IN VITRO STUDIES OF ACULEACIN A, A NEW ANTIFUNGAL ANTIBIOTIC

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Aculeacin A is a cyclopeptide-containing long-chain fatty acid, representing a new class of antibiotics. It has a relatively narrow antifungal spectrum *in vitro* and is highly active against some groups of yeasts. Of 31 strains of *Candida* and *Torulopsis* tested, the majority were susceptible to aculeacin A at 0.31 μg per ml or less. On the other hand, the antibiotic was scarcely active or inactive against other yeasts, such as *Cryptococcus neoformans*, and all filamentous and dimorphic fungi tested. A distinct inoculum effect has been observed *in vitro* with a number of strains of *C. albicans*; minimum growth-inhibitory concentrations (MIC) have tended to increase with increased incubation time. MIC values were also increased in the presence of serum. Aculeacin A is fungicidal for growing cells of *C. albicans*. It was most lethal against sensitive yeasts at 0.08 to 0.31 μg per ml, and increases in the concentration of the drug above this range reduced, rather than increased, its lethal effect.

Aculeacin A is the major component of a mixture of new antifungal antibiotics produced by *Aspergillus aculeatus*.[1] The chemical structure of aculeacin A is closely related to that of echinocandin B which is a polypeptide with a fatty acid residue.[2,3] Preliminary information revealed that aculeacin A is highly active *in vitro* against *Candida albicans* and several other yeasts, while it has very slight activity against a number of fungi and is inactive against bacteria.[1]

Studies therefore were undertaken to further define the *in vitro* antifungal spectrum and other *in vitro* properties of this antibiotic. Where appropriate, the antifungal activity of aculeacin A was compared with clotrimazole, a drug of choice used in treatment of several groups of superficial mycotic infections.

Materials and Methods

Drugs

Aculeacin A (Toyo Jozo Co., Ltd., Tokyo) and clotrimazole (bis-phenyl-(2-chloro-phenyl)-1-imidazolylmethane; Bayer-Yakuhin Co., Ltd., Osaka) were dissolved in 100% dimethyl sulfoxide. Final stock drug solutions of the two drugs were further diluted to contain 8 mg of active material per ml.

Organisms

One hundred and thirteen strains of fungi, most of which are of medical interest, were tested (Tables 1 ~ 3). These consisted of clinical isolates and stock cultures in our laboratory.

Preparation of Inocula

Stock cultures of monomorphic yeasts (*Candida, Torulopsis* and *Cryptococcus*) were grown at 27°C for 3 days on Sabouraud glucose (SG) agar medium, and those of filamentous and dimorphic fungi were grown for 3 weeks on the same medium. Viable-cell suspensions of monomorphic yeasts were prepared by suspending viable cells harvested from 3-day-old SG agar slants in sterile saline. Spore suspensions

of filamentous fungi or dimorphic fungi were prepared by rubbing on the surface of 20-day-old SG agar slants with a loop after the addition of sterile saline containing 0.1% (w/v) Tween 80. Then they were filtered through two layers of gauze or allowed to stand at room temperature for settle down large blocks of cell aggregates or mycelia. All inocula were adjusted by nepherometry with sterile saline with or without 0.1% Tween 80 so as to contain from 10^6 to 10^7 cells or spores per ml as confirmed by viable plate counts.

Media

SG agar medium and SG broth were used. The pH was 5.6.

Susceptibility Testing

The agar dilution method was used. Drugs were diluted in the SG agar medium over a final concentration range of 0.01 to 80 μg per ml. Here 0.003 ml of cell or spore suspensions was inoculated by making a 2 cm-long streak with the calibrate loop, and the plate was incubated at 27°C, except for *Candida* spp., which preferred 37°C, for the indicated period of time that sufficed to show appreciable growth of colonies on drug-free control plates. The minimum growth-inhibitory concentration (MIC) was defined as the lowest concentration of drug at which there was no visible growth.

