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THE NATURE OF THE SUSCEPTIBILITY OF PATHOGENIC DERMATOPHYTES TO MONOFLUOROMETHYLAGMATINE (MFMAg)

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SUMMARY

Polyamines are essential aliphatic cations required for normal growth and develepment in plants, animals, bacteria and fungi. *Microsporum*, and *Trichophyton* were in-vitro susceptible to growth inhibition by difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA), suicide inhibitors of the ornithine (ODC) and arginine decarboxylase (ADC) respectively. However, the basis for the susceptibility to DFMA was unclear as initial studies failed to find detectable ADC, arginase nor agmatine ureohydrolase (AUH) activities in extracts from either genus. We have re-examined the arginase, AUH and ADC activities as well as dermatophytes' susceptibilities to MFMAg, a suicide inhibitor of AUH. The data demonstrated existence of arginase as well as AUH' in these dermatophytes and found the (Lineweayer-Burke) K_{ms} and K_{1s} of arginase and AUH with respect to DFMA and MFMAg in both Microsporum, and Trichophyton.

Key words: Decarboxylase, monofluoromethylagmatine, *Microsporum*, polyamines

PATOJENİK DERMAFİTLERİN MONOFLOROMETİLAGMATİNE DUYARLILIK MEKANİZMASININ ARAŞTIRILMASI

ÖZET

Poliaminler, bitki, hayvan, bakterileri ve mantarların normal büyüme ve gelişmesi için gerekli olan temel alifatik katyonlardır. *Microsporum* ve *Trichophyton* sırasıyla ornitin (ODC) ve arginin-dekarboksilazın (ADC) suicide inhibitörleri olan diflorometilarjinin (DFMA) ve diflorometilornitin (DFMO) büyüme inhibisyonuna in-vitro duyarlılık göstermişlerdir. Bununla birlikte DFMA'ya olan duyarlılığı destekleyecek bilgiler net değildir. Bu konuda yapılan başlangıç çalışmalarda, her iki mantarın ekstrelerinde gözlenebilir. ADC, arginaz veya agmatin ürehidrolaz (AUH)'a olan aktiviteyi saptayamamışlardır. Bizim çalışmamızda arginaz, AUH ve ADC aktivitelerinin tekrar incelenmesinin yanı sıra dermatofitlerin AUH'ı yok edici bir inhibitör olan Monoflorometilagmatine tithylagmatine olan duyarlılıkları incelenmiştir. Elde ettiğimiz bulgular dermatofitlerde AUH'ın olduğu gibi arginazın da mevcudiyetini göstermiş ve ayrıca *Microsporum*, *Trichophyton*'da DFMA ve DFMAg açısından her iki mantarın arginaz ve AUH enzimlerinin K_{ms} ve K_{is} (Kinetiği=Lineweawer-Burke) değerleri bulunmuştur.

Anahtar kelimeler: Dekarboksilaz, monoflorometilagmatin, mikrosporum, poliamamin sentezi

INTRODUCTION

Though the use of mutants and specific inhibitors, polyamines (putrescine spermidine, and spermine) have been shown to be essential for normal growth and development of fungi, bacteria, green plants and mammals (1). In bacteria and plants, polyamine synthesis initially involves the production of putrescine via either the ornithine decarboxylase (ODC) or arginine

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decarboxylase (ADC) pathways (2). ADC and ODC are two key enzymes in the synthesis of polyamines.

ADC catalyzes the conversion of arginine to agmatine which is then hydrolyzed by agmatine ureohydrolase (AUH) to produce putrescine and urea (3).

