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Synthesis and Biological Evaluation of Itraconazole Derivatives: Design in an Old Scaffold

Shuai Zhong,^a Apollonia Kalamatianou,^a Gonzalo Scalese,^{b,c} Cillian O'Beirne,^a Marta Redrado,^a Olivier Blacque,^d Carine Picot,^e Christophe Tomasoni,^e Cédric Logé,^e Marjorie Albassier,^e Isabelle Ourliac-Garnier,^e Fabrice Pagniez,^e Patrice Le Pape,^e Leticia Pérez-Díaz,^f Dinorah Gambino,^b Marcelo A. Comini,^c Kevin Cariou,^{a,} and Gilles Gasser,^{a,*}*

^a Chimie ParisTech, PSL University, CNRS, Institute of Chemistry for Life and Health Sciences, Laboratory for Inorganic Chemical Biology, 75005 Paris, France.

^b Área Química Inorgánica, Facultad de Química, Universidad de la República, 11800 Montevideo, Uruguay.

^c Laboratory Redox Biology of Trypanosomes, Institut Pasteur de Montevideo, 11400 Montevideo, Uruguay.

^d Department of Chemistry, University of Zurich, 8001 Zurich, Switzerland.

^e Nantes Université, CHU de Nantes, Cibles et Médicaments des Infections et de l'Immunité, IICiMed, UR 1155, F-44000 Nantes, France.

^f Sección Genómica Funcional, Facultad de Ciencias, Universidad de la República, 11400 Montevideo, Uruguay.

* Corresponding Authors:

Email: kevin.cariou@chimieparistech.psl.eu, gilles.gasser@chimieparistech.psl.eu; WWW: www.gassergroup.com

Abstract

Antimicrobial resistance is a major global problem for public health, indicating the need for the development of new anti-infective drugs, among other actions (i.e., better stewardship, diagnostics, etc.). A common strategy in medicinal chemistry is to modify existing drugs with an organometallic moiety to enhance their efficacy or overcome resistance. One notable example is ferroquine, an organometallic derivative of chloroquine. Here, we describe the design, in-depth characterization and *in vitro* evaluation of seven new derivatives of the antifungal drug itraconazole (ITZ) against parasitic and fungal pathogens. ITZ was selected as a privileged scaffold because it targets ergosterol biosynthesis, which is an essential component of cell membranes in fungi and trypanosomatid parasites. While none of the compounds were active against *Trypanosoma cruzi* and *Leishmania infantum*, the ferrocenyl derivatives proved to be 1.5- to 1.9-fold more potent than ITZ towards *Trypanosoma brucei*. Of particular interest, all the compounds exhibited high antifungal activity against the azole-susceptible clinical isolates. Furthermore, the ferrocenyl-containing compound was the most active against *Aspergillus*. Despite showing 10-fold lower activity than ITZ, these organometallic derivatives constitute an interesting starting point for further pharmacomodulation since we confirmed that they blocked the ERG11 enzyme, the main target of azoles.

Keywords: Antifungal agents; Antitrypanosomatid agents; Antimicrobial resistance; Ferrocene; Itraconazole; Medicinal Organometallic Chemistry.

Introduction

Antimicrobial resistance (AMR) occurs when microorganisms such as bacteria, fungi, viruses or parasites survive and continue multiplying despite being exposed to anti-infective agents. Today, the World Health Organization (WHO) considers AMR as a leading threat to global public health, associated with major implications for human health and millions of deaths worldwide.¹ AMR can be intrinsic when a specific pathogenic strain is naturally resistant to a certain type of medicine, or acquired when the resistance occurs over time due to gene mutations. Although the latter is a natural consequence, in the case of antifungal azoles for example, the clinical misuse and agricultural overuse of azole-based fungicides during the past decades have accelerated its emergence.²⁻⁵ Therefore, there is a pressing need to develop novel and effective antifungal agents that will target emerging pathogens while bypassing resistance mechanisms.

In most eukaryotic cells, sterols are essential components of the plasma membrane, though their specific structures vary across different kingdoms. In animal cells, cholesterol is the main sterol, whereas in fungi and trypanosomatid parasites (genus *Trypanosoma* and *Leishmania*) predominates ergosterol, which plays a crucial role in maintaining membrane integrity and supporting various cellular functions.⁶ About twenty different enzymes are involved in ergosterol biosynthesis.⁷ Structurally, ergosterol differs from cholesterol by having an additional double bond in ring B, imparting some aromatic character, another double bond in the short aliphatic chain, and a branched methyl group at C24. Despite these relatively minor structural differences, cholesterol and ergosterol have distinct effects on lipid membrane properties.⁸

The difference in membrane composition between mammals, trypanosomatids, and fungi, along with the essential function of ergosterol in trypanosomatids and fungi plasma membranes,⁹ makes this pathway a promising target for the development of new drugs to combat these infections. Several drugs targeting sterol biosynthesis have been used to treat fungal infections, and some have also been tested for their antiparasitic potential against different trypanosomatids species.¹⁰

Itraconazole (ITZ) is an azole-bearing antifungal drug administered both orally and intravenously to treat fungal infections. This second-generation of antifungal drugs inhibits the development of several species of pathogenic fungi, including *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., etc.¹¹⁻¹⁴ The term Cytochrome P450

(CYP450) encompasses a class of enzymes involved in a variety of metabolic processes, especially in detoxification of drugs and exogenous substances. Human and fungal CYP450 differ in structure, function and regulation.^{15,16} Like other azoles, ITZ exerts its activity by disrupting ergosterol synthesis, leading to defective fungal and trypanosomatid cell membranes, increased membrane permeability and ultimately cell death.¹⁷ ITZ specifically inhibits the enzyme lanosterol 14 α -demethylase (also known as CYP51A, a member of the CYP450 family), which catalyzes the demethylation of lanosterol, a precursor of ergosterol.^{6,18} ITZ's scaffold consists of: 1) the triazole with *m*-dichlorophenyl group-containing dioxolane, 2) a triazolone-side chain and 3) a central phenyl-piperazine-phenyl linker (**Figure 1A**). ITZ's triazole moiety is essential for its antifungal activity, also charged with adverse side effects,¹⁹ for instance, the inhibition of human CYP3A4 (a subtype of CYP450).

