HIGH SUSCEPTIBILITY OF *CANDIDA ALBICANS* ATCC 10231 TO TETRAHYDROFURANOSYL-1,2,3-TRIAZOLES OBTAINED BY CLICK CHEMISTRY

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ABSTRACT

Tetrahydrofuranosyl-1,2,3-triazoles, synthesized by "Click chemistry", were tested as novel antifungal compounds. The results show a remarkable activity against Candida albicans ATCC 10231 expressed in a high MIC50 and MIC90 compared to traditional antifungals such as fluconazole.

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INTRODUCTION

Copper-Catalyzed Alkyne-Azide Cycloaddition (CuAAC) is the best known example of an ideal reaction for "Click Chemistry", which is a modular approach to attach distinct molecules in order to enhance diverse properties and to increase the applications for different purposes, in particular, in materials science and medicinal chemistry [1,2]. "Click chemistry" is a synthetic chemistry subarea based on the optimization of small materials and simple reactions [3-5], which has already proven to be a powerful tool in the preparation of ideal "building blocks" involving heteroatoms [6,7] and including simple reaction conditions in presence of oxygen, water, materials, reagents affording a simple product isolation. These conditions are fully provided by CuAAC that is reliable for different types of components and functional groups [5].

On the other hand, invasive fungal infections in immunocompromised patients have increased in recent years [8], with several variables contributing to this problem; fundamentally those affected with AIDS, patients undergoing cancer chemotherapy and treated with steroids [9]. Furthermore, the emerging in vitro intrinsic fungal resistance against classic antimycotics is an important factor in the HIV-infected patients with *Candida albicans* [10]. In addition, the widespread use of classic antimycotics such as fluconazole in the treatment and prevention of Yeast infections has put in evidence the detection of decreased susceptibility to this drug [11]. The number of available antifungal agents for systemic use is rather limited [12] and synergistic actions have been explored [13]. In this concern, a new kind of antifungals with 1,2,3-triazole moiety [2] may be an improvement for clinical options in parallel with less toxicity.

The triazole antifungal agents belong to the Sterol Biosynthesis Inhibitors (SBI's); a class of fungicides widely used to treat mycotic diseases in humans and animals. The molecular spatial conformation of the fungicide is a crucial factor which can increase or reduce the activity of the molecule. Conventional triazoles as fluconazole have decreased effectiveness indices in these infections.

The elements above-mentioned motivated us to initiate an extensive investigation in order to explore the possibility to use 1,2,3-triazoles as novel antifungals. The aim of this study was to evaluate the susceptibility of *Candida albicans* ATCC 10231 to tetrahydrofuranosyl-1, 2, 3-triazoles obtained via "Click chemistry".

RESULTS AND DISCUSSION

Comparing the results with the Yeast microdilution method, triazol C showed statistical significant difference with triazol A (p = 0.007), triazol B (p = 0.008) and triazol D (p = 0.007), while, with the microdilution method triazol C showed statistical significant differences with triazol A (p = 0.005), triazol B (p = 0.005) and triazol D (p = 0.005) (Table 1).

Table 1. *In vitro susceptibly Candida albicans ATCC 10231 to tetrahydrofuranosyl-1, 2, 3-triazoles obtained via "click chemistry" (Sensititre vs Yeast microdilution method)*

Candida species	Antifungal agent	MIC range (µg/ml) Sensititre	Yeast microdilution method	p
Candida albicans ATCC 10231	Fluconazole	0.125 - 256	0.125 - 256	≤ 0.01* †
	Triazole A	1-4	1-4	
	Triazole B	1-32	1-32	
	Triazole C	0.125 - 256	0.250 - 256	
	Triazole D	1-4	1-32	

*: with the Yeast microdilution method between triazol C and triazol A, triazol B and triazol D (p = 0.007). †: with the microdilution method between triazol C and triazol A, triazol B and triazol D.