In fungi and most mammalian cells, arginine metabolism involves the urea cycles in which arginine is hydrolyzed by arginase to ornithine and urea. Although fungi can grow in the absence of arginine, exogenously applied arginine can be transported, stored and when necessary used as a nitrogen source fungal and mammalian cells appear to lack the later enzyme (2). The ODC inhibitors α-difluoromethyl ornithine (DFMO) and monofluoromehyl dehydroornithinemethylester (MFMOme) and the ADC inhibitor α-difluoromethylornithine (DFMA) have been shown to inhibit the growth of various species of *Trichophyton* and *Microsporum*, the causal agents of dermatophytoses of human and animals. The growth inhibition was specific as it could be reversed by abdition of either putrescine or spermidine (4). DFMO also inhibits growth and synthesis of polyamine in clinical isolates of the pathogenic yeast *Candida* (5). Studies with plant pathogenic fungi have demonstrated that DFMO and DFMA inhibit germination of fungal propagules, vegatitive growth or sporulation (6-10). In the plant pathogens *Rhizoctonia solan*i (11), *Botrytis* (12) and *Monilia*, *Helminthosporium* and *Uromyces phaseoli* (9). DFMO aparently directly inhibits ODC while DFMA can be converted to DFMO by fungal arginase (12,13).

There have been no reports of the existence of ADC and AUH involved in the synthesis of putrescine in fungi

In the present study, we have re-examined the arginase, ODC and ADC activities as well as examined the susceptibilities of the dermatophytes to monofluoromethylagmatine, a suicide inhibitor of AUH.

MATERIAL AND METHODS

The fungi used in this study were maintained at 4°C in Saboraud's agar as part of the Mycolgy culture Collection at Virginia Polytechnic Institute and State University.

Microsporum canis was provided by Wiley Schell from Duke University School of Medicine Durham, North Carolina, U.S.A.

The species of *Microsporum canis* and *Trichophyton mentagrophytes* used in this study maintained in Saboraud's agar medium (pH 6.0) at 30°C in the dark (4,14).

Enzyme Assays

Fungal cultures were grown in 100 ml of Palmer's medium (without or with 500 mg arginine) at 30°C for 7-10 days. The ratio of medium volume to flask size was never greater than 1:5 in order to provide a maximal surface area for hyphal growth. Mats of fungal tissues were removed from cultures after 10 days of incubation and rinsed with cold buffer and homogenized in cold extraction buffer (100 mM HEPES, 1 mM $MgCl_2$, pH 7.4) (1 ml/g fresh weight) in a pre-chilled mortar and pestle. The homogenate was centrifuged at 10.000xg for 20 min at 4°C and the supernatant were kept on ice or frozened at –70°C and used for enzyme assays (AUH activity) (15).

Protein determination were performed using the Biorad protein reagent (Biorad Lab) and bovine serum albumin as a standard.

Growth of Inhibition Studies

0.5 cm diameter plugs were removed from the leading edge of growth of cultures grow in Palmer's agar and transferred to Palmer's agar containing various concentrations of the inhibitors (MFMAg, DFMA, DFMO).

The inhibitors were supplied by Merrell Dow Research Institute, Merrell Dow Pharmaceuticals Inc., Cincinnati, Ohio. DFMA (MDL 71.897) and DFMO (MDL 71.782A) and MFMAg (MDL 27382A) were dissolved in sterile water, filtered and sterilized solutions of the inhibitors were added to sterile Palmer's medium cooled at 48°C

and 20 ml medium poured into sterile petri dishes. Each petri dish was inoculated at the center with a plug of mycelium and incubated in the dark at 30°C. The colony diameter was measured in two dimension at 2 day intervals, each treatment involving five replicates and each experiment was repeated at least twice.

The range of inhibitors and effectors tested were stated in Table 1 with DFMO+DFMA used together, MFMAg used alone (4,14).

Table 1. Effects of MFMAg on growth of dermatophytes

		Colony diameter in cm days after inoculation*			
Species	Treatment	2d	4d	6d	8d
M.canis	None (Control)	1.25	1.74	2.35	3.05
	5 mM MFMAg	1.07	1.65	2.08	2.85
	5 mM MFMAg + 1 mM Agmatine	1.17	1.16	2.07	2.95
	1 mM Agmatine	1.25	1.60	2.15	2.97
T.mentagrophytes	None (Control)	1.49	2.20	2.90	2.90
	5 mM MFMAg	0.85	1.18	1.62	1.30
	5 mM MFMAg + 1 mM Agmatine	0.77	0.77	1.16	2.50
	1 mM Agmatine	1.40	2.15	2.70	2.85

* Averages of five replicates plugs each measured along two axes at 90° from each other.