ITZ became widely used after its introduction, particularly in treating infections caused by *A. fumigatus*. The first clinical case of ITZ-resistance in *Aspergillus* was reported approximately 10 years after the commercial availability of ITZ, emphasizing the severe problem of antifungal resistance.²⁰ Although *Aspergillosis* can be treated with other antifungal agents, such as voriconazole and posaconazole, the latter display a plethora of adverse effects.²¹

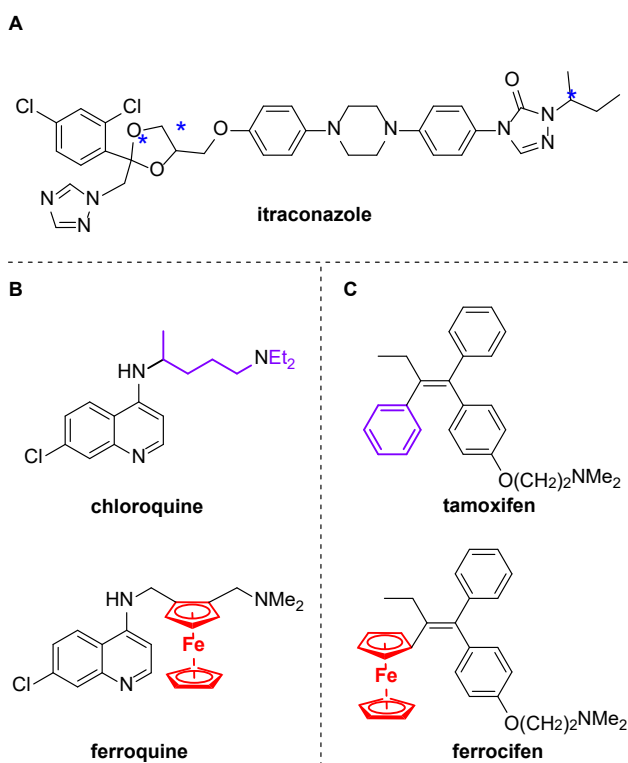


Figure 1. A) Chemical structure of itraconazole (ITZ), **B)** comparison between the structures of antimalarial drugs chloroquine/ferroquine, and **C)** comparison between the structures of anticancer drugs tamoxifen/ferrocifen.

Coordination of azoles to metal centers has been explored as a strategy to enhance antifungal and antiparasitic activity. Several studies demonstrated that metal-based derivatives of ketoconazole, clotrimazole, and ITZ exhibit improved biological potency compared to their parent compounds, often with increased selectivity. Despite promising results, further research is needed to fully understand the structure-activity relationship and to elucidate their multiple mechanisms of action.²²

Scaffold modification of FDA-approved drugs and re-evaluation of their biological activity has emerged as a viable strategy to accelerate drug discovery. This is because the drug scaffold possesses an optimized pharmacokinetic and safety profile. An example of such successful modifications is the derivatization of drugs with metallocenyl moieties to create organometallic molecular hybrids with a high therapeutic potential.²³ This concept was pioneered in the late 1990s, and since then has been exploited by many research groups, including ours, to design various types of therapeutic agents.^{22,24} Notably, the incorporation of the ferrocenyl moiety to a drug has gained interest as an approach to transfer the new molecule some of its prominent physicochemical properties and biological effects (such as lipophilicity, redox activity and lack of toxicity).^{25,26} Ferroquine and ferrocifen, which are derivatives of the antimalarial drug chloroquine and the anticancer drug tamoxifen, respectively, are the two most famous and successful examples of ferrocene-based medicinal agents (**Figure 1B-C**).^{27,28} The improved pharmacological properties of these drug candidates compared to their parent molecules are directly linked to the presence of the ferrocenyl moiety and have been mechanistically ascribed, among others, to its capacity for generating reactive oxygen species *via* the reversible ferrocene/ferrocenium redox couple.²⁹ Another interesting metallocene is ruthenocene, which is the ruthenium analogue of ferrocene,³⁰ and has been introduced in some anti-infective drugs with some success.^{31,32}

Herein, we report the design, synthesis and evaluation of the biological activity of seven novel ITZ analogues on fungal clinical isolates (*Candida* spp. and *A. fumigatus*) and protozoan parasites (e.g., *T. cruzi*, *T. brucei* and *L. infantum*) that are pathogenic

to mammals. Furthermore, their cytotoxicity was evaluated on mammalian cell lines (VERO cells, murine macrophages and human fibroblasts).

Results and Discussion

Synthesis and characterization

Pursuing the efforts in designing new organometallic anti-microbial agents led by several groups,^{33,34} including ourselves,^{35–37} we now report the synthesis and full characterization of seven ITZ derivatives. The design of our compounds relied on the formation of an amide-linker bridging the organometallic or organic groups and the ITZ core. To streamline the synthesis, the triazolone nucleus (highlighted in blue) at the opposite side of the key heme-interacting triazole moiety (highlighted in pink) was discarded and the aniline moiety was chosen as the linchpin to attach the metallocene scaffold through an amide bond (**Figure 2**). In order to avoid manipulating mixtures of stereoisomers, these ITZ analogues were prepared starting from the (2*R*, 4*S*) dioxolane core.

