact that *C. albicans* did not show direct matches between the residues identified by docking and the sites described in UniProt, suggests that the active site of enolase in this species does not appear to be affected by fluconazole. Regarding human enolases, the gamma isoform of *H. sapiens* showed similarity with nine residues, the alpha isoform with three, and the beta isoform with only one. The joint analysis of the results obtained shows that, even though the affinity energy obtained in the molecular docking is highly similar for all the enzymes analyzed, the type of interactions varies depending on the species. Therefore, based on this last criterion, it can be suggested that fluconazole shows adequate affinity towards *Candida* spp. enolases, mainly in *C. krusei* and *C. tropicalis*, compared to human isoforms, where the matches were limited.

Interaction between nystatin and enolases

Nystatin had the lowest total number of interactions (Table 6) among the antifungals analyzed. In fungal species, the values ranged from 5 to 8 interactions, while in human enolases they ranged from 4 to 8. The number and type of interactions identified indicate a less consistent binding pattern compared to the number of interactions found for fluconazole. Furthermore, there appears to be a minimal difference between fungi and humans, suggesting a non-specific affinity, which could be due to their primary mechanism of action based on membrane disruption (Lazić *et al.*, 2024). It should be noted that, although some interactions were identified in *C. albicans*, *C. krusei*, and *C. parapsilosis* (Fig. 4) as well as in human isoforms (Fig. 5), these were rare, and no unfavorable interactions were observed. However, the low total number of residues involved, and the lack of differentiation between fungal and human species, suggest that enolase is of little relevance as a therapeutic target for this drug. On the other

Tabla 3. Interacciones entre el fluconazol y las enolasas.**Table 3.** Interactions between fluconazole and enolases.

Organism	Amino acid	Atom from ligand	Type of interaction	Distance
<i>C. albicans</i>	Lys A:432	N-H	Conventional bond hydrogen	2.80
	Asp A:433	N	Attractive charge	2.30
	Phenyl A:434	O	Pi cation	4.33
	Lys A:318	F	Halogen (Fluorine)	3.60
	Val A:294	F	Carbon-Hydrogen bond	2.80
	Gln A:435	N-H	Conventional hydrogen bond	2.30
	Gly A:316	C-H	Carbon -H	3.40
<i>C. glabrata</i>	Asp A:321	H	Salt bridge, attractive charge	2.19
		N	Salt bridge, attractive charge	4.73
		N	Salt bridge, attractive	3.91
		O	Salt bridge, attractive charge	4.60
		N	Salt bridge, attractive charge	3.70
		H	salt bridge, attractive charge	2.43
		Fluor	Carbon-hydrogen bond	3.60
	Asp A:322	H	Salt bridge, attractive charge	2.10
		N	Salt bridge, attractive charge	2.26
		C	Salt bridge, attractive charge	5.13
		C	Salt bridge, attractive charge	5.32
		O	Salt bridge, attractive charge	5.13
	Glu A:169	N	Salt bridge, attractive charge	4.40
		N	Salt bridge, attractive	3.02
		N	Salt bridge, attractive charge	4.35
		O	Salt bridge, attractive charge	4.67
	Glu A:212	N	Salt bridge, attractive charge	4.64
	Asp A:247	O	Salt bridge, attractive charge	4.90
		N	Salt bridge, attractive charge	4.55
		N	Salt bridge, attractive charge	3.76
		N	Salt bridge, attractive charge	3.88
	His A:160	O	Unfavorable positive-positive	5.13
	Wing A:249	F	Carbon-hydrogen bond	3.61
	GluA:252	F	Carbon-hydrogen bond	3.24
	Asp A:322	F	Carbon-hydrogen bond	3.60
	Arg A:375	H	Unfavorable positive-positive	2.20
	Ser A:40	H	Conventional hydrogen bond	2.20

Organism	Amino acid	Atom from ligand	Type of interaction	Distance
<i>C. krusei</i>	His A:160	N	Pi cation	4.77
		N	Pi cation	4.62
		N	Pi cation	3.54
	Glu A:212	O	Attractive charge	4.90
		N	Attractive charge	3.23
		N	Attractive charge	3.18
		N	Attractive charge	3.26
	Glu A:169	N	Attractive charge	5.26
		O	Attractive charge	5.45
		N	Attractive charge	4.09
		N	Attractive charge	4.94
		N	Attractive charge	4.20
	Gln A:168	H	Conventional hydrogen bond	2.41
	Asp A:247	H	Attractive charge	4.90
	Ser A:376	F	Conventional hydrogen bond	2.89
	Arg A:375	F	C-H	2.70
	His A:374	F	C-H(F)	3.16
	Lys A:346	O	Hydrogen bridge	2.35
	Asp A:321	N	Attractive charge	4.00
		N	Attractive charge	4.33
		N	Attractive charge	4.93
		O	Attractive charge	4.89
	Asp A :322	N	Attractive charge	5.06
		N	Attractive charge	3.81
		N	Attractive charge	5.34
<i>C. parapsilosis</i>	Asp A:247	N	Attractive charge	5.47
		N	Attractive charge	5.22
	Asp A:324	F	C-H(F)	3.38
	Ser A:378	F	Hydrogen bridge	2.79
	Lys A:348	O	Hydrogen bridge	2.37
	Glu A:212	N	Attractive charge	8.00
		N	Attractive charge	3.86
		N	Attractive charge	3.27
		N	Attractive charge	3.73
		N	Attractive charge	4.00
		N	Attractive charge	3.30
		N	Attractive charge	3.00
	Glu A:169	N	Attractive charge	3.78
		N	Attractive charge	4.55
		N	Attractive charge	3.62
		N	Attractive charge	1.95