The MIC determination by the microdilution method used in this study, allowed us to comparatively determine the effectiveness of triazoles versus fluconazole. In this respect, the studied Candida strain showed decreased susceptibility to fluconazole (range 0.125-256 µg/ml), in contrast, the triazoles, synthesized via "Click chemistry" showed a tendency for a better effectiveness in terms of MIC than fluconazole (used as an internal control), although we did not reach significant statistical difference due to the number of samples. Being more specific, triazoles A and D showed, in vitro, an important index of effectiveness to inhibit the growth of *Candida albicans*.

It is recognized that the percentages of *Candida albicans* resistance to fluconazole have increased in the past decade, reducing the chances of effective treatment in patients affected by this agent [14]. It is, therefore, imperative to develop new antifungal drugs with higher levels of effectiveness than the actual antimycotics, in order to cut the death rates in immunocompromised patients.

Overall, antifungal susceptibility testing results of clinically significant Candida species are of interest for empiric and prophylactic therapies. In fact, in vitro antifungal susceptibility testing is now standardized internationally [15] and is becoming essential in patient management and resistance surveillance [16].

Several studies have compared the antifungal susceptibility profiles of fluconazol and other antimycotics [17,18] but until now the experience with triazoles via "Click Chemistry" is scarce and in vitro [19,20]. With the CuAAC technique we are reaching the required functionality to potentiate the biological activities of new compounds. Copper complexes derived from the propenditioic acid are stable to the environmental conditions of temperature and humidity showing an excellent catalytic activity in the azide-alkyne cycloadditions as the binder of the catalyst stabilizes the oxidation state of copper, making unnecessary, in principle, adding a reducing agent or an organic base to stabilize the transition metal [21].

Much attention has been focused on the antimicrobial [22] and antiparasitic [23] actions of triazoles. In our study we used the synthesis of novel 1,2,3-triazoles containing rings of five members with or without oxygen as substituents, in order to prepare in a simple way compounds which mimic nucleotides with possible antimycotic activity.

A deeper study of the compounds tested here is required to verify their clinical effect in order to corroborate their pharmacological perspective use since "Click Chemistry" synthesis offers great expectations for the development of new effective antifungal agents.

EXPERIMENTAL

Molecules synthesis

General Remarks

The four tested triazoles, were provided by Dr. Erick Cuevas-Yañez, from CIQS, UAEM-UNAM (Table 2 and Figures 1 and 2), and they were synthesized using CuAAC according to previous reports [2,24].