AUH/Arginase Assays

Extracts were preincubated for 3 min at 37°C. The reaction was initiated by the addition of substrate (agmatine sulphate for AUH assay and L-arginase for arginase assay). At 1 min intervals (over 5 min) 20 μl portions of the reaction mixture were transferred to 0.1μl solution of urease buffer reagent (# 640-5; Sigma Chemical Co., St Louis) at 0°C; the AUH and arginase reaction were stopped by this treatment. The urea content in the fractions was measured by the diagnostic urea nitrogen determination procedure (#640; Sigma Chemical Co.).

For inhibition of enzyme activity by DFMA MFMAg the mixture was preincubated at 37°C for 30 min in the presence of inhibitors before adding the substrate (16). Arginase and AUH activities were assayed at various concentrations of MFMAg and DFMA to determine their (Kinetics) K_m or K_i by the method of Lineweaver-Burke (17).

Ornithine Decarboxylase / Arginine Decarboxylase

The enzymatic reaction was started by adding 175 μl of fungal extract to a test tube containing 120 μl isolation buffer and 5 μCi of either L-[1-^{14}C] ornithine hydrochloride (49.1 mCi mmol^{-1}) or 0.25 μCi of L-[^{14}C (U)] arginine hydrochloride (324 mCi mmol^{-1}). The tubes were capped with rubber stoppers fitted with plastic centre wells (Kontes Glass Company) containing 0.15 ml Protosol on a Whatman No. 1 paper wick. The reaction was allowed to proceed for 45-60 min at 32°C and terminated by injection of 0.2 ml 10% trichloracetic acid (TCA) into the reaction mixture. After an additional 15 min the centre wells removed and placed into vials in which 3 ml of toluene-PPO-POPOP scintillation mixture were added. Blank values were obtained by omitting the fungal extract from the reaction mixture. Enzyme activities were presented as pmol $^{14}CO_2$ min^{-1} mg^{-1} protein (18,19).

RESULTS

We tested the effect of DFMO+DFMA and MFMAg, on selected species of *M.canis* and *T.mentagrophytes*. By comparing growth in treated cultures relative to control cultures over a 8 day interval, a semi-quantitative assessment was made as to the growth inhibitory effects of DFMO+MFMAg or MFMAg, Table 1 summarized the effects of DFMO+DFMAg and MFMAg on *M.canis* and *T.mentagrophytes.*

It appears that the combination of DFMA and DFMO inhibitors depressed growth sharply, as compared with the control or either inhibitor used alone; cell length were much reduced and cell diameters increased. Mycelia also contained sharply, distinct septa, crinkled branches with a ribboned appearance. Only *T.mentagrophytes* is inhibited by MFMAg; the inhibition appears to be specific as agmatine eliminates any measurable growth effect.

In order to explain the susceptibility of these species to MFMAg or DFMA , cell free extracts were assayed for either ADC, AUH or arginase activity (1,2). Both species had significant ADC

activity when assayed at pH 8.2 compared to pH 7.0 (Table 2). In addition, both species exhibited AUH and arginase activities which were inducible by arginine (Table 3). The K_m of arginase for DFMA measured in extracts of *M.canis* or *T.mentagrophytes* was 61 mM and 101 mM respectively. The K_m (Kinetics) of AUH for agmatine measured in extracts of *M.canis* or *T.mentagrophytes* was 5.3 mM and 58.5 mM respectively.