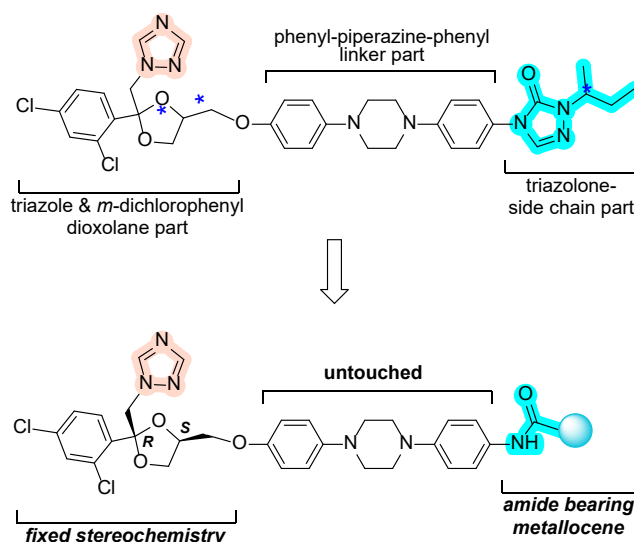
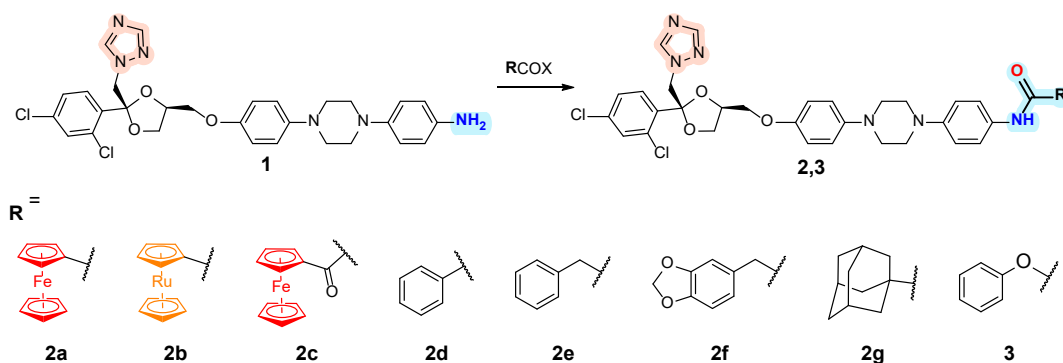


Figure 2. Design of the itraconazole analogues.

ITZ aniline precursor **1** could readily be obtained in three steps from commercially available starting materials following known procedures (see Supplementary Information). From **1**, acylation under various conditions provided a straightforward access to the analogues (**Scheme 1**). The ferrocenyl (Fc) **2a** and ruthenocenyl (Rc) analogues **2b** were obtained by an amidation reaction between the metallocene acyl chlorides and the aniline. The acyl chloride species were generated in situ from the corresponding carboxylic acid **S2a**, **S2b** using an excess of oxalyl chloride in degassed dichloromethane (anhydrous). After reacting with the ITZ amine, the target amide was

obtained with 45% and 30% yields, respectively. Interestingly, during the reaction between ferrocenylacetic and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylammonium tetrafluoroborate (TBTU), the homologated Fc analogue **2c** was accidentally produced and could be isolated, albeit with a low yield (21%). For comparison purposes, we have prepared some fully organic derivatives to help us obtain more insight into the effect of the metallocene moieties. Specifically, we synthesized three aryl derivatives: phenyl **2d**, benzyl **2e** and piperonyl **2f** as well as an adamantyl analogue **2g**. The former three serve as aromatic analogues of the metallocene that are flat, while the adamantane is a non-aromatic scaffold with a 3D spatial structure, just like the metallocene. It is worth noting that adamantane derivatives have been found to possess excellent activities when appended to anti-infective scaffolds.^{38,39} **2d** was obtained under the same procedures described for **2a** and **2b**. The third method we employed for the formation of the amide bonds involved the use of a condensation reagent with enhanced reactivity, O-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU). After generating the activated esters derived from the corresponding carboxylic acids, an addition-elimination reaction with various amines allowed for the obtention of **2e-2g** with yields ranging from 42% to 63%. Additionally, using phenyl chloroformate as an acylation agent gave carbamate **3** (an intermediate in the actual synthesis of ITZ), which was also included in the analogue panel.

All newly synthesized compounds were unambiguously characterized by ¹H and ¹³C NMR spectroscopy, HRMS/MS and IR, while their purities were confirmed by microanalysis. Details on synthesis and characterization of the products can be found in Supporting Information.



Scheme 1. Synthesis of itraconazole derivatives **2a-2g**. Reagents and conditions: for **2a-2b**, **2d**: Oxalyl chloride, RCOOH , dichloromethane (DCM), 0 °C to r.t., 14-16 h; for **2c**: RCOOH , TBTU, DIPEA, *N,N*-dimethylformamide (DMF), ice bath to 45 °C, 14-16 h; for **2e-2g**: RCOOH , HATU, 4-methylmorpholine, DMF, r.t., 14-16 h. Synthesis of itraconazole derivative **3**: Pyridine, ClCOOPh , acetonitrile, ice bath then 40 °C, 16 h.

X-ray crystallography

The single crystal structure determination was carried out for one ITZ derivative synthesized in this study (**2a**, **Figure 3**). Suitable crystals were obtained by slow diffusion of diethyl ether into a dichloroethane solution of **2a**. The molecule crystallized in a monoclinic system, with a $C2/c$ space group and the metallocene displaying the standard sandwich geometry with nearly parallel cyclopentadienyl rings.⁴⁰