Table 2. *1, 2, 3-triazoles synthesis (modified from Velasco, B.; et al [2])*

Triazole	Found		
	C	**H**	**N**
4-{1-[5-Chloro-2-(2,4-dichloro-phenoxy)-phenyl]-1,2,3-triazol-4-ylmethoxy}-benzoic acid methyl ester (28).	54.62%	3.29%	8.37%
White solid, m.p. 157 °C. IR (ATR, cm^{-1}): 1700, 1600, 1350, 1260. ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.78 (s, 3H), 5.30 (s, 2H), 7.02 (d, 1H, J=9 Hz), 7.13 (m, 2H), 7.22 (d, 1H, J=9 Hz), 7.41 (d, 1H, J=2.5Hz), 7.56 (d, 1H, J=2.5 Hz), 7.70 (d, 1H, J=2.5 Hz), 7.86 (m, 2H), 7.94 (d, 1H, J= 2.5 Hz), 8.68 (s, 1H). ^{13} C NMR (125 MHz, $CDCl_3$): δ 52.2, 61.3, 115.2, 120.1, 122.673, 123.0, 126.1, 126.7, 127.0, 128.3, 128.5, 129.4, 130.2, 130.7, 131.3, 131.6, 142.9, 147.8, 149.7, 162.1, 166.2. MS [EI+] m/z(%): 418 (23), 324 (64), 288 (100), 290 (80), 254 (70), 161 (54). HRMS (EI+): for $C_{23}H_{16}Cl_3N_3O_4$ calcd. 503.0206, found 503.0209. Anal. Calcd for $C_{23}H_{16}Cl_3N_3O_4$: C, 54.73%; H, 3.20%; N, 8.32%.			
4-[5-Chloro-2-(2,4-dichlorophenoxy)-phenoxymethyl]-1-(4-chloro-phenyl)-1H-[1,2,3]triazole (21).	54.87%	2.89%	9.12%
White solid, m.p. 160.0 °C. IR (ATR, cm-1): 3100, 1550, 1500, 1250. 1H NMR (500 MHz, CDCl3): δ 5.27 (s, 2H), 6.67 (d, 1H, J=9Hz), 6.95 – 6.99 (m, 1H), 7.09 - 7.11 (d, 1H, J=3Hz), 7.18 – 7.19 (d, 1H, J=3Hz), 7.26 (s, 1H), 7.41 – 7.442(d, 1H, J=3Hz), 7.51 – 7.52 (d, 1H, J=3Hz), 7.63 - 765 (m, 1H), 7.67 (s, 1H). 13C NMR (125 MHz, CDCl3): δ 63.6, 115.966, 118.2, 120.4, 121.5, 122.1, 122.3, 124.4, 127.8, 128.1, 130.0, 130.1, 130.7, 134.7, 135.3, 143.3, 144.5, 149.8, 152.3. MS [EI+] m/z(%): 479 [M]+ (5), 318 (8), 290 (17), 164 (100), 128 (27), 111 (41). HRMS (EI+): for $C_{21}H_{13}Cl_4N_3O_2$ calcd. 478.9762, found 478.9764. Anal. Calcd for $C_{21}H_{13}Cl_4N_3O$: C, 54.22%; H, 2.82%; N, 9.03%.			
4-[5-Chloro-2-(2,4-dichlorophenoxy)-phenoxymethyl]-1-(3,4-dichloro-phenyl)-1,2,3-triazole	48.86%	2.39%	8.19%.
White solid, m.p. 163 °C. IR (ATR, cm^{-1}): 3100, 1550, 1500, 1250, 1000. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.17 (s, 2H), 5.56 (s, 2H), 6.69 (d, 1H, J=9Hz), 7.023 (s, 2H), 7.20 – 7.25 (m, 3H), 7.33 - 7.35 (m, 3H), 7.47 – 7.47 (m, 1H,), 7.54 – 7.55 (m, 1H), 7.95 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 53.3, 62.7, 116.1, 119.1, 121.9, 122.4, 123.8, 124.9, 127.5, 128.3, 128.6, 128.7, 129.2, 129.9, 130.1, 136.2, 142.8, 143.1, 150.3, 152.1. MS [EI+] m/z(%): 513 [M]+ (15), 145 (100). HRMS (EI+): for $C_{21}H_{12}Cl_5N_3O_2$ calcd. 512.9372, found 512.9375.			