Organism	Amino acid	Atom from ligand	Type of interaction	Distance
<i>C. tropicalis</i>	Asp A:325	N	Attractive charge	4.69
	Asp A:248	N	Attractive charge	5.08
	Asp A:324	N	Attractive charge	4.30
		N	Attractive charge	4.44
		N	Attractive charge	4.93
		O	Attractive charge	4.96
	Glu A:170	N	Attractive charge	4.97
		N	Attractive charge	5.25
		O	Attractive charge	5.19
		N	Attractive charge	3.55
		N	Attractive charge	4.41
		H	Attractive charge	1.86
	Glu A:213	O	Attractive charge	5.21
		N	Attractive charge	3.91
		N	Attractive charge	3.30
		N	Attractive charge	3.10
	Gln A:169	H	Unfavorable positive-positive	2.15
	Lys A:400	F	Carbon-hydrogen bond	2.68
	His A:377	F	Carbon-hydrogen bond	2.99
	Lys A:349	O	Conventional hydrogen bond	2.13
	Arg A:378	O	Conventional hydrogen bond	3.08
		F	Conventional hydrogen bond	2.70
	Be A:379	F	Conventional hydrogen bond	2.70
<i>H. sapiens alpha</i>	Asp A:209	H	Conventional hydrogen bond	2.41
	Cys A:399	H	Conventional hydrogen bond	2.59
	Ser A:373	H	Conventional hydrogen bond	2.32
	Arg A:372	H	Conventional hydrogen bond	2.50
	Thr A:376	F	Carbon-hydrogen bond	3.60
<i>H. sapiens Beta</i>	Tyr A:44	N	Conventional hydrogen bond	2.70
		C	Conventional hydrogen bond	3.71
	Gln A:346	H	Conventional hydrogen bond	2.57
	Lys A:103	N	Unfavorable positive-positive	4.90
	Glu A:352	O	Attractive charge	5.20
	Lys A:36	C	Rent	4.81
		C	Carbon hydrogen	2.17
	PRO A:325	C	Alkyl	4.89
	Glu A:101	O	Attractive charge	5.30
		H	Conventional hydrogen bond	2.12
		N	Attractive charge	3.65
		N	Attractive charge	3.75
	Asp A:383	O	Attractive charge	3.14

Organism	Amino acid	Atom from ligand	Type of interaction	Distance
<i>H. sapiens Gamma</i>	Ser A:40	C	Conventional hydrogen bond	2.00
	Wing A:39	F	Conventional hydrogen bond	2.50
	Ser A:373	F	Conventional hydrogen bond	2.92
	Arg A:372	F	Conventional hydrogen bond	2.91
	Asp A:209	N	Attractive charge	5.00
	Asp A:245	N	Attractive charge	5.20
	His A:371	N	Pi cation	4.10
	Glu A:167	N	Attractive charge	4.20
		C	Carbon-hydrogen	3.06
		H	Attractive charge	2.40
	Glu A:210	H	Attractive charge	2.58
		N	Attractive charge	3.56
		N	Attractive charge	4.54
		O	Attractive charge	4.77
		N	Attractive charge	3.27

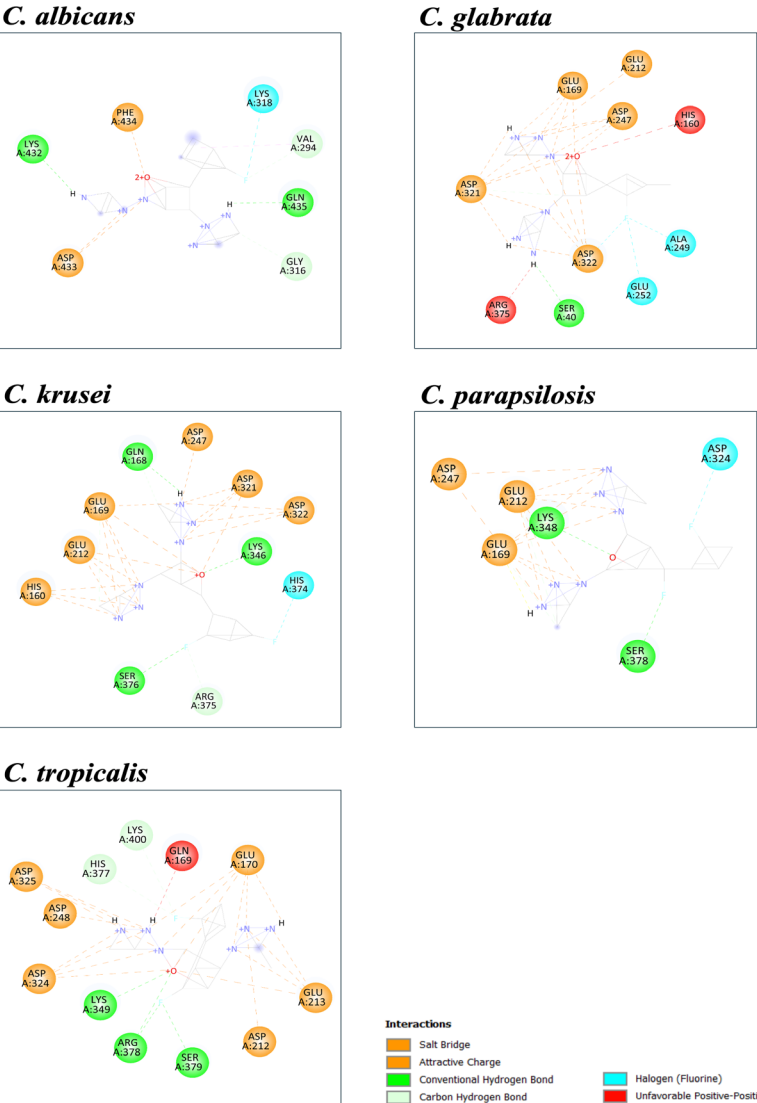


Figura 1. Interacciones moleculares entre las enolasas de diferentes especies y el fluconazol.
Figure 1. Molecular interactions between enolases of different species and fluconazole.