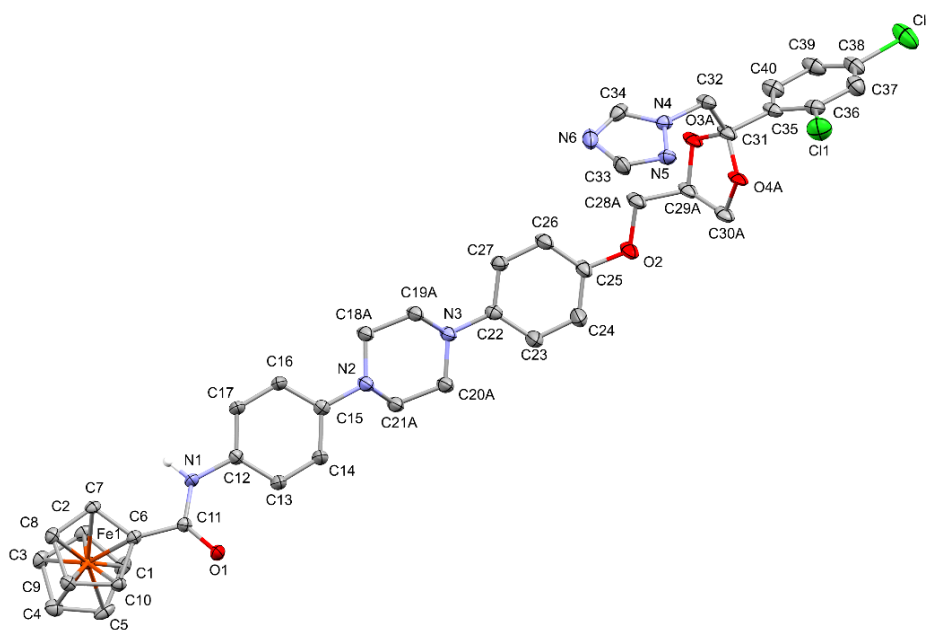


Figure 3. Pov-ray representation of complex **2a**. The most relevant crystallographic data are collected in ESI, see **Table S4-S9**.

Stability of compounds in DMSO

Dimethyl sulfoxide (DMSO) is used for the preparation of stock solution for bioassays.⁴¹ As potential drug candidates, the stability of our compounds in DMSO is an important parameter that needs to be investigated since it was shown that it could coordinate metal complexes⁴² and lead to ligand dissociation. Therefore, we dissolved all the newly synthesized ITZ derivatives in DMSO and monitored their UV-Visible spectra at 0, 3, 6, 24, 30 and 48 h. No shifted peaks or new peaks (which would be a sign of compounds' alteration) were found for **2**, **3**, and **2a-2g** over 2 days. Also, we have measured compounds **2a**, **2d** and **2g** by NMR in DMSO- d_6 at 0, 6, 24, 30 and 48 h and did not detect shifted peaks or new peaks in the spectra. Taking together, these

results suggest the compounds are stable in DMSO. All UV-Visible spectra or ¹H NMR spectra of these studies can be found in Supporting Information (**Figures S36-47**).

In vitro* activity against *Candida* spp. and *Aspergillus fumigatus

Considering that ITZ has clinically demonstrated its efficacy for the treatment of *Aspergillosis* or, as rescue therapy, in case of candidiasis, the activity of the compounds **2a**, **2b**, **2c**, **2d**, **2e**, **2f**, **2g** and **3** was evaluated, *in vitro*, against *Aspergillus fumigatus* and *Candida* spp. strains, including azole-resistant clinical isolates.

The *Candida* species were selected according to the following criteria: major representative species of the epidemiology of candidiasis in developed countries, an azole(s)-resistance strain within each species for which the underlying resistance mechanism is known. The survival assay consisted of incubating the fungi strains with the compounds for 24-hour at 35 °C and measuring of viability with a fluorimetric method.⁴³

As shown in **Table 1**, all compounds showed potent growth inhibition, as high as itraconazole against the ITZ- and fluconazole (FLC)-susceptible strain (CAAL93). All compounds were then selected for evaluation against azole (ITZ and/or FLC)-resistant *Candida albicans* strains. Because of the low solubility of the compounds, the results are limited by the highest concentration at which no crystals were observed in the culture wells. Only compounds **2a** and **2c** retained activity against the FLC-resistant but ITZ-susceptible *C. albicans*, though, in most cases, with a potency lower than ITZ. None of the compounds was active against most FLC- and ITZ-resistant *C. albicans*, except for the strain CAAL67. The mechanism of resistance of this strain is ascribed to overexpression of efflux pumps unlike the other *Candida* whose antifungal resistance is associated with point mutations in the Cyp51A gene. This proves that metallocenyl moieties have a real impact on restoring susceptibility to ITZ (minimum inhibitory concentration or MIC of 0.22±0.01 μM and 0.33±0.08 μM for **2a** and **2b**, respectively) by limiting drug leakage. The compounds **2a**, **2b**, **2f**, **2g** and **3** were more potent than ITZ while the activity of **2c** was similar to that of ITZ. For the other FLC-resistant and ITZ-susceptible *Candida* species, only **2f** displayed an activity similar to ITZ. Compounds **2a** and **2c** exhibited a significant activity against *Pichia kudriavzevii* (*Candida krusei*) (CAKR) and *C. parapsilosis* (CAPA).

Table 1. Anti-*Candida* activities (MIC, μM) evaluated on fluconazole/itraconazole-susceptible or -resistant *Candida* spp.

