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Antifungal Spectrum, *in vivo* Efficacy and Structure Activity Relationship of Ilicicolin H

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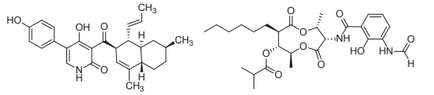
Abstract

Ilicicolin H is a polyketide –Non Ribosomal Peptide Synthase (NRPS)-natural product isolated from *Gliocadium roseum* which exhibits potent and broad spectrum antifungal activity, with sub micro g/mL MICs against *Candida* spp, *Aspergillus fumigatus* and *Cryptococcus* spp. It showed a novel mode of action, potent inhibition (IC_{50} 2-3 ng/mL) of the mitochondrial cytochrome bc1 reductase, and over 1000-fold selectivity relative to rat liver cytochrome bc1 reductase. Ilicicolin H exhibited *in vivo* efficacy in murine models of *Candida albicans* and *Cryptococcus neoformans* infections, but efficacy may have been limited by high plasma protein binding. Systematic structural modification of ilicicolin H was undertaken to understand the structural requirement for the antifungal activity. The details of the biological activity of ilicicolin H and structural modification of some of the key parts of the molecule and resulting activity of the derivatives are discussed. These data suggest that the β-keto group is critical for the antifungal activity.

Introduction

Infections caused by pathogenic fungi (e.g., *Candida albicans* and *Aspergillus fumigatus*) are life-threatening particularly to immunocompromised populations.[1] Three main therapeutic options exist for the treatment of such infections including azoles (e.g., fluconazole)[2] macrocyclic polyenes (e.g., amphotericin)[3] and candins (e.g., caspofungin, micafungin and anidulafungin).[4] Each treatment option has limitations to its utility thus creating a need for new antifungal agents.



Ilicicolin H (1)

Antimycin A

Results and Discussion

Identification of Ilicicolin H

- Screening Assay: Whole cell wild type *Candida albicans*
- Isolated from *Gliocadium roseum* by extract screening
- Structure was confirmed by NMR and Mass spectral analysis
- Originally isolated from *Cylindrocladium ilicicola* in 1971
  - No antifungal spectrum was reported

Antifungal Spectrum of Ilicicolin H

Ilicicolin H shows broad spectrum antifungal activity with MIC ranges of 0.04-0.31 μg/mL against *C. albicans* including fluconazole resistant strains. It showed potent activities against *Cryptococcus neoformans* and *Aspergillus fumigatus* (**Table 1**).

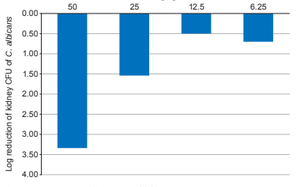
Table 1. Antifungal spectrum (MIC, μg/mL) of ilicicolin H (1) and clinical comparators

	MIC (μg/mL)				
		Ilicicolin H	Caspofungin	Amphotericin B	Fluconazole
Strain	Strain number	24 hr	24 hr	24 hr	24 hr
Candida albicans	MY 1055	0.04	0.25	0.25	0.5
Candida albicans	MY 2301	0.31	0.5	0.5	>64
Candida albicans	SC 53124	0.04	NT	NT	NT
Candida glabrata	CLY 574	0.63	0.5	0.4	>64
Candida glabrata	MY 1381	1.3	0.5	0.5	8
Candida guilliermondii	CLY 308	2.5	0.5	0.25	4
Candida guilliermondii	CLY 346	5	1	0.13	4
Candida krusei	CLY 549	0.01	1	0.5	16
Candida lusitaniae	MY 1396	3.1	0.25	0.06	1
Candida parapsilosis	ATCC 22019	0.16	1	0.13	2
Aspergillus flavus	MF 383	>100	>64	1	>64
Aspergillus fumigatus	MF 5668	0.08	64	0.5	>64
		48 hr	48 hr	48 hr	48 hr
Cryptococcus tropicalis	MY 1012	0.1	0.25	2	>64
Cryptococcus neoformans	H 99	1.56	NT	NT	NT
Cryptococcus neoformans	MY 2061	0.2	16	0.13	2

NT = Not tested. Antifungal activity was determined using NCCLS protocols. Antifungal activity of ilicicolin H was determined in glycerol based media while the antifungal activity of comparator antifungals was determined in glucose based media. All MICs were read after 24 hr, at 37°C except for *C. neoformans* and *C. tropicalis* which were read after 40 hr.

