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Elucidation of antimicrobial activity and mechanism of action by *N*-substituted carbazole derivatives

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ABSTRACT

Compounds belonging to a carbazole series have been identified as potent fungal plasma membrane proton adenosine triphophatase (H^+-ATPase) inhibitors with a broad spectrum of antifungal activity. The carbazole compounds inhibit the adenosine triphosphate (ATP) hydrolysis activity of the essential fungal H^+-ATPase, thereby functionally inhibiting the extrusion of protons and extracellular acidification, processes that are responsible for maintaining high plasma membrane potential. The compound class binds to and inhibits the H^+-ATPase within minutes, leading to fungal death after 1–3 h of compound exposure *in vitro*. The tested compounds are not selective for the fungal H^+-ATPase, exhibiting an overlap of inhibitory activity with the mammalian protein family of P-type ATPases; the sarco(endo)plasmic reticulum calcium ATPase (Ca^{2+}-ATPase) and the sodium potassium ATPase (Na^+,K^+-ATPase). The ion transport in the P-type ATPases is energized by the conversion of ATP to adenosine diphosphate (ADP) and phosphate and a general inhibitory mechanism mediated by the carbazole derivative could therefore be blocking of the active site. However, biochemical studies show that increased concentrations of ATP do not change the inhibitory activity of the carbazoles suggesting they act as allosteric inhibitors. Furthermore decreased levels of intracellular ATP would suggest that the compounds inhibit the H^+-ATPase indirectly, but *Candida albicans* cells exposed to potent H^+-ATPase-inhibitory carbazoles result in increased levels of intracellular ATP, indicating direct inhibition of H^+-ATPase.

Introduction

Each year, 2 million people contract an invasive fungal infection (IFI) worldwide, and with mortality rates reaching 95% depending on the pathogen and underlying risk factors, IFIs represent a significant public health problem.[1] Despite a rising number of emerging invasive fungal pathogens, the majority of IFI's are caused by the yeast *Candida albicans* and the mold *Aspergillus fumigatus*. Candidemia (caused by *Candida* spp.) is among the top 5 most common nosocomial blood-borne infections in the US.[2] Individuals most at risk of developing life-threatening fungal infections are those on immunosuppressive therapies, aggressive chemotherapies, HIV-infected patients and those with congenital immunodeficiencies (e.g. chronic granulomatous disease).[3] Continuous advances in surgical procedures, organ transplantation medicine and chemotherapeutic regimens, as well as ongoing resistance development coincides with an increase in the incidence of difficult-to-treat invasive fungal disease.[4,5] Current antifungal treatments represent 3 main compound classes; the polyenes (e.g. amphotericin B), the azoles (e.g. voriconazole, fluconazole), and the echinocandins (e.g. caspofungin). These current therapies collectively suffer from shortfalls such as toxicity, drug-drug interactions, narrow spectrum of activity, and resistance development. Furthermore, the emergence of multidrug-resistant *Candida* and *Aspergillus* isolates is an increasing concern.[6] Besides the inherent limitations in the current compound classes, initiation of appropriate treatment is often delayed by challenges in diagnosis.[7] Therefore, new classes of safe, well-tolerated broad-spectrum antifungal drugs without drug-drug interactions and a propensity for resistance are urgently needed.[8]

The fungal plasma membrane H^+-ATPase is essential for fungal growth and survival.[9] The H^+-ATPase is a proton pump, generating the electrochemical gradient across the fungal plasma membrane by transporting protons from the cytoplasm to the extracellular site. This process is energized by the conversion of ATP to ADP and phosphate. Fungal plasma membrane proton pumps belong to the P_{III}-type ATPase family,[10] and the H^+-ATPase is highly

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conserved across the fungal kingdom, with 80–90% sequence identity between the H^+-ATPase in different *Candida* species. In mammalian cells, the functionally related P_{II}-type Na^+,K^+-ATPase is responsible for maintaining the ion gradient across the plasma membrane.[11,12] The sequence identity of the H^+-ATPase to the mammalian P_{II}-type ATPases, Na^+,K^+-ATPase and the sarcoplasmic reticulum Ca^{2+}-ATPase, is less than 30%. The activity of the Na^+,K^+-ATPase is targeted with cardiotonic steroids (CTS) in the treatment of congestive heart failure.[13] The Ca^{2+}-ATPase has been identified as a promising anticancer target using a pro-drug approach with the highly potent and specific Ca^{2+}-ATPase inhibitor thapsigargin.[14] The essential nature of the conserved fungal H^+-ATPase, which is absent from mammalian cells, makes it an attractive target for the development of novel broad-spectrum antifungal agents.