Table 2. Arginine decarboxylase (ADC) activity in extracts from dermatophytes grown in Palmer's medium

	pmoles $^{14}CO_2$ min^{-1} mg^{-1}	
Treatment	pH 7.0	pH 8.2
M.canis	105	6908
T.mentagrophytes	96	1816

Table 3. Agmatine ureohydrolase (AUH) and arginase activities in extracts from dermatophytes grown in Palmer's medium

	pmoles $^{14}CO_2$ min^{-1} mg^{-1}		
Species	Supplement	AUH	Arginase
M.canis	None	35.5	38.0
	Arginine*	65.12	60.6
T.mentagrophytes	None	14.5	25.0
	Arginine*	58.5	101.0

* 500 g ml^{-1} supplement in culture

DISCUSSION

Fungal infections in animals and humans are largely as a result of opportunistic situations in which the host is predisposed either genetically or as a result of a primary infection, e.g. HIV infections. There are a limited number of antimycotic agents which are effective and have minimal unwanted side effects on the patient. Therefore, it is important to assess any mechanism of action as well as the chemotherapeutic value of any new antimycotic compaund. This is the case with the fluorinated analogues, DFMA, DFMO and MFMAg, as they are very soluble in water, do not bind to plasma proteins, and are nontoxic at maximum tolerated doses (1).

The polyamine pathway has been shown to be a potential target for antimicrobial therapy for a variety of microorganisms (1).

Although much of the work on the function and biosynthesis of polyamines has been done in *Escherichia coli,* trypanosomes and mammalian cells , polyamines have also been studied in non-phytopathogenic fungi s as *Saccharomyces cerevisiae* and in the phytopathogenic fungi (1,3,19-21). There have been no reports of the existence of ADC and AUH involved in the synthesis of putrescine in fungi. Putrescine is made in plants and bacteria through decarboxylation of arginine (ADC) and ornithine (ODC). Although in the case of the wild fungus verticullium, ADC activity was reported (22).

In addition, it has been demonstrated that the substrate analogue. DFMA inhibited the mycelia growth of four phytopathogenic fungi cultured on a defined medium. This growth inhibition was specific as it could be reversed by arginine (1,8,9). It has been reported that a number of species of *Microsporum* and *Trichophyton* are susceptible to growth inhibition by DFMA or DFMO, an inhibitor of ornithine decarboxylase (3,4,19). However, it was not clear from these studies the basis for the susceptibility to DFMA as there was no detectable ADC activity. Additionally, no arginase activity, which can convert DFMA to DFMO, was detectable.

These studies reveal both *M.canis* and *T.mentagrophytes* as synthesizing ADC and AUH and therefore possess a putrescine biosynthetic pathway characteristic of bacteria and plants (2,22). The presence of these two polyamine biosynthetic enzymes explains why these two species are susceptible to growth inhibition by DFMA or MFMAg. The inability of previous worker to detect ADC appears to be the result of measuring activity at pH 7.0 at which ADC has 18-60 fold less activity than at pH 8.2 (14,15). The K_m of AUH measured in extracts from *M.canis* was 10-fold lower than that measured from *T.mentagrophytes.* The affinity differences

can not explain the different susceptibility of these two dermatophytes to MFMAg. For example, if intracellular pool of endogenously synthesized agmatine competes with MFMAg for AUH, one would expect that AUH in *T.mentagrophytes* would bind strongly more agmatine than MFMAg relative to AUH in M.canis. It seems more likely that differences in species susceptibility are due to the rate uptake of the MFMAg into the cells. It still remains to be resolved why these two dermatophytes (*M.canis and T.mentagrophytes*) are peculiarly susceptible to either DFMO, DFMA or MFMAg when only one of the two putrescine biosynthetic pathways is inhibited. It seems appropriate that trails be initiated to determine if these inhibitors of polyamine synthesis would be useful antifungal agents in treating dermatophytes (*M.canis, T.mentagrophytes*) infections in animals. In addition the differential susceptibility of *Trichophyton* and *Microsporum* species to these inhibitors could prove to be a useful characteristic in the identification of these two genera.

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