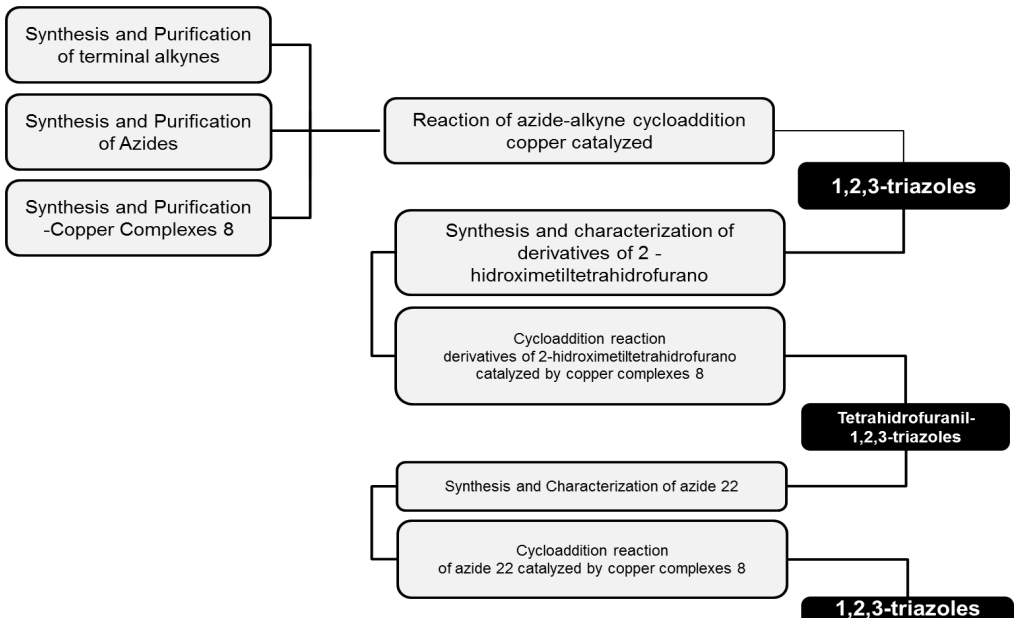

Figure 1: *Model of tetrahydrofuranosyl-1, 2, 3-triazoles synthesis (modified from Velasco, B.; et al [2])*

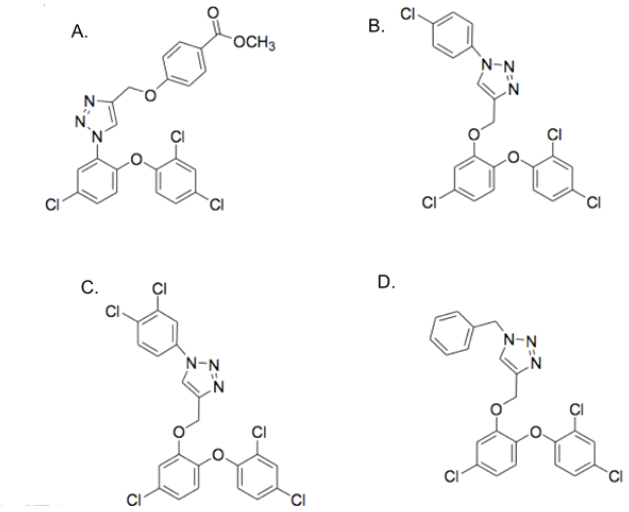

Figure 2: *Tetrahydrofuranosyl-1, 2, 3-triazoles synthesized via "click chemistry" by reactivity of azides and alkynes front various copper salts and complexes (I).* ***A.*** *4-{1-[5-Chloro-2-(2,4-dichloro-phenoxy)-phenyl]-1,2,3-triazol-4-ylmethoxy}-benzoic acid methyl Ester.* ***B*** *4-[5-Chloro-2-(2,4-dichlorophenoxy)-phenoxymethyl]-1-(4-chloro-phenyl)-1H-[1,2,3]triazole (21).* ***C.*** *4-[5-Chloro-2-(2, 4-dichlorophenoxy)-phenoxymethyl]-1-(3,4-dichloro-phenyl)-1,2,3-triazole.* ***D****.4-[3-Chloro-2-(2,4-dichlorophenoxy)]-1,2,3-triazole (modified from Velasco, B.; et al [2])*

The starting materials were used without further purification and the solvents were distilled before use. Silica plates of 0.20 mm thickness were used for thin layer chromatography. Melting points were determined with a Fisher-Johns melting point apparatus and they are uncorrected. 1H and 13C NMR spectra were recorded using a Varian 500. The chemical shifts (δ) are given in ppm relative to TMS as internal standard (0.00). For analytical purposes the mass spectra were recorded on a Shimadzu GCMS-QP2010 Plus in the EI mode, 70 eV, 200 °C via direct inlet probe. Only the molecular and parent ions (m/z) are reported. IR spectra were recorded on a Bruker Tensor 27 equipment.