Effect of pH on Activity

The MIC of aculeacin A against fungi on medium with different pH values was determined by the agar dilution method on SG agar medium adjusted to a final pH value ranging from 3.0 to 8.0 with 0.1 M glycine-HCl buffer (pH 3~5) and 0.1 M phosphate buffer (pH 5~8). Six strains of *C. albicans* were used as the test organism with an inoculum size of 0.03 ml of 10^6 viable cells per ml.

Effect of Inoculum Size on Activity

The MIC of aculeacin A against *C. albicans* with different inoculum sizes was determined by the agar dilution method on SG agar medium (pH 5.6) with an inoculum size of 0.003 ml of 10^4, 10^5, 3×10^5, 10^6, 10^7, and 10^8 viable cells per ml.

Effect of Human Serum on Activity

The effect of serum on the MIC of aculeacin A against *C. albicans* was determined by the agar dilution method on SG agar medium containing 10% human serum with an inoculum size of 0.003 ml of 10^6 viable cells per ml.

Fungicidal Activity Test

A series of L-tubes containing 9 ml of SG broth incorporating from 0 to 20 μg of aculeacin A per ml was prepared, and inoculated with 0.1 ml of viable cell suspensions of *C. albicans* to give final cell concentrations of approximately 10^6, 10^7 and 10^8 colony forming units (CFU) per ml. After incubation at 37°C for 6 hours on a shaker, samples were taken from each tube, and aliquots (0.1 ml) from serial dilutions were plated on SG agar plates with the aid of spreaders for counts of CFU. No significant drug carry-over was detected at the lowest dilution used.

Results

In Vitro Antifungal Spectrum

The *in vitro* antifungal activity of aculeacin A and clotrimazole against a variety of pathogenic yeasts and filamentous fungi is shown in Tables 1, 2 and 3. Data for clotrimazole, which are consistent with previously published results,[4~6] show that clotrimazole indeed had a broad spectrum of activity against yeasts and filamentous fungi. By contrast, the antifungal spectrum of aculeacin A was much less in wideness. As shown in Table 1, aculeacin A was highly active against *C. albicans* and other *Candida* species as well as *Torulopsis glabrata*; the MIC range was $\leq$0.04 to 5 μg per ml, and the order of activity appeared to be much greater than with clotrimazole. In particular, *C. albicans* was inhibited by aculeacin A at levels around 0.1 μg per ml or less. Contrary to these aculeacin A-sensitive yeasts, *Cryptococcus neoformans* lacked the sensitivity to this antifungal antibiotic; the MIC for all strains of *C. neofor-*

Table 1. Cumulative percentage of medically important yeasts inhibited by varying concentrations (MIC) of aculeacin A and clotrimazole.

Organism (No. tested)	Antifungal agent	Cumulative % of strains inhibited at drug concentration (μg/ml) of:											
		≤0.04	0.08	0.16	0.31	0.63	1.25	2.5	5	10	20	40	>80
Candida albicans (25)	Aculeacin A	96	100										
	Clotrimazole							18	45	73	100		
C. tropicalis (4)	Aculeacin A		50	100									
	Clotrimazole									50	100		
C. pseudotropicalis (4)	Aculeacin A							50	100				
	Clotrimazole		25				50	75	100				
C. krusei (2)	Aculeacin A			50	100								
	Clotrimazole			50			100						
C. parapsilosis (2)	Aculeacin A						50	100					
	Clotrimazole								50	100			
C. stellatoidea (2)	Aculeacin A	50		100									
	Clotrimazole									100			
C. guilliermondii (2)	Aculeacin A					50			100				
	Clotrimazole						50				100		
Torulopsis glabrata (4)	Aculeacin A		25	100									
	Clotrimazole		25						75	100			
Cryptococcus neoformans (9)	Aculeacin A												100
	Clotrimazole				33	78	89	100					

All MIC values were read after 4 days.