Compound	MIC* \pm SD (μM)												
	CAAL93	CAAL146	CAAL28	CAAL111	CAAL117	CAAL2	CAAL67	CAAL76	CAKR7	CAKR8	CAGL2	CAPA1	CAPA2
2a	0.0041 \pm 0.0012	0.031 \pm 0.010	0.14 \pm 0.01	2.9 \pm 2.5	5.1 \pm 1.5	>100	0.22 \pm 0.01	>100	>100	3.08 \pm 0.34	>100	0.13 \pm 0.04	1.23 \pm 1.09
2b	0.0031 \pm 0.0011	0.036 \pm 0.003	>10	>100	>100	>100	0.33 \pm 0.08	>100	>100	>100	>100	4.05 \pm 3.97	>10
2c	0.013 \pm 0.0008	0.032 \pm 0.004	0.21 \pm 0.04	0.35 \pm 0.13	5.73 \pm 2.72	>100	1.58 \pm 0.42	>100	1.61 \pm 0.25	0.35 \pm 0.13	>10	0.18 \pm 0.06	0.20 \pm 0.11
2d	0.0039 \pm 0.0002	0.045 \pm 0.011	>100	>100	>100	>100	>100	>100	>100	>100	>100	37 \pm 20	43 \pm 19
2e	0.0037 \pm 0.0011	0.028 \pm 0.003	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2f	0.0036 \pm 0.0002	0.023 \pm 0.001	0.25 \pm 0.03	>100	>100	>100	0.31 \pm 0.12	>10	0.88 \pm 0.63	0.19 \pm 0.12	0.79 \pm 1.74	0.04 \pm 0.03	0.13 \pm 0.01
2g	0.0039 \pm 0.0009	0.031 \pm 0.008	1.60 \pm 0.52	11 \pm 8	>100	>100	0.21 \pm 0.02	>100	>100	2.96 \pm 0.19	2.07 \pm 0.66	2.28 \pm 0.88	2.34 \pm 0.64
3	0.0045 \pm 0.0013	0.037 \pm 0.018	6.43 \pm 0.74	>10	>10	>10	0.33 \pm 0.01	4.7 \pm 3.3	3.15 \pm 0.65	2.08 \pm 0.34	0.42 \pm 0.01	0.31 \pm 0.04	0.71 \pm 0.06
ITZ**	0.0040 \pm 0.0003	0.010 \pm 0.002	0.25 \pm 0.08	0.26 \pm 0.18	0.42 \pm 0.07	>100	3.0	>100	0.42 \pm 0.07	0.22 \pm 0.08	0.36 \pm 0.20	0.09 \pm 0.07	0.17 \pm 0.04
FLC**	0.30 \pm 0.07	0.47 \pm 0.26	>100	>100	>100	>100	-	-	>100	49.59 \pm 2.90	4.75 \pm 0.64	>100	>100

*Minimal Inhibitory Concentration (MIC) is the concentration that inhibited 50% of growth. CAAL (*C. albicans*), CAKR (*Pichia kudriavzevii*- *C. krusei*), CAGL (*Nakaseomyces glabrata* - *C. glabrata*) and CAPA (*C. parapsilosis*). ** ITZ (Itraconazole) and FLC (Fluconazole).

The results of the evaluation of the compounds against *A. fumigatus* are presented in **Table 2**. At first, antifungal activity was evaluated against an ITZ-susceptible strain (ASFU7). Only compounds **2a**, **2c** and **2d** showed growth inhibition, but their MIC was 10 or 100 times higher than the ITZ-MIC. Even if these activities were quite low, these compounds were selected for further evaluation against ITZ-resistant strains. However, none of the compounds exhibited any activity against these resistant strains. It is important to note that the resistance mechanism of all TR (tandem repeat on CYP51 promoter) strains consists on molecular target overexpression.

Table 2. Anti-*Aspergillus* activities (MIC, μM) evaluated on ITZ-susceptible or -resistant *A. fumigatus*.

Compound	MIC* \pm SD (μM)							
	ASFU7	AF23 (TR34/L98H)	AF1897 (TR34/L98H)	AF2226 (TR46/Y121F, T289A)	AM AF17 (TR46/Y121F, T289A)	AF84 (TR53)	AF112 (TR53)	AF71183 (M220T)
2a	6.64\pm0.67	>100	>100	>100	>100	>100	>100	>100
2b	>100	-	-	-	-	-	-	-
2c	53.33\pm3.61	>100	>100	>100	>100	>100	>100	>100
2d	>100	-	-	-	-	-	-	-
2e	>100	-	-	-	-	-	-	-
2f	>100	-	-	-	-	-	-	-
2g	21.09\pm1.17	>100	>100	>100	>100	>100	>100	>100
3	>100	-	-	-	-	-	-	-
ITZ	0.65\pm0.04	>10	>10	>10	>10	>10	>10	>10

*MIC is the concentration that inhibited 90% of growth.

Sterol experiments

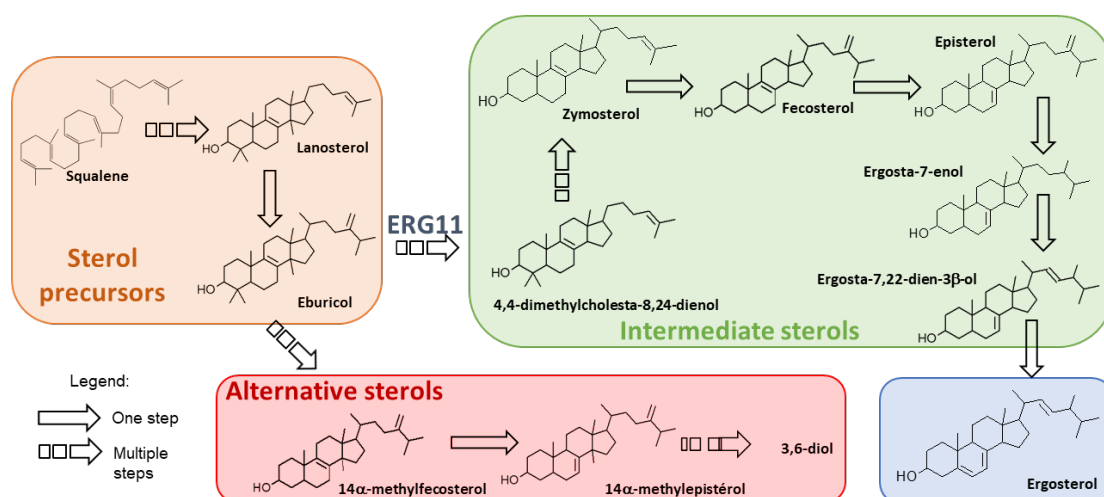
The target of the azole compounds is the sterol 14 α -demethylase cytochrome P450 (CYP51 also known as ERG11). As shown in **Figure 4A**, this enzyme is located upstream in the ergosterol biosynthesis cascade enabling the conversion of sterol precursors into intermediate products demethylated at C14 position. When the ERG11

enzyme is inhibited or dysfunctional, a secondary sterol synthesis pathway, the toxic sterol pathway, is activated. In this alternative pathway, the sterols produced retain the methyl at position C14 and the last sterol produced, the 3,6-diol, is toxic and leads to cell death.⁴⁴

In the two azole-susceptible *C. albicans* strains studied (CAAL93 and CAAL146), treatment with ITZ resulted in an accumulation of lanosterol and eburicol (two sterol precursors) as well as of 14 α -methylated sterols from the alternative pathway. Furthermore, this trend positively correlated with ITZ concentration (**Figure 4B**), confirming the on-target effect (i.e. fungal CYP51) of the drug.