Synthesis of 1,2,3-triazoles

The appropriate alkyne (1.05 mol) was added in one portion to a solution of the corresponding azide (1 mol) and the catalyst (3-Hydroxy-3-phenyl-2-propenedithioate-S,S')bis(triphenylphosphine-P)copper(I) (0.005 mmol) in acetonitrile (30 mL) [2,24]. The resulting mixture was stirred at 60° C for 2 h or at room temperature for 10 h. The mixture was cooled to room temperature and the solvent was removed *in vacuo*. The reaction product was extracted with toluene (40 ml) and treated with activated charcoal (0.5 g). The mixture was filtered and evaporated under vacuum, and the product was crystallized from hot ethyl acetate and n-heptane.

Yeast microdilution method

Tetrahydrofuranosyl-1, 2, 3-triazoles, synthesized by "Click chemistry", were probed in RPMI agar (Sigma Aldrich, St Louis MO, USA) with *Candida albicans* ATCC 10231, following the next steps:

a. Solution preparation: it was done weighing sufficient powder to obtain a concentration at least 100 times the highest concentration of the antifungal drug tested [25].

b. Fluconazole (Pfizer, Mexico) (concentration of 2 mg/ml). The diluent used was sterile distilled water. For preparation of fluconazole dilutions we followed the method of additive double serial dilutions. The prepared dilutions differed according to whether being soluble or not in water [26]. Dilution of fluconazole (soluble in water) was prepared at a dilution series concentration 10 times higher than the desired final concentration, using RPMI 1640 (Sigma Aldrich, St Louis MO, USA) as diluent. Subsequently a 1/5 dilution was added to all tubes (4 ml of RPMI),

so that the antifungal concentration was twice the desired final concentration (128 µg/ml to 0.25 µg/ml). The final concentration obtained in the plate after inoculation was between 64 and 0.12 µg/ml.

c. Triazoles dilution (insoluble in water): From the stock solution, dilution series concentration 100 times the desired final concentration was prepared, using DMSO (Sigma Aldrich, St Louis MO, USA) as diluent. Each tube was transferred to another tube with RPMI (4.9 ml), whereupon the antifungal obtained concentration was twice the desired final concentration (32 µg/ml - 0.06 µg/ml) and 2% DMSO.

d. Filling plates: 100 µl per well in column two were taken from tube two and so on to column No. 11; wells in column No. 12 were filled, in the case of the fluconazole dilution, with 100 µl of RPMI (growth control) and in the case of triazoles dilution with 100 µl of the dilution resulting from 4.9 ml of RPMI and 100 µl of 2% DMSO. Wells in column No. 1 were filled with 200 µl of RPMI (sterile control). Once filled, plates were closed and frozen at -70°C until use.

e. Inoculum preparation: before preparing the inoculum we sowed with Saboraud Dextrose Agar (SDA) (Sigma Aldrich, St Louis MO, USA) for 24 hours at 30°C. In the case of Candida spp strains used in our study, the inoculum was prepared taking a sterile loop of culture (about five colonies ≥ 1 mm) after 24 hours of growth on SDA.

f. A plate was suspended in a tube with sterile distilled water. Stirring well it was adjusted to an optical density of 0.5 McFarland. The addition of the required amount of sterile distilled water was done with the aid of a spectrophotometer (wavelength at 530 nm).

g. Inoculation: plates were taken from the freezer and left at room temperature until completely thawed.

h. Incubation: plates were incubated at 35°C for 48 hours [27].

i. Reading and interpreting the results: Visual readout was performed using an inverted mirror. For azoles, such as fluconazole and triazoles, the minimum inhibitory concentration (MIC) was defined as the lowest antifungal concentration that produced a ≥ 50% reduction of yeast growth as compared with the drug free control growth [28].