Table 2. Cumulative percentage of filamentous and dimorphic fungal pathogens inhibited by varying concentrations (MIC) of aculeacin A and clotrimazole.

Organism (No. tested)	Antifungal agent	Cumulative % of strains inhibited at drug concentration (μg/ml) of:											
		≤0.04	0.08	0.16	0.31	0.63	1.25	2.5	5	10	20	40	>80
Aspergillus fumigatus (11)	Aculeacin A												100
	Clotrimazole					9		73	100				
Dematiaceous fungi (10)*	Aculeacin A												100
	Clotrimazole						10	40	50	100			
Histoplasma capsulatum (2)	Aculeacin A												100
	Clotrimazole		100										
Blastomyces dermatitidis (2)	Aculeacin A												100
	Clotrimazole					100							
Paracoccidioides brasiliensis (2)	Aculeacin A												100
	Clotrimazole					100							
Sporothrix schenckii (5)	Aculeacin A												100
	Clotrimazole											100	
Geotrichum candidum (2)	Aculeacin A												100
	Clotrimazole											100	

* Tested were: 3 strains of *Fonsecaea pedrosoi*, 2 strains of *F. compactum*, 1 strain of *Phialophora verrucosa*, 2 strains of *P. dermatitidis*, and 1 strain each of *Cladosporium carrionii* and *C. bantianum*.
All MIC values were read after 14 days, except for those against *A. fumigatus* which were read after 7 days.

Table 3. Cumulative percentage of dermatophytic fungi inhibited by varying concentrations (MIC) of aculeacin A and clotrimazole.

Organism (No. tested)	Antifungal agent	Cumulative % of strains inhibited at drug concentration (μg/ml) of:											
		≤0.04	0.08	0.16	0.31	0.63	1.25	2.5	5	10	20	40	>80
Trichophyton mentagrophytes (7)	Aculeacin A												100
	Clotrimazole				14	71	100						
T. rubrum (5)	Aculeacin A												100
	Clotrimazole		20	60	80	100							
T. tonsurans (2)	Aculeacin A												100
	Clotrimazole					100							
T. violaceum (2)	Aculeacin A												100
	Clotrimazole					50	100						
Microsporum audouinii (2)	Aculeacin A												100
	Clotrimazole		50			100							
M. canis (2)	Aculeacin A												100
	Clotrimazole			50			100						
M. gypseum (3)	Aculeacin A												100
	Clotrimazole						100						
Epidermophyton floccosum (2)	Aculeacin A												100
	Clotrimazole				100								

All MIC values were read after 14 days.

mans tested was more than 80 μg per ml.

The spectrum of activity against some opportunistic filamentous fungi, such as *Aspergillus fumigatus*, and dematiaceous fungi is presented in Table 2 and that against dermatophytic fungi is given in Table 3. With the only exception of a strain of *Trichophyton tonsurans*, all these varying groups of fungi were not inhibited by aculeacin A at any levels up to 80 μg per ml, although partial inhibition was observed with almost all strains at much lower drug concentrations.

Influence of pH, Inoculum Size, Incubation Time, and Serum on *In Vitro* Activity

The results of experiments which were carried out using 6 laboratory strains of *C. albicans* on SG agar medium with a pH value of 3 to 8 show that the MIC value was virtually unaffected over the entire pH range tested (Table 4).