Treatment with compounds **2a** and **2b** also gave the same results (**Table S2** and **S3**), i.e. an accumulation of precursor sterols, a reduction in the amount of ergosterol and an accumulation of 14 α -methylated sterols, thus confirming that the bulky presence of a metallocene moiety does not interfere with this enzymatic inhibition.

A



B

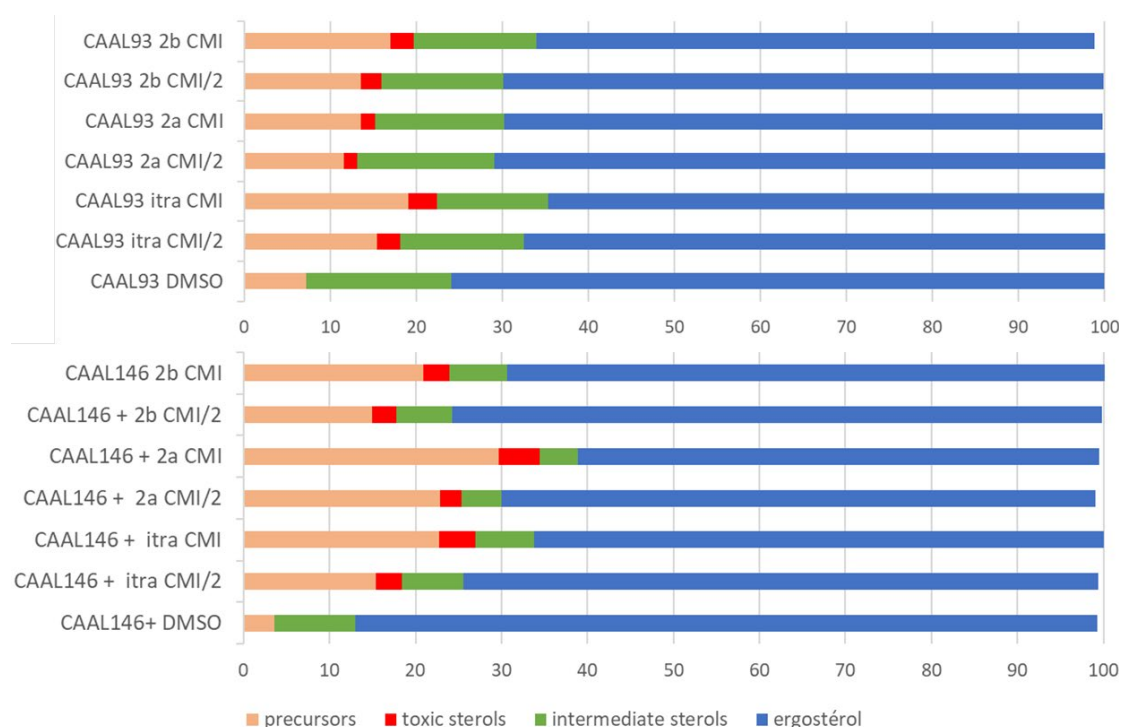


Figure 4. A) Simplified ergosterol biosynthesis pathway with sterol precursors in the orange box, sterol intermediates in the green box, end products in the blue box and toxic sterols pathway in the red box. **B)** Relative membrane sterol composition (%) of *C. albicans* strains (CAAL93 and CAAL146) untreated (DMSO) and treated with ITZ (CMI (CAAL93) = 0.0040 μ M and CMI (CAAL146) = 0.010 μ M or CMI/2) or **2a** (CMI (CAAL93) = 0.0041 and CMI (CAAL146) = 0.0031 μ M or CMI/2) or **2b** (CMI (CAAL93) = 0.0031 μ M and CMI (CAAL146) = 0.0036 μ M or CMI/2).

Docking study and molecular modeling

Docking of **2a** into the active site pocket of *C. albicans* CYP51 shows that it binds in a similar fashion to posaconazole (PCZ), a structural analogue of ITZ (**Figure 5**). The interaction of **2a** with the enzyme involves several van der Waals / aromatic contacts with Phe58, Ala61, Ala62, Tyr64 (helix A'), Leu88 (β 1- β 2 turn), Tyr118, Leu121, Phe126 (helix B'), Ile131, Tyr132 (B'' helical turn), Phe228, Pro230, Phe233 (helix F''), Leu376 (K/ β 1-4 loop), Phe380 (β 1-4 strand) and Met508 (β 4 hairpin) but none H-bonds. The bulky ferrocenyl moiety with unique spatial requirements is positioned at the surface entrance of the channel and overlaps the terminal 2-hydroxypentan-3-yltriazol-3-one group of PCZ. In this conformation, this substituent does not interfere neither contribute to the binding of the ITZ moiety to the enzyme active site.

***In vitro* activity against protozoa**

Azole antifungal drugs (such as PCZ and ITZ) have demonstrated very promising activities against *Trypanosomatids* parasites (especially *T. cruzi* and *Leishmania*).^{32,37,45,46} Several studies also point to an activity of ITZ against other pathogenic protozoan.^{47–49} Therefore we tested the activity of all seven new analogues (**2a-g**), compounds **1** and **3** as well as the parent drug ITZ against three major *trypanosomatid* species of clinical and veterinary relevance.