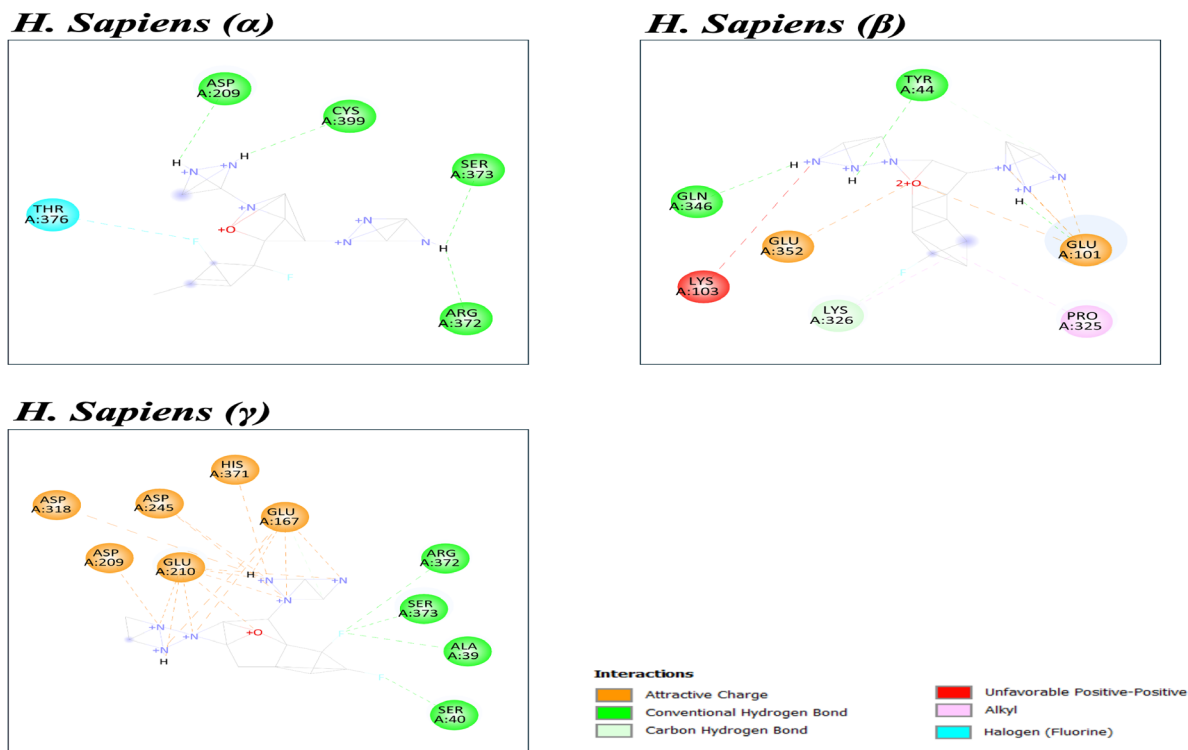


Figura 2. Interacciones moleculares entre las enolasas humanas y el fluconazol.
Figure 2. Molecular interactions between human enolases and fluconazole.

Tabla 4. Energía de Afinidad para los diferentes antifúngicos analizados.
Table 4. Affinity energy for the different antifungals analyzed.

Species	Affinity energy		
	Nystatin	Fluconazole	Caspofungine
<i>C. albicans</i>	-11.51	-6.41	-6.43
<i>C. glabrata</i>	-11.05	-6.92	-6.23
<i>C. tropicalis</i>	-11.58	-7.24	-6.62
<i>C. krusei</i>	-11.00	-7.12	-6.63
<i>C. parapsilosis</i>	-10.83	-7.76	-7.41
Alpha enolase <i>H. sapiens</i>	-10.88	-7.03	-6.51
Beta enolase <i>H. sapiens</i>	-11.11	-6.84	-7.76
Gamma enolase <i>H. sapiens</i>	-11.24	-6.98	-7.96

hand, the comparative analysis (Table 7) performed to determine whether the enolase-nystatin interaction directly or indirectly affects the active site showed no coincidence in any of the species evaluated, including human isoforms. Therefore, this result suggests that the possible interaction of nystatin does not occur at or near the active site and, although it showed the most significant affinity values (Table 4), this does not necessarily imply a relevant functional interaction, demonstrating that the analysis of the type of interactions in a molecular coupling is indispensable to determine the quality of the possible complex, and affinity energy should not be considered alone as a predictor of a potential protein-ligand interaction.

Interaction of enolases with caspofungin

The results obtained in the enolase-caspofungin interaction interestingly showed a large number of interactions between caspofungin and human isoforms of enolase (Table 8), with 14 interactions for the gamma isoform, 13 for the beta isoform, and 10 for the alpha isoform. However, of the total interactions, there are several unfavorable ones, which would coincide with the fact that this type of drug does not have significant side effects and has been one of the most widely used in recent years due to its safety profile (Rosanova *et al.*, 2016). On the other hand, it is noteworthy that there are no unfavorable interactions for *C. parapsilosis*, which has the highest number of interactions with this drug (Fig. 6). When comparing the

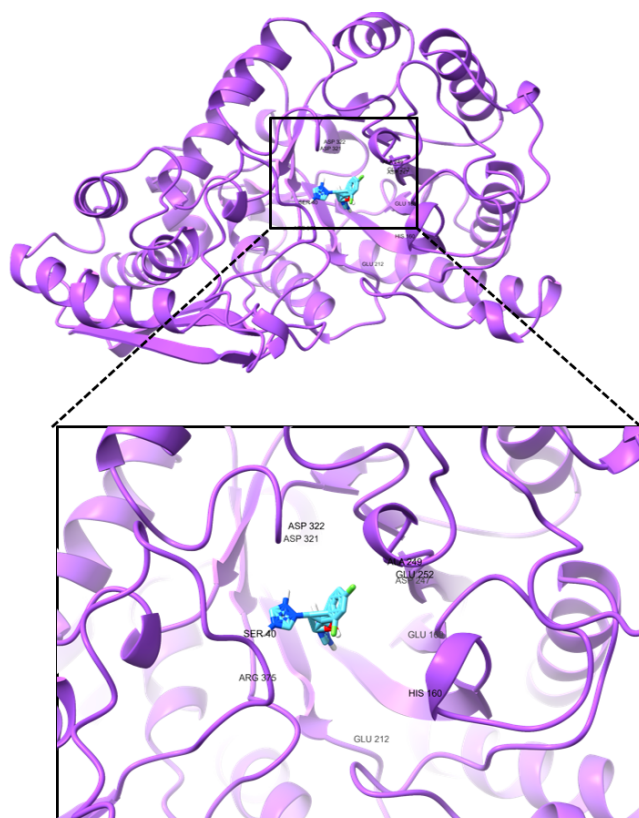


Figure 3. Estructura tridimensional de la enolasa de *C. glabrata* acoplada con fluconazol.