In vivo Efficacy:

Figure 1. *In vivo* activity of ilicicolin H in disseminated *C. albicans* (MY 1055) murine infection model



Dosed twice daily orally in 10% aqueous DMSO for 2 days.
DBA/2N mice were challenged i.v. with *Candida albicans* MY1055 at 5.4 x 10^4.

C. albicans (po, bid): ED_{90} = 15.45 mg/kg/dose; ED_{99} = 30.75 mg/kg/dose
C. albicans (iv, tid): ED_{90} = 22.3 mg/kg/day
Cryptococcus neoformans (speen model, ip): ↓one log cfu/g spleen

Mechanism of action:

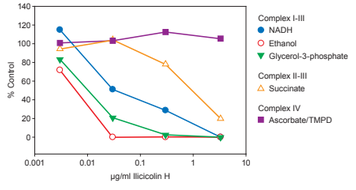
Table 2. MIC of ilicicolin H with different carbon source

	MIC μg/ml		Concentration (μg/ml) to inhibit whole cell O_2 consumption 100%
	Glucose	Glycerol	
S. cerevisiae MY 2141	>50.0	0.012	0.003
C. albicans MY 1055	>50.0	0.025	0.003

Table 3. Effect of ilicicolin H on substrate dependent rates of oxygen consumption by coupled mitochondria from *S. cerevisiae* MY 2141

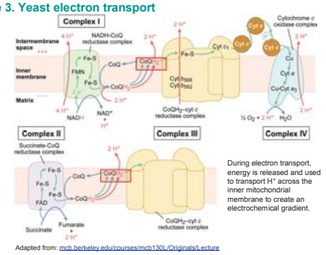
	IC_{50} (μg/ml)
Complex I - III	
NADH	0.08
Ethanol	0.008
Glycerol-3-phosphate	0.02
Complex II- III	1.0
Complex IV	10.0

Figure 2. Graphical representation of oxygen comsumtion rate by coupled *S. cerevisiae* mitochondria



- Antifungal activity of ilicicolin H dependent on carbon source in the test media.
- Respiration inhibited in *C. albicans* and *S. cerevisiae*
- Measurements of the effect of ilicicolin H on substrate dependent rates of oxygen consumption by coupled mitochondria from *S. cerevisiae* MY 2141 revealed that complex I – III was the most sensitive complex in the respiratory chain
- Inhibition of complex I – III accounted for by inhibition of NADH:cytochrome c oxidoreductase; IC_{50} of 0.8 and 1.0 ng/mL (1.85 nM and 2.31 nM); *C. albicans* and *S. cerevisiae*, respectively.
- Ilicicolin H was shown to inhibit the center N (Qn site, also called Qi site) of the cytochrome bc1 complex with an IC_{50} of 3-5 nM in the ubiquinol:cytochrome c reductase of *S. cerevisiae*.[5]

Figure 3. Yeast electron transport



Adapted from: mcb.berkeley.edu/courses/mcb130L/Originals/Lecture

Selection of ilicicolin H resistant *C. albicans* mutants:

- Isolated with a frequency of 10^{-7}
- Comared to WT:
  - Growth rates varied between each mutant and WT
    - MICs were 250-1000-fold higher
    - Not cross-resistant to myxothiazol and antimycin
  - NADH:cytochrome c reductase: IC_{50} 8-66-fold higher
  - Ubiquinol:cytochrome c reductase IC_{50} 12-222-fold higher
- *C. albicans* mutants showed five single amino acid changes at four sites in the cytochrome b gene identical to *S. cerevisiae* mutations (**Table 3**)
- G37 mutations (G37D, G37S, D37C) most frequent in both yeast strains (**Table 3**)