In the search for novel H^+-ATPase inhibitors, a library screen of 20,240 small molecule compounds was conducted by screening for H^+-ATPase inhibitory activity at a compound concentration of 20 μM. Four compounds containing an *N1*-substituted carbazole moiety were identified from the library screening as novel H^+-ATPase inhibitors (Fig. 1), and the ATP hydrolysis IC_{50} was determined together with antifungal activity against *S. cerevisiae* and *C. albicans* (Table 1). Compound **4** was the most potent antifungal compound, which displayed H^+-ATPase inhibitory activity.

It seemed plausible from the limited structure-activity relationship available that the chloro substitutions of R^1 and R^2, possibly in combination with some size exclusion in R^4, were driving the cellular activity (comparing compound **4** with **1** and **2**, and compound **4** with compound **3**, respectively), given that the ATP hydrolysis IC_{50} was similar for all four compounds. Based on this hypothesis fifteen compounds were synthesized to further explore the structure-activity relationship (Figs. 2–4). The compounds were characterized for H^+-ATPase inhibition and antifungal activity by means of an ATP hydrolysis assay and a fungal growth inhibition assay, respectively. Furthermore a study was conducted to investigate if the binding site of the compounds overlapped with the nucleotide-binding site within the H^+-ATPase, and to investigate possible effects on the intracellular ATP level in *C. albicans*. Additionally, the ability of selected compounds to inhibit acidification of the surrounding media of fungal cells after addition of glucose was investigated. Finally, we determined whether the compounds acted in a fungistatic or a fungicidal manner.

To investigate the H^+-ATPase inhibitory effect of the compounds we isolated plasma membranes containing H^+-ATPase from *S. cerevisiae* and *C. albicans* cells.[15] Plasma membranes isolated from pig kidney as well as endoplasmic reticulum membranes from rabbit hind leg muscle were used to counter-screen compounds for activity against mammalian Na^+,K^+-ATPase and Ca^{2+}-ATPase, respectively. Prior to their use, fungal plasma membrane batches were

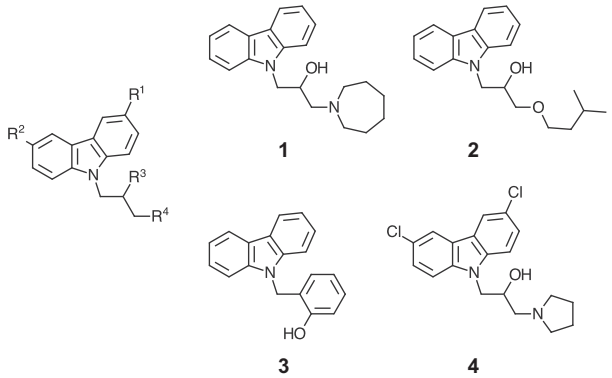


Fig. 1. Carbazole scaffold (left) and structures of initial H^+-ATPase inhibitor hits **1**–**4**.

Table 1
IC_{50} determination of the H^+-ATPase inhibition and minimal inhibitory concentration determination (MIC) of fungal growth by initial hit compounds **1**–**4**.

	ATP hydrolysis IC_{50} [μM]	Fungal Growth Inhibition MIC [μM]	
	S. cerevisiae H^+-ATPase	*S. cerevisiae*	*C. albicans*
1	18.8 ± 7.3	>200	>200
2	5.5 ± 0.7	50	>200
3	9.7 ± 0.6	50	100
4	17.3 ± 5.5	10	30

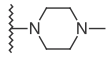
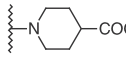
Compound	R^1	R^2	R^3	R^4	pK_a
5	-Cl	-Cl	-OH		7.1
6	-Cl	-Cl	-OH		2.7
7	-Cl	-Cl	-OH		6.6
8	-Cl	-Cl	-OH		7.6
9	-Cl	-Cl	-OH		zwitterionic
10	-Cl	-Cl	-OH		9.2

Fig. 2. Structures of compounds **5**–**10**.

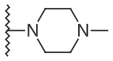
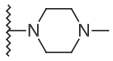
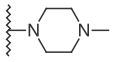
Compound	R^1	R^2	R^3	R^4
11	$-OCH_3$	$-OCH_3$	-OH	
12	-CN	-CN	-OH	
13	-OH	-OH	-OH	

Fig. 3. Structures of compounds **11**–**13**.

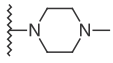
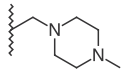
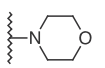
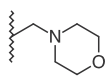
Compound	R^1	R^2	R^3	R^4
14	-Cl	-Cl	-H	
15	-Cl	-Cl	-H	
16	-Cl	-Cl	-H	
17	-Cl	-Cl	-H	
18	-Cl	-Cl	-H	
19	-Cl	-Cl	-H	

Fig. 4. Structures of compounds **14**–**19**.