In *T. cruzi*, the activity was assayed against both extracellular forms of the parasite: epimastigotes (non-infective form, proliferative) and cell-derived trypomastigotes (infective form, non-proliferative). For *L. infantum*, compound activity was evaluated against the promastigote stage of the parasite (extracellular, replicative). The methods were used to evaluate parasite viability consisted of: i) a 24-hour incubation of the compounds at different concentrations with parasites and a resazurin-based fluorescence readout^{22,50–52} and ii) a 5-days incubation of compounds with the replicative forms of the parasites and measurement of optical density.^{53,54} During all experiments, Nifurtimox and Amphotericin B were used as positive controls for *T. cruzi* and *L. infantum*, respectively. Unfortunately, none of the compounds showed activity against both parasites at the maximum concentration they could be dissolved (50 μ M for **1** and **3**, 10 μ M for **2a** and **2g**, 5 μ M for ITZ, **2c**, **2d** and **2f** and 1 μ M for **2b**). The lack of activity against extracellular forms may reflect limited compound-CYP51 interaction due to interspecies differences in enzyme structure. While *T. cruzi* and *L. infantum* share more than 70% CYP51 identity,⁵⁵ similarity to fungal orthologs drops below 25%,¹⁷ potentially affecting binding affinity. In addition, variations in drug uptake, efflux, or metabolism may limit compound efficacy in non-intracellular stages. Although no docking studies were performed in this work, in silico modeling is planned to support future compound optimization.

The activity of the compounds against the bloodstream stage of *T. b. brucei* was investigated using a bioluminescent viability assay.⁵⁶ The compounds dissolved well when prepared at 10 mM in 100% v/v DMSO (stock solution), and no solid was detected immediately upon diluting them to achieve a 10 μ M concentration in the assay medium (HMI-9 culture medium + 1% v/v DMSO). However, after 24 hours of incubation at 37 °C and 5% CO₂, crystals were observed in almost all samples under the light microscope, indicating a limited solubility of the compounds in aqueous medium. The exceptions were **1** and **3**. To overcome this problem, all compounds

were also tested at a 10-fold lower concentration (1 μ M). The EC₅₀ (half maximal effective concentration) was determined for compounds reducing parasite viability to < 50% at 10 μ M (**Table 3**).

Compounds **2b**, **2d**, **2e**, **2f** and **2g**, resulted inactive or marginally active, respectively, at both concentrations tested. ITZ, **2a** and **2c**, significantly impaired pathogen viability (5-28% parasite viability) when tested at 10 μ M and lacked or showed a minor anti-trypanosoma effect at 1 μ M. These compounds presented one-digit EC₅₀ values (3.5-6.8 μ M, Figure S1), similar to that of the clinical drug Nifurtimox, whereas the more soluble derivatives **1** and **3** proved less active with estimated EC₅₀ values of 11 and 50 μ M, respectively.

Based on these results, we can conclude that: i) although active, the ferrocene moiety present in **2a** and **2c** reduces by 1.5- to 1.9-fold the intrinsic activity of the ITZ pharmacophore; ii) exchanging Fe by Ru in the ferrocene moiety proved detrimental for compound solubility; iii) the replacement of the ITZ alkyl chain by a free amine (**1**) or a acetylphenol moiety (**3**) improved the solubility of the parental compound but lowered its potency 3- to 14-fold, respectively; iv) the inclusion of (hetero)aromatic groups linked to ITZ by non-ester bonds affected negatively the activity of ITZ independently of the chemical nature of the substituent.

Table 3. Compounds' activity against the infective stage of *T. b. brucei*.

Compound	Bloodstream <i>T. b. brucei</i>			
	% viability		EC ₅₀ ± SD(μM)	Selectivity index ^c
	at 10 μM	at 1 μM		
ITZ	5.2 ± 1.8*	83.7 ± 1.1	3.4 ± 0.2	>1.5
1	51.4 ± 4.6	100.9 ± 4.5	11.3 ± 1.5	<0.4
3	104.3 ± 3.2	99.4 ± 3.3	≈50 ^a	>0.1
2a	28.1 ± 5.0*	97.8 ± 3.3	5.5 ± 0.5	>1.8
2b	88.7 ± 3.9*	86.1 ± 4.7*	ND	ND
2c	23.4 ± 4.5*	102.4 ± 2.7	6.8 ± 0.2	>0.7
2d	109.6 ± 1.3*	106.0 ± 2.3	ND	ND
2e	104.3 ± 2.0*	100.2 ± 4.3	ND	ND
2f	78.0 ± 2.3*	90.1 ± 3.2	ND	ND
2g	106.7 ± 5.4*	102.2 ± 4.2	ND	ND
Nifurtimox	ND	ND	6.0 ± 0.4 ^b	23

*Crystals were detected at the corresponding concentration.

^a value inferred because crystals were observed at concentrations close to 50 μM.

^b value previously reported using the same technique.⁵⁷

^c values calculated as the quotient CC₅₀ (half maximal cytotoxic concentration in μM units) macrophages (cell line J774)/EC₅₀ *T. b. brucei*.

***In vitro* cytotoxicity on mammalian cells and selectivity**

In order to explore the cytotoxicity of the synthesized ITZ analogues, different mammalian cell lines were incubated for 24 h with the compounds and viability was assessed with different colorimetric methods (see Materials and Methods).

VERO cells (kidney epithelial cells of African green monkeys) were selected as mammalian cell model, because they were used as host cells for obtaining infective trypomastigotes of *T. cruzi* and is widely used for phenotypic drug screening, including antiparasitic agents.⁵⁸ The most cytotoxic compounds of the series were **1**, **3** and **2e** (Table 4). ITZ, **2a**, **2b**, **2c**, **2d**, **2f** and **2g** showed percentages of viability higher than 50 % at the highest tested concentrations where crystals were not microscopically observed (10 μM for **2a** and **2g**, and 5 μM for **2b**, **2c**, **2d**).

Table 4. Compounds' cytotoxicity against VERO cells.

Compound	ITZ	1	3	2a	2b	2c	2d	2e	2f	2g
CC ₅₀ ± SD (μM)	>5 ^a	2.4 ± 0.9	2.9 ± 0.2	>10 ^a	>5 ^a	>5 ^a	>5 ^a	1.0 ± 0.1	>5 ^a	>10 ^a

^a Higher compound's concentrations could not be tested due to solubility issues.