Table 3. Cytochrome b mutations generating ilicicolin H resistance in *S. cerevisiae* and *C. albicans*

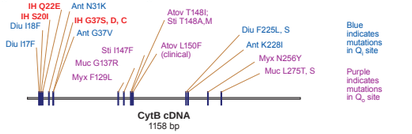
Position	Mutations	
	S. cerevisiae	*C. albicans*
20	ND S20L (1) S20T (1)	S20I (1) ND ND
22	Q22E (3) Q22T (2)	Q22E (2) ND
37	ND G37D (2) G37S (2)	G37C (3) G37D (4) G37S (6)
198	L198F (1)	ND

ND is not detected. *S. cerevisiae* mutations identified by Ding et al[6]; *C. albicans* mutations identified current study. The numbers of independent isolates are listed in parentheses.

Table 4. Biological profile of *C. albicans* Ilicicolin H resistant mutants

Strain	Fold change in ilicicolin H			Cyt c1 peak		Growth	
(cyt b aa substitution)	*MIC	*NADH: cyt c reductase IC_{50}	*Ubiquinol:cyt c reductase IC_{50}	0.1 mg/ml ilicicolin H	1 μg/ml ilicicolin H	Glycerol	Glucose
Parent	1	1	1	-	-	Average	Average
Ca2 (G37D)	500	8	222	+	-	♦Slow lag	Average
Ca3 (G37D)	250	13	12	+	-	♦Slow lag	Average
Ca9 (G37D)	250	13	20	+	-	Slow	Average
Ca7 (G37D)	250	21	30	+	-	♦Slow lag	Average
Ca10 (S20I)	250	33	17	+	-	Slow	♦Slow lag
Ca15 (G37S)	500	8	23	+	-	Average	Fast
Ca5 (G37S)	500	20	12	+	-	Average	Fast
Ca8 (G37S)	500	25	25	+	-	Average	Slow-Ave
Ca4 (G37S)	500	25	12	+	-	Fast	Fast
Ca6 (G37S)	500	35	26	+	-	Fast	Fast
Ca12 (Q22E)	500	50	128	+	+	Average	Fast
Ca16 (Q22E)	500	33	89	+	+	Average	Fast
Ca14 (G37S)	500	33	111	+	+	Fast	Fast
Ca1 (G37C)	1000	41	23	+	+	Fast	Fast
Ca11 (G37C)	1000	66	26	+	+	Fast	Fast
Ca13 (G37C)	500	66	47	+	+	Fast	Fast

Figure 4. Cytochrome b drug resistant mutations



Fungal Specificity:

Ilicicolin H showed exquisite NADH:cytochrome c oxidoreductase specificity for *C. albicans enzyme* compared to rat or rhesus liver enzymes.

Table 5. NADH:cytochrome c oxidoreductase specificity of ilicicolin H and antimycin

	NADH:cytochrome c oxidoreductase IC_{50} (nM)	
	Ilicicolin H	Antimycin
C. albicans	1.8	1.1
Rat liver	3464.0	1.1
Rhesus liver	1154.0	0.2

Selectivity rationale:

- Differences due to differences in ubiquinol isoprene side chains between fungal (n = 4-6) and mammalian (n = 9-10) systems
- Differences in sequence homology Qi-site amino acids
  - 76% identity and 90% similarity (*S. cerevisiae* vs *C. albicans*)
  - 51% identity and 72% similarity (*S. cerevisiae* vs bovine)
- These differences suggest binding differences of the substrate, ubiquinone and the inhibitor, ilicicolin H