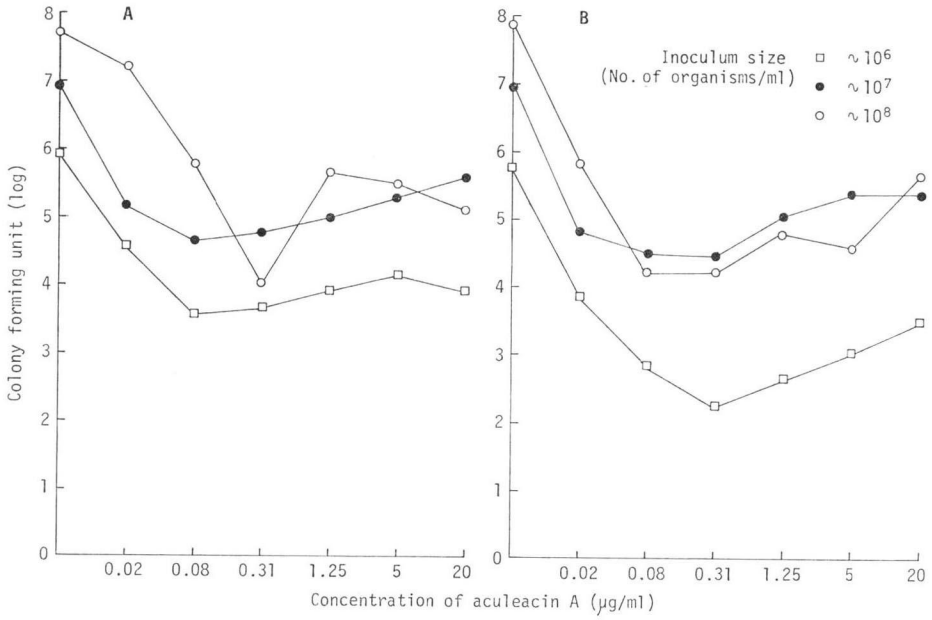
Table 4. Effect of pH of the medium on *in vitro* inhibitory activity of aculeacin A.

Strain		MIC (μg/ml) at various pH values					
		pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
C. albicans	MTU 12021	0.08	0.04	0.04	0.04	0.04	0.08
〃	MTU 12063	0.01	0.01	0.02	0.01	0.01	0.01
〃	MTU 12077	0.04	0.04	0.04	0.04	0.04	0.04
〃	MTU 12084	0.01	0.02	0.04	0.04	0.02	0.02
〃	MTU 12086	0.08	0.08	0.08	0.08	0.08	0.08
〃	MTU 12090	0.08	0.08	0.16	0.08	0.08	0.08

Table 5. Effect of inoculum size, incubation time and human serum on *in vitro* inhibitory activity of aculeacin A.

Strain	Inoculum size (No. of organisms/ml)	MIC (μg/ml) at various time of incubation in SG agar with or without human serum					
		Without human serum			With human serum		
		2 days	4 days	7 days	2 days	4 days	7 days
C. albicans MTU 12084	10^4	0.02	0.02	0.02	1.25	1.25	1.25
	10^5	0.01	0.01	0.01	1.25	1.25	1.25
	3×10^5	0.02	0.02	0.02	1.25	1.25	1.25
	10^6	0.02	0.02	0.02	1.25	1.25	1.25
	10^7	0.02	0.02	0.02	1.25	1.25	1.25
	10^8	0.02	0.02	0.02	1.25	1.25	1.25
C. albicans MTU 12021	10^4	0.02	0.04	2.5	2.5	2.5	2.5
	10^5	0.02	0.04	2.5	2.5	2.5	2.5
	3×10^5	0.04	0.04	5	2.5	2.5	2.5
	10^6	0.04	0.08	10	2.5	2.5	2.5
	10^7	0.16	0.16	>80	2.5	2.5	2.5
	10^8	1.25	10	>80	2.5	2.5	5

Fig. 1. Fungicidal action of aculeacin A toward *C. albicans* strains MTU 12021 (A) and MTU 12084 (B). Viable counts were determined after 6 hours of incubation at 37°C.