Next, we studied the cytotoxicity on macrophages (murine-derived cell line J774), because this cell type plays an important defensive role during pathogen infection and can serve as host for the intracellular stages of both *T. cruzi* and *Leishmania sp.* parasites.^{59,60} Based on the poor solubility observed for most compounds, the CC₅₀ values could not be determined, and cytotoxicity was inferred from viability data obtained at the highest concentration at which the compounds proved soluble in the assay medium (**Table 5**).

Table 5. Compounds' cytotoxicity against murine macrophages (cell line J774).

Compound	% cell viability		
	10 μM	5 μM	1 μM
ITZ	ND	103.3 ± 23.7	ND
1	ND	5.6 ± 10.6	74.5 ± 6.3
3	ND	65.8 ± 1.2	ND
2a	98.6 ± 17.7	ND	ND
2b	ND	ND	108.0 ± 18.5*
2c	ND	101.3 ± 2.2	ND
2d	ND	115.7 ± 2.8	ND
2e	ND	103.0 ± 2.5	ND
2f	ND	82.7 ± 11.0	ND
2g	101.3 ± 18.3	ND	ND

* Crystals were observed at the working conditions. ND: not determined.

2d, 2e, 2f, 2g and **3**, which were not active against *T. brucei* up to 10 μM, were also not cytotoxic to macrophages at 5 or 10 μM, depending on the compound. The

cytotoxicity of the Ru-containing compound **2b** could not be assessed due to solubility issues at 1 μM , the lowest concentration tested. The more soluble derivatives **1** and **3** reduced macrophage viability to 6% and 66%, respectively, when tested at 5 μM . Based on this, it can be inferred that their CC_{50} is below or close to 5 μM , respectively, and hence that they lack selectivity towards the parasite. Finally, ITZ, **2a** and **2c** were not cytotoxic at the maximum concentration at which they proved soluble (*i.e.* 5, 10, and 5 μM , respectively). This data suggests that these compounds display a certain selectivity towards the pathogen.

The cytotoxicity of the compounds was also evaluated against human fibroblasts (cell line MRC5; **Table S1**). Despite its physiological relevance for being a human-derived cell line, it is routinely used as host cell in drug screening campaigns against the intracellular stage of *T. cruzi*.⁶¹ This evaluation was limited by the solubility of the compounds, therefore, the CC_{50} values were inferred from viability assays performed at the highest concentration where no compound' crystals were observed in the wells. All compounds demonstrated low cytotoxicity ($\text{CC}_{50} >10$ or >100 μM). Compared to the activities against fungi and parasites, the compounds **2a** and **2c** exhibited a high selectivity index against *Candida*.

Conclusion

This study sought to design and test organometallic (or organic) ITZ derivatives that could potentially have a unique mechanism of action due to the attached organometallic portions. Seven new derivatives were synthesized via amination reaction from carboxylic acid and amine. All the prepared compounds demonstrated high *in vitro* activity against azole-susceptible *C. albicans*. Furthermore, compounds **2a** and **2c** displayed a potent *in vitro* activity against most of the azole-resistant *C. albicans* and non-*albicans* species. The study of ergosterol biosynthesis showed that the itraconazole-metallocene (ferrocene and ruthenocene) compounds retain the mode of action of ITZ. The observed accumulation of sterols precursors (lanosterol and eburicol), the reduction in the amount of ergosterol and the appearance of 14 α -methylated sterols, which are precursors of the toxic 3,6-diol sterol, strongly suggests that ERG11 is the molecular target of these compounds. Concerning *A. fumigatus*, the best compound (**2a**) showed an activity that was 10-fold lower than that of ITZ. Unfortunately, none of the compounds showed activity against the extracellular stages of *T. cruzi* and *L. infantum*. By contrast, compounds with ferrocenyl moiety, **2a** and **2c**, affected the viability of the infective stage of *T. brucei*.

Independent of the mammalian cell type used as reference model, both compounds exhibited an interesting selectivity toward this pathogen. Possibly, the poor solubility of the compounds limits their *in vitro* bioavailability for acting against pathogens. Nonetheless, these compounds represent a reasonable starting point for structure-activity relationship (SAR) studies with organometallic ITZ derivatives. Further studies would be necessary to improve their solubility or to develop new formulations, to elucidate additional mechanisms of actions, and to assess whether they have the potential to be applied against azole-resistant fungal pathogens and *trypanosomatid* parasites.

Experimental Section

Materials and Methods

All the reagents were purchased from commercial sources and used without further purification. The solvents were dried according to standard procedures. The compounds were prepared as depicted in **Scheme 1** and the detailed synthetic procedures and characterizations are given in the Supporting Information.

Computational Methods

Analysis of membrane sterol composition was conducted by GC–MS. Molecular modeling studies were performed using SYBYL-X 2.1.1 software¹⁸ running on a Dell precision 7680 workstation. (more details are given in the Supporting Information).

Supporting Information

Experimental details: chemical synthesis, biological experiments, methods and procedures, instrumentation and characterization; additional data: NMR spectra, copies of the IR, stability studies, selected crystal data and structure refinement parameters (PDF).

Author Contributions: S.Z. prepared and characterized the compounds. A.K., G.S. and L.P. performed and confirmed data *in vitro* experiments on *T. cruzi*, *L. infantum*, *T. brucei*, VERO cells and murine macrophages. C.P., C.T., F.P. and P.L.P. performed and confirmed data *in vitro* experiments on fungal pathogens and C.L. performed docking study. I.O.G. and M.A. performed the sterol experiments and analysis. M.R. and O.B. undertook crystallographic studies. I.O.G., F.P., P.L.P., G.S., L.P.D., M.A.C. and D.G.

revised the manuscript. K. C. and G. G. revised and supervised the manuscript. All authors edited the manuscript.

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Data and materials Availability: All data associated with this study are present in the paper or the Supporting Information.

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