Figure 5. Amino acid sequence alignments of cytochrome c Qi site

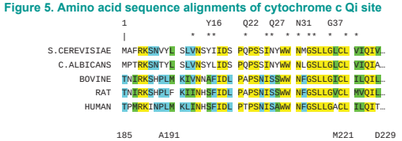
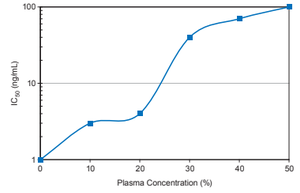
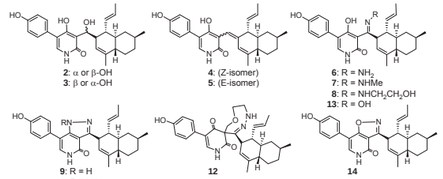


Figure 6. Effect of mouse plasma on ilicicolin H enzyme activity



- Mouse plasma significantly reduced the activity of ilicicolin H
- >1000 fold (>1000 ng/mL) *C. albicans* MIC shift in the presence of 10% mouse serum
- Likely responsible for poor *in vivo* activity

Chemical modification and SAR



2: α or β-OH
3: β or α-OH

4: (Z-isomer)
5: (E-isomer)

6: R = NH_2
7: R = NHMe
8: R = $NHCH_2CH_2OH$
13: R = OH

9: R = H
10: R = Me
11: R = CH_2CH_2OH

12

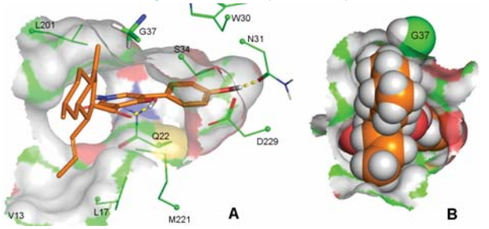
14

Table 6. *C. albicans* antifungal and NADH: cytochrome c1 oxidoreductase activity as well as selectivity against rat liver NADH: cytochrome c1 oxido reductase of analogs of ilicicolin H

Cpd #	MY1055[a] (MIC, ng/mL)	MY1055[b] (IC_{50}, ng/mL)	Rat[c] (IC_{50}, ng/mL)
1	20-40	2-3	2000-5000
2	>5000	12	140
3	>5000	94	375
4	>5000	21	100
5	5000	48	190
6	250	250	NT
7	500	NT	NT
8	500	NT	NT
9	250	3	>1000
10	250	NT	NT
11	250	NT	NT
12	>1000	NT	NT
13	>1000	NT	NT
14	>1000	NT	NT

[a]*C. albicans* MY1055, [b]*C. albicans* MY1055 NADH: cytochrome c reductase, [c]Rat liver NADH:cytochrome c reductase, NT = not tested.

Figure 7. Binding Model of Ilicicolin H: Homology model based on the crystal structure of *S. cerevisiae* cytochrome bc1 complex with ubiquinol in Qi site



(**A**) The binding mode of ilicicolin in *C. albicans* model. Several key residues are shown as thin sticks. G37 is shown in thicker sticks. (**B**) To better appreciate the steric constraints of the binding pocket, ilicicolin H is shown in CPK representation. The necessity for a perpendicular orientation of the left- and right-hand side of ilicicolin H is obvious. G37 Cα carbon with hydrogens shown is also displayed in CPK.

Summary and Conclusion

- Ilicicolin H is a natural product produced by an imperfect fungus, *Cylindrocladium ilicicola* which showed broad spectrum antifungal activity.
- It imparts its activity by selectively inhibiting fungal cytochrome c1 oxidoreductase activity and respiration.
- It demonstrated modest *in vivo* activity in a Candida and Cryptococcus infection mouse model.
- The *in vivo* activity was limited by high plasma protein binding. Preliminary medicinal chemistry efforts pointed out the criticality of the β-diketone feature of the molecule and lead to mostly less active or inactive compounds.
- The homology model suggests that its binding mode has some similarities but also differences relative to antimycin binding, and provides valuable insight to SAR and fungal specificity.
- These studies open the window for future work on ilicicolin H and the development of new mode of action antifungal agents.
- Details of this work including methods is in press[7]

References

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