Figure 3. Three-dimensional structure of *C. glabrata* enolase bound to fluconazole.

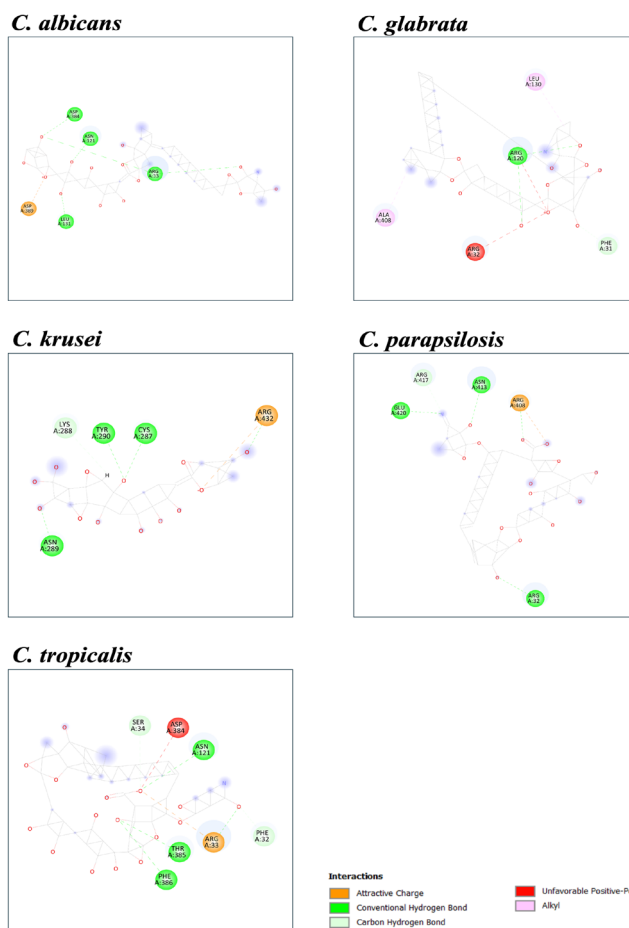


Figure 4. Interacciones moleculares entre las enolasas de diferentes especies y la nistatina.

Figure 4. Molecular interactions between enolases from different species and nystatin.

Tabla 5. Comparación de residuos de interacción con Fluconazol (docking) y sitios activos y de unión en UniProt.

Table 5. Comparison of interaction residues with fluconazole (docking) and active and binding sites in UniProt.

Species	UniProt		Docking *interaction
	Active Site	Binding site	
<i>C. albicans</i>	349 and 213	161,170,248,297,297,324,324,376-379 and 400	N/C
<i>C. glabrata</i>	212,346	160, 169, 247, 296, 321, 373-376 and 397	Asp A:321, Asp A:322, Glu A:169, Glu A:212, Asp A:247, His A:160, Ala A:249, Asp A:322, Arg A:375
<i>C. krusei</i>	212 and 346		His A:160, Glu A:212, Gln A:168, Asp A:247, Lys A:346, Asp A:321, Asp A:322, Arg A:375, His A:374, Ser A:376
<i>C. parapsilosis</i>	348 and 212		Asp A:247, Asp A:324, Ser A:378, Lys A:348, Glu A:212, Glu A:169
<i>C. tropicalis</i>	349		Asp A:325, Asp A:248, Glu A:170, Glu A:213, His A:377, Lys A:349, Arg A:378, Ser A:379, Lys A:400
<i>H. sapiens</i> Alpha enolase	210,343		Asp A:209, Ser A:373, Arg A:372
<i>H. sapiens</i> Beta enolase	210,342		Gln A:346
<i>H. sapiens</i> Gamma enolase	167,343		Ser A:40, Ala A:39, Ser A:373, Arg A:372, Asp A:209, Asp A:245, His A:371, Glu A:167, Glu A:210

N/C = Does not match.