The effect of inoculum size and incubation time on the *in vitro* activity of aculeacin A was tested against several strains of *C. albicans*. These strains were divided into two groups on the basis of their response to the effect of inoculum size. Data obtained with a typical strain of each group are given in Table 5. Some strains, like MTU 12021, showed a distinct inoculum effect; increased MIC values were observed with increased inoculum size and also with increased incubation time. By contrast, MIC

values against other strains, like MTU 12084, appeared to be not influenced by inoculum size nor incubation time. Table 5 also demonstrates that, in the presence of 10% human serum, there was a 50-fold or greater increase in the MIC, which was read after 2 days of incubation, for both types of *C. albicans* strains. Moreover, on the serum-supplemented medium, not only the inoculum effect but the incubation time effect on the MIC of aculeacin A against the former type of strains, such as MTU 12021, was cancelled.

Fungicidal Action of Aculeacin A

Experiments were conducted using two strains of *C. albicans* to examine whether aculeacin A primarily acts on sensitive yeasts as a fungistatic agent or a fungicide. The survival of *C. albicans* strains MTU 12021 and MTU 12084 after 6 hours of exposure to various concentrations of aculeacin A is shown in Fig. 1. It can be seen that both strains behaved in a similar fashion, irrespective of different inoculum sizes, in that 0.08 to 0.31 μg of aculeacin A per ml was most lethal; more than 99% kill occurred within the experimental period of time, and that further increases in the concentration of the antibiotic progressively reduced its fungicidal effect. Essentially the same dose-response pattern was obtained for other strains of *C. albicans*, as well as strains of other species of sensitive yeasts such as *C. tropicalis* and *T. glabrata*. Thus the results show that the dose-response pattern elicited by aculeacin A was neither strain nor species specific.

Discussion

The results of this expanded *in vitro* study indicate that aculeacin A is highly active against *Candida* and *Torulopsis* yeasts while it is poorly active or inactive against *Cryptococcus* yeasts and all the pathogenic filamentous fungi tested including various dimorphic, dematiaceous and dermatophytic fungi. Thus the antibiotic shows a considerably restricted spectrum of activity against several yeasts. A very similar antifungal spectrum has been reported with the structurally related antibiotics echinocandin B[7] and papulacandins.[8,9] For some unknown reasons, the aculeacin A-sensitivity of some strains of sensitive yeasts was markedly influenced by inoculum size and incubation time. It is desirable, therefore, to express the MIC of the antibiotic against *Candida* and other yeasts as a value determined after 2 days or, at most, 4 days of incubation using relatively small inoculum sizes.

Previous studies made by other investigators on the biochemical and cytological effects of aculeacin A revealed that it preferentially inhibits *de novo* synthesis of cell wall, particularly wall glucan, eventually leading to cell lysis in growing cells of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.[10,11] This may explain the basal mechanism by which aculeacin A exerts potent fungicidal action toward sensitive yeasts.

Aculeacin A appears unusual among antifungal agents in respect to the dose-response pattern elicited by aculeacin A-sensitive yeasts. Data are presented to illustrate that when aculeacin A was used *in vitro* to treat *C. albicans*, it was most lethal at a concentration range between 0.08 and 0.31 μg per ml, above which concentration range the drug became decreasingly lethal. This paradoxic effect of aculeacin A would be analogous to that previous described for the action of penicillin against some Gram-positive and -negative bacteria[12,13] and for the action of nalidixic acid against *Escherichia coli* and some other Gram-negative bacteria.[14,15] Our current interest is in elucidating a mechanism that explains the paradoxic dose-response behavior of *C. albicans* to aculeacin A, along with a mechanism of its antifungal action.

The therapeutic implications of the paradoxic effect of aculeacin A is difficult to assess. Probably it has no serious clinical implication, since it is shown that this anomalous effect is cancelled by serum, and the antibiotic is highly bound to serum. Studies are needed to assess the relevancy of this serum binding phenomenon to the utility of aculeacin A in chemotherapy.

Apart from therapeutic applications as an antifungal agent, the selectivity of the fungicidal action

of aculeacin A suggests its usefulness for taxonomical grouping of yeasts. In this expectation, aculeacin A-sensitivity of a wider range of yeast species is being explored.

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