Sensititre yeast one method

This system is based on a technique of RPMI 1640 glucose broth microdilution. It contains an indicator of redox growth, which detects pH variations (Alamar blue), allowing the determination of in vitro sensitivity, and by colorimetric changes also quantifies different triazoles, including fluconazole. The steps were the next:

a. With the help of a sterile loop, we took in SDA five colonies less than 1 mm, and evaluated the growth of *Candida albicans* ATCC 10231 after 24 hours.

b. Emulsify the colonies in a tube of distilled water, after stirring we adjusted a spectrophotometer (wavelength 530 nm) to an optical density of 0.5 on the McFarland scale (1-5 × 106 Colony Forming Units (CFUs)/ml).

c. Inoculate 20 ml of this suspension in the 10 ml tube of culture medium (working dilution: 1.5 to 8 × 103 CFU/ml).

d. The panel was rehydrated by dispensing 100 µl of the inoculum suspension in each well.

e. The plates, previously sealed with self-adhesive paper, were aerobically incubated at 35°C for 24-48 hours for *Candida albicans* ATCC 10231. After this period of time, we counted the number of CFUs, registering between 15 and 18 colonies.

For validation of results, a control shift (pink) growth was first checked. The interpretation of the results and the MIC calculation were determined after 24 hours of incubation. The MIC was the lowest antifungal concentration showing no color change (the first blue well). However, in the azoles it may appear an intermediate purple color (due to trailing effects) (Figure 3); in this case MIC was the concentration of the first purple well. For those genera and species with more than five tested strains, MIC_{50} and MIC_{90} were calculated.

Statistical analysis was performed using SPSS version 19. We estimated the differences between the study groups by Mann Whitney U test. A $p \leq 0.05$ was considered significant.

CONCLUSIONS

The antimycotic activity determination of 1,2,3-triazoles compounds synthesized from CuAAC represents significant importance in the continuing development of new pharmacological alternatives to treat diseases of multiple etiologies. The antifungal resistance indices have presented an exponential behavior especially in yeast causing opportunistic infections in patients with immunosuppressant status. The Yeast microdilution is an effective method to semiquantitatively measure the effectiveness indexes of triazoles calculation based on the MIC_{50} and

MIC_{90} for *Candida albicans* ATCC 10231. This strain showed high sensitivity to antifungal agents A, C and D and poor sensitivity to antifungal agent B. Therefore, the effective compounds must be used in other studies for determining susceptibility and clinical viability in inhibiting fungal growth in hospital strains isolates where such microorganisms are causing severe infections.

The proposed advantages by the synthesis of pharmacologically active compounds through "Click chemistry" is cost-effective in the development of new drug therapies that might contribute to reduce the actual resistance rising rates. However, there is still a large route to follow up until finally apply the triazoles in a broad clinical setting.

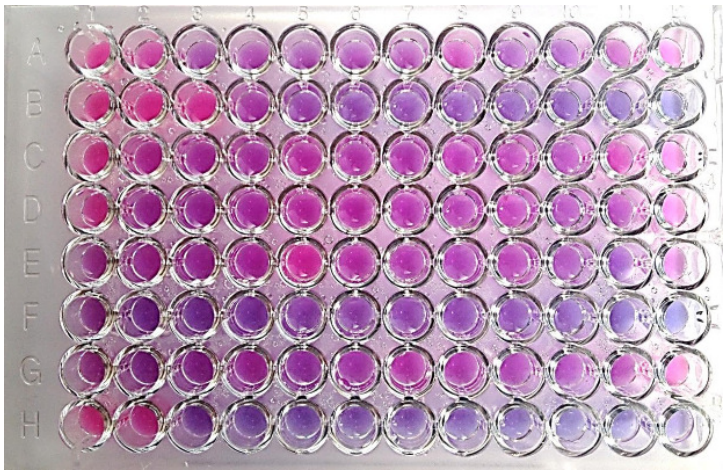

Figure 3: *Sensititre Yeast One Panel with trailing effect in azole antifungals*

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