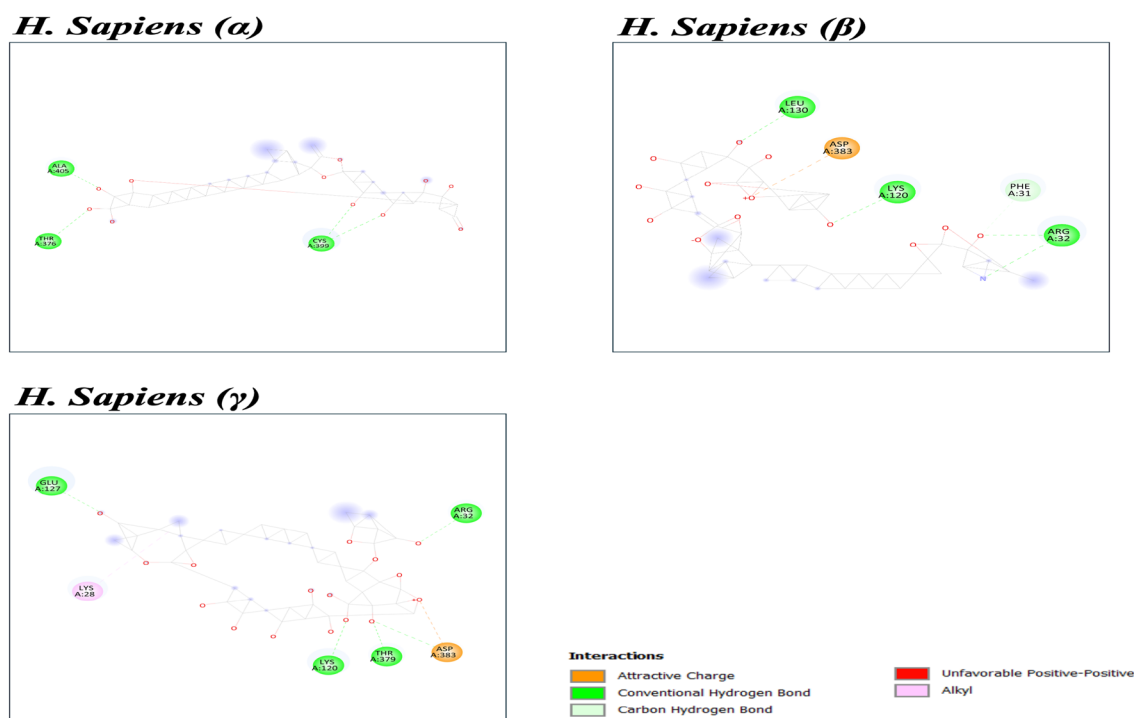


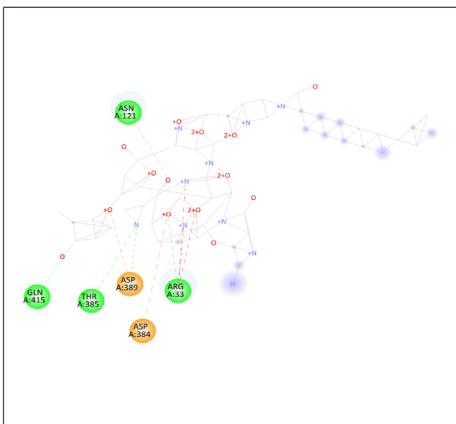
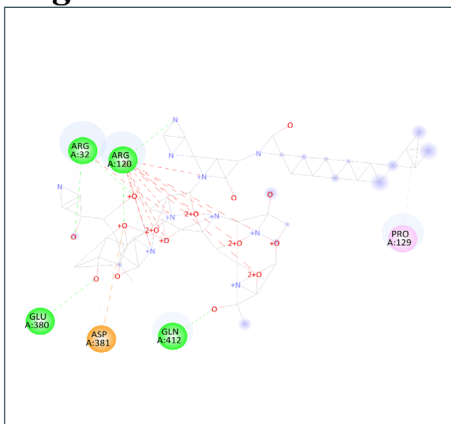
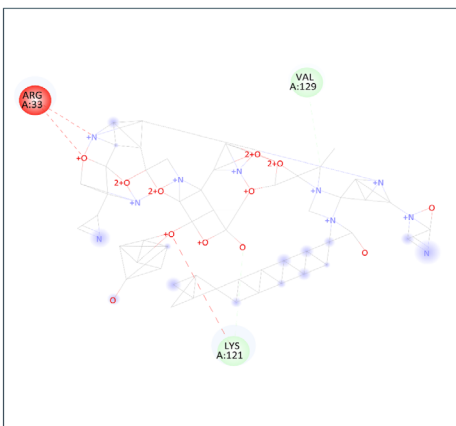
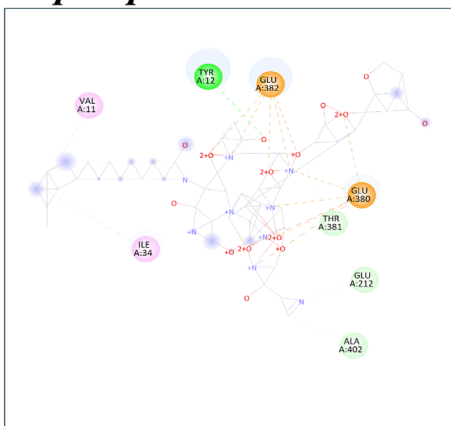
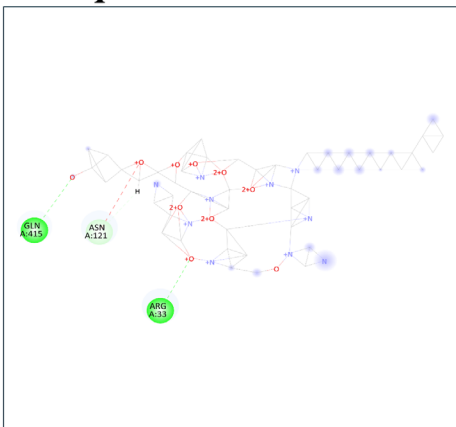
Figura 5. Interacciones moleculares entre las enolasas humanas la nistatina
Figure 5. Molecular interactions between human enolases and nystatin.

residues identified by *docking* with those described in the active and binding sites (Table 9), no amino acids involved in *C. albicans*, *C. glabrata*, or *C. krusei* were found, even when considering nearby residues (± 3 positions). In contrast, in *C. parapsilosis*, a single relevant match was identified, the residue Glu212 (Fig. 8), which is involved in the active site. Likewise, in the human alpha isoform, a close match was observed between residue 209 identified in molecular *docking* and residue 210 reported in UniProt. However, the significance of a single residue at the active site being affected must be evaluated experimentally to determine whether it is essential to the enzyme's activity or insignificant. With these results taken together, fluconazole is of particular relevance due to its high selectivity towards *Candida spp.* and its low incidence of unfavorable interactions in humans, since Nystatin has a generalized and nonspecific binding pattern and Caspofungin has high affinity but is accompanied by a higher risk of binding to human structures.

The bioinformatic findings obtained in this study suggest that fluconazole exhibits a comparatively higher and more specific affinity toward *Candida* enolases, particularly those of *C. krusei* and *C. parapsilosis*. From a biological standpoint, these results may indicate that, beyond its canonical target, fluconazole could also influence metabolic or virulence-associated processes involving enolase. Recent studies have shown that exposure to fluconazole can trigger adaptative metabolic responses and remodeling of the cell wall in different *Candida* species, processes closely linked to

antifungal tolerance and pathogenicity (Shahi *et al.*, 2022; Torres-Tirado *et al.*, 2025). Enolase, being a moonlighting protein located both in the cytosol and on the fungal cell wall, plays essential roles in adhesion, invasion, and immune evasion (Satala *et al.*, 2020). Therefore, a potential interaction between fluconazole and enolase might indirectly influence virulence-related pathways or cell wall dynamics, contributing to strain-dependent variations in susceptibility observed among *Candida* species. These observations reinforce the notion that conventional antifungals may exert additional effects on noncanonical targets that influence fungal physiology and pathogenesis.

In contrast, the nonspecific interaction pattern observed for nystatin supports its well-established mechanism of action through membrane disruption rather than specific enzyme inhibition. The apparent lack of interaction near enolase active sites in both fungal and human isoforms, suggests that any binding detected *in silico* is likely incidental and biologically irrelevant. Caspofungin, on the other hand, showed higher binding energies and a greater number of contacts with human enolases, yet many of these were unfavorable, consistent with its low cytotoxicity and selective inhibition of b-(1,3)-D-glucan synthesis in fungal cell walls (Perlin *et al.*, 2017). Altogether, these results highlight the importance of interacting computational analyses with biological context: strong docking affinities do not necessarily translate to functional inhibition but can reveal potential off-target interactions worth exploring experimentally. Future *in*

C. albicans*C. glabrata**C. krusei**C. parapsilosis**C. tropicalis***Interactions**

- Attractive Charge
- Conventional Hydrogen Bond
- Carbon Hydrogen Bond

- Unfavorable Positive-Positive
- Alkyl

Figura 6. Interacciones moleculares entre las enolasas y la caspofungina.
Figure 6. Molecular interactions between enolases and caspofungin.

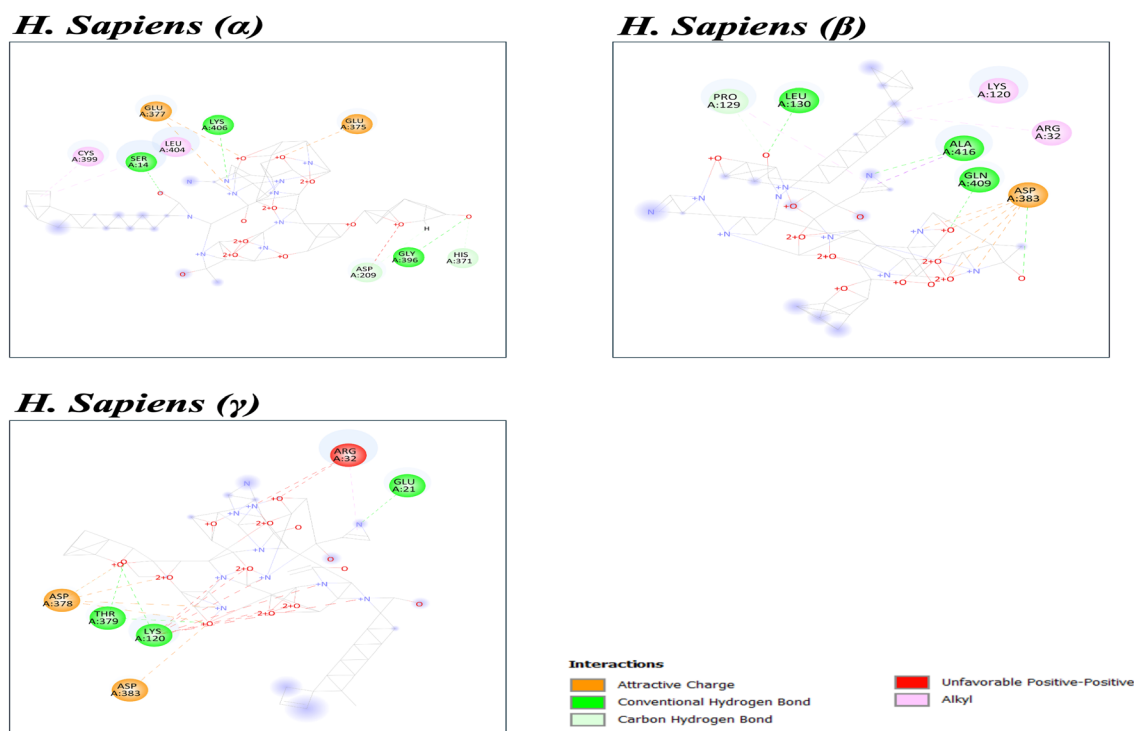


Figura 7. Interacciones moleculares entre las enolasas humanas y la caspofungina.

Figure 7. Molecular interactions between human enolases and caspofungin.

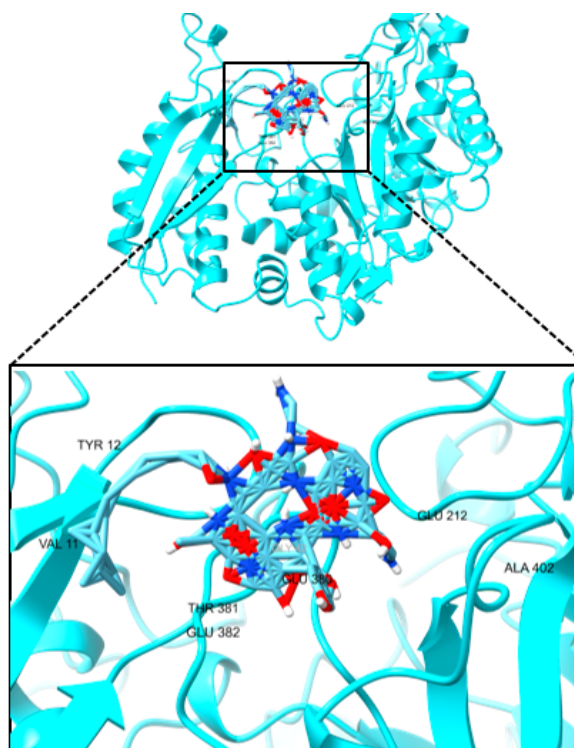


Figura 8. Estructura tridimensional de la enolasa de *C. parapsilosis* acoplado con Caspofungina.

Figure 8. Three-dimensional structure of *C. parapsilosis* enolase coupled with Caspofungin.

Table 6. Interacciones entre la nistatina y las enolasas.**Table 6.** Interactions between nystatin and enolases.

Organism	Amino acid	Atom from ligand	Type of interaction	Distance
<i>C. albicans</i>	Asp A:384	O	Conventional hydrogen bond	3.00
	ASN A:121	O	Conventional hydrogen bond	3.20
	Arg A:33	O	Conventional hydrogen bond	2.40
	Leu A:131	O	Conventional hydrogen bond	2.70
	Asp A:389	O+	Attractive charge	2.80
<i>C. glabrata</i>	Wing A:408		Rent	4.56
	Leu A:130		Rent	4.58
	Arg A:120	O	Conventional hydrogen bond	2.99
	Arg A:120	O	Conventional hydrogen bond	3.60
	Arg A:32	O	Unfavorable Positive-positive	
	Arg A:32	O	Conventional hydrogen bond	1.70
<i>C. krusei</i>	ASN A:289	O	Conventional hydrogen bond	3.20
	Lys A:288	H	Carbon hydrogen bond	2.60
	Tyr A:290	O	Conventional hydrogen bond	3.00
	Cys A:287	O	Conventional hydrogen bond	2.60
	Arg A :432	O	Conventional hydrogen bond	2.29
		O-	Attractive charge	5.48
<i>C. parapsilosis</i>	Glu A:420	N	Conventional hydrogen bond	2.80
	Arg A :417	N	Carbon hydrogen bond	2.92
	ASN A:413	O	Conventional hydrogen bond	2.95
	Arg A :408	O	Conventional hydrogen bond	2.50
		O	Attractive charge	4.69
	Arg A :32	O	Conventional hydrogen bond	3.00
<i>C. tropicalis</i>	Asp A:384	O	Unfavorable positive-positive	2.95
	Ser A:34	O	Carbon hydrogen bond	2.00
	Asn A:121	O	Conventional hydrogen bond	2.40
	Phe A:32	O	Carbon hydrogen bond	2.12
	Arg A:33	O	Attractive charge	5.40
		O	Conventional hydrogen bond	2.03
		O	Conventional hydrogen bond	2.14
	Phe:386	O	Conventional hydrogen bond	2.57
<i>H. sapiens</i> Alpha enolase	Thr A:376	O	Conventional hydrogen bond	3.30
	Ala A:405	O	Conventional hydrogen bond	2.00
	Cys A:399	O	Conventional hydrogen bond	3.60
		O	Conventional hydrogen bond	3.60
<i>H. sapiens</i> Beta enolase	Leu A:130	O	Conventional hydrogen bond	2.44
	Asp A:383	O	Attractive charge	2.90
	Lys A:120	O	Conventional hydrogen bond	2.43
	Phe A:31	O	Carbon hydrogen bond	2.68
	Arg A:32	O	Conventional hydrogen bond	2.62
		C	Conventional hydrogen bond	2.77
<i>H. sapiens</i> Gamma enolase	Glu A:127	O	Conventional hydrogen bond	3.00
	Lys A:23	C	Alkyl	5.30
	Arg A :32	O	Conventional hydrogen bond	2.70
	Lys A :120	O	Conventional hydrogen bond	2.43
	Thr A:379	O	Conventional hydrogen bond	1.80
		C	Conventional hydrogen bond	2.90
	Asp A:383	O	Attractive charge	3.10

Tabla 7. Comparación de residuos de interacción con Nistatina(docking) y sitios activos y de unión en (UniProt).**Table 7.** Comparison of interaction residues with Nystatin (docking) and active and binding sites in (UniProt).

Species	UniProt		Docking * interactions
	Active site	Binding site	
<i>C. albicans</i>	349 and 213	161,170,248,297,297,324,324,376-379 and 400	N/C
<i>C. glabrata</i>	212,346	160,169,247,296,321,373-376 and 397	N/C
<i>C. krusei</i>	212 and 346	160,169,247,296,212,321,373-376,397	N/C
<i>C. parapsilosis</i>	348 and 212	160,169,247,296,323,375-378,399,348	N/C
<i>C. tropicalis</i>	349,213	161,170,248,297,324,375,376-379,400	N/C
<i>H. sapiens</i> Alpha enolase	210,343	40,158,167,245,293,318,370-373	N/C
<i>H. sapiens</i> Beta enolase	210,342	158,167,245,293,318,370-373,394	N/C
<i>H. sapiens</i> Gamma enolase	167,343	40,158,210,245,293,318,370-373,394	N/C

N/C = Does not match.

Tabla 8. Interacciones entre la caspofungina y las enolasas.**Table 8.** Interactions between caspofungine and enolases.

Organism	Amino acid	Atom from ligand	Type of interaction	Distance
<i>C. albicans</i>	Asn A:121	O	Conventional hydrogen bond	2.61
	Gln A:415	O	Conventional hydrogen bond	2.10
	Thr A:385	N	Conventional hydrogen bond	3.20
	Asp A:389	N	Conventional hydrogen bond	3.00
		O	Attractive charge	4.48
	Asp A:384	O	Attractive charge	4.74
	Arg A:33	O	Unfavorable positive-positive	5.32
		N	Unfavorable positive-positive	5.58
<i>C. glabrata</i>	Gln A:412	O	Conventional hydrogen bond	1.81
	Asp A:381	O	Attractive charge	5.15
		O	Carbon-hydrogen bond	2.65
	Glu A:380	O	Conventional hydrogen bond	2.90
	Pro A:129	C	Alkyl	4.20
	Arg A: 120	N	Conventional hydrogen bond	2.90
		N	Unfavorable positive-positive	3.50
		O	Unfavorable positive-positive	3.15
	Arg A:32	O	Conventional hydrogen bond	2.20
		O	Conventional hydrogen bond	2.58
		C	Carbon-hydrogen bond	3.77
<i>C. krusei</i>	Arg A:33	O	Unfavorable positive-positive	4.40
		N	Unfavorable positive-positive	5.23
	Val A:129	C	Carbon-hydrogen bond	3.26
	Lys A:121	O	Unfavorable positive-positive	4.00

Organism	Amino acid	Atom from ligand	Type of interaction	Distance
<i>C. parapsilosis</i>	Val A:11	C	Alkyl	4.10
	ILE A:34	C	Rent	5.46
	Tyr A:12	O	Conventional hydrogen bond	2.87
	Glu A:382	O	Attractive charge	5.18
		N	Attractive charge	5.07
		O	Attractive charge	5.49
		N	Attractive charge	5.43
		O	Attractive charge	4.84
	Glu A:380	O	Attractive charge	3.00
		N	Attractive charge	5.28
		N	Attractive charge	5.15
		N	Attractive charge	3.95
		O	Attractive charge	5.11
	THR:381	O	Carbon-hydrogen bond	2.90
	Glu A:212	N	Carbon-hydrogen bond	2.79
<i>C. tropicalis</i>	Ala:402	C	Carbon-hydrogen bond	3.50
	Gln A:415	O	Conventional hydrogen bond	3.14
	Asn A:121	O	Unfavorable positive-positive	2.70
	Arg A:33	O	Conventional hydrogen bond	2.70
<i>H. sapiens Alpha enolase</i>	Asn A:121	H	Carbon-hydrogen bond	2.64
	Cys A:399	C	Alkyl	4.39
	Leu A:404	C	Rent	5.14
	Ser A:14	O	Conventional hydrogen bond	3.29
	Glu A:377	N	Attractive charge	4.60
		O	Attractive charge	4.21
	Lys A:406	N	Conventional hydrogen bond	2.73
	Glu A:375	O	Attractive charge	4.80
	Asp A: 209	O	Unfavorable positive-positive	2.80
		H	Carbon-hydrogen bond	2.60
	Gly A:396	O	Conventional hydrogen bond	2.30
	His A:371	O	Carbon-hydrogen bond	2.69

Organism	Amino acid	Atom from ligand	Type of interaction	Distance
<i>H. sapiens</i> Beta enolase	Pro A:129	O	Carbon hydrogen bond	3.02
		N	pi -alkyl	5.26
	Leu A:130	O	Conventional hydrogen bond	2.40
	Lys A:120	C	Alkyl	4.91
	Arg A:32	C	Rent	5.07
	Wing A:416	N	Conventional hydrogen bond	3.11
		C	pi-sigma	2.82
	Gln A:409	O	Conventional hydrogen bond	2.10
	Asp A:383	N	Attractive charge	4.60
		O	Attractive charge	3.97
		O	Attractive charge	4.89
		O	Attractive charge	4.81
		N	Attractive charge	4.96
		O	Conventional hydrogen bond	3.20
<i>H. sapiens</i> Gamma enolase	Asp A:383	O	Attractive charge	3.14
	Asp A:383	O	Attractive charge	4.90
	Glu A:21	N	Conventional hydrogen bond	2.95
	Arg A:32	N	Conventional hydrogen bond	4.50
		O	Unfavorable positive-positive	3.80
		O	Unfavorable positive-positive	4.82
	Lys A:120	O	Conventional hydrogen bond	2.70
	Thr A:379	O	Conventional hydrogen bond	2.32
		O	Conventional hydrogen bond	2.80
	Asp A:378	O	Attractive charge	3.70
		O	Attractive charge	5.27
		N	Attractive charge	5.12
		O	Attractive charge	5.23
	Lys A:120	N	Unfavorable positive-positive	4.12
		O	Unfavorable positive-positive	5.13
		N	Unfavorable positive-positive	5.54
		N	Unfavorable positive-positive	4.22
		O	Unfavorable positive-positive	3.30

Tabla 9. Comparación de residuos de interacción con Caspofungina (docking) y sitios activos y de unión en (UniProt).**Table 9.** Comparison of interaction residues with caspofungin (docking) and active and binding sites in (UniProt).

Species	UniProt		Docking *interactions
	Active site	Binding site	
<i>C. albicans</i>	349 and 213	161,170,248,297,297,324,324,376-379 and 400	N/C
<i>C. glabrata</i>	212,346	160,169,247,296,321,373-376 and 397	N/C
<i>C. krusei</i>	212 and 346	160,169,247,296,212,321,373-376,397	N/C
<i>C. parapsilosis</i>	348 and 212	160,169,247,296,323,375-378,399,348	Glu A:212
<i>C. tropicalis</i>	349,213	161,170,248,297,324,375,376-379,400	N/C
<i>H. sapiens</i> Alpha enolase	210,343	40,158,167,245,293,318,370-373	Asp A :209
<i>H. sapiens</i> Beta enolase	210,342	158,167,245,293,318,370-373,394	N/C
<i>H. sapiens</i> Gamma enolase	167,343	40,158,210,245,293,318,370-373,394	N/C

vitro assays, such as enzyme inhibition kinetics or proteomic binding studies, would be essential to validate whether fluconazole or other antifungals exert secondary effects on *Candida* enolase that may modulate virulence or resistance mechanisms.

CONCLUSIONS

Molecular docking analyses revealed distinct interaction patterns between clinically used antifungal agents and *Candida* enolases. Among the compounds tested, fluconazole displayed the highest number of favorable interactions, particularly with the enolases of *C. krusei* and *C. parapsilosis*, where several binding residues were located within or near the active site, suggesting potential functional relevance. Nystatin, in contrast, showed few and nonspecific contacts in both fungal and human isoforms, while caspofungin exhibited higher affinity but also a greater proportion of unfavorable interactions, especially with human enolases.

Although these antifungal agents were not originally designed to target enolase, the results suggest that fluconazole may interact with this moonlighting protein, potentially influencing metabolic or virulence-related processes in certain *Candida* species. These findings highlight the importance of exploring noncanonical targets to understand antifungal activity more comprehensively. Future experimental studies will be necessary to validate these computational predictions and to evaluate the feasibility of enolase as a complementary antifungal drug target.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

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