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Enhancement of the Antifungal Activity of Rapamycin by the Coproduced Elaiophylin and Nigericin

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Streptomyces hygroscopicus ATCC 29253 produces rapamycin, elaiophylin and nigericin. Although elaiophylin has no activity against *Candida albicans* ATCC 11651, it markedly enhances rapamycin's antifungal activity. Nigericin has only weak activity on its own but it also enhances rapamycin action. Surprisingly, elaiophylin does not enhance nigericin activity on *C. albicans*.

During our studies on the biosynthesis of rapamycin and the nutrition of its producer *Streptomyces hygroscopicus*[1~6], we observed that antifungal bioassays of fermentation extracts against *Candida albicans* by the agar diffusion method did not agree quantitatively with the more accurate method of HPLC analysis. In all cases, bioassay data were higher than HPLC values. Since this strain of *S. hygroscopicus* also produces the polyketide elaiophylin[7~10] (a 16-membered macrodiolide with C2-symmetry[11,12]) and the polyether antibiotic nigericin[10], we suspected that one or both of these antibiotics were affecting the rapamycin bioassay. We expected nigericin to be the main interfering compound since it was reported to inhibit *C. albicans*[13] whereas elaiophylin, an antibacterial, does not[14,15]; we were surprised to find that both antibiotics increase the antifungal activity of rapamycin.

Materials and Methods

Agar-diffusion Assay

Candida albicans ATCC 11651, the assay microorganism, was preserved at −80°C in 30% glycerol. The GP agar assay medium consisted of (g L^{-1}) peptone 2, glucose 5 and agar 8. Fifty μl of thawed cell suspension was added to 100 ml of GP agar; 9 ml of seeded agar was used for each Petri dish. Fifteen μl of antibiotic solution (in 50% methanol) was added to each paper disc. When testing mixtures, the discs were dried between applications of the different antibiotics. The plates were incubated for 30 minutes at 4°C to allow diffusion of the antibiotics into the agar. Plates were then incubated for 16~18 hours at 37°C before measuring diameters of the resulting inhibitory zones surrounding the discs.

Liquid Assay

The frozen cell suspension of *C. albicans* was thawed and diluted 10-fold with sterile double distilled water. Twenty μl was used to inoculate each 15×150 mm test tube which contained 4 ml GP broth with and without different concentrations of the antibiotics. The compounds were sterilized by filtration through 0.2 μm Acrodiscs (Gelman Sciences, Ann Arbor, MI). The tubes were shaken at 300 rpm at 37°C and growth was measured with the Klett-Summerson colorimeter (Klett Manufacturing Co, Inc, New York, NY) using a red filter.

Results

The reported lack of activity of elaiophylin against *C. albicans*[15,16] was confirmed. Table 1 shows that elaiophylin produced no inhibitory zone on *C. albicans* plates when 15 μl of a 10 μg/ml concentration was placed

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Table 1. Agar diffusion assay of elaiophylin and rapamycin against *Candida albicans* alone and in combination.

Compound*	Zone Diameter (mm)
10 μg/ml elaiophylin	<6.5**
10 μg/ml rapamycin	17.10
10 μg/ml elaiophylin + 10 μg/ml rapamycin	18.62

*All solutions were added at 15 μl per disc.

**The diameter of the disc itself is 6.5 mm; thus <6.5 mm signifies the lack of any inhibitory zone.

Table 2. Enhancement of activity of rapamycin against *Candida albicans* in agar diffusion assays by elaiophylin.

Elaiophylin (μg/ml)	Zone Diameter (mm)	Rapamycin (μg/ml)	Zone Diameter (mm)	Elaiophylin + Rapamycin Zone Diameter (mm)	"Apparent Rapamycin" Assay (μg/ml)	Enhancement Factor (%)
		1	12.45			
		5	16.25			
		10	17.10			
		50	22.17			
		100	23.75			
1	<6.5					
5	<6.5					
10	<6.5					
50	<6.5					
100	<6.5					
1		10		17.75	12.6	26
5		10		18.70	17.3	73
10		10		18.70	17.3	73
50		10		19.73	24.3	143
100		10		20.05	31.9	219
10		1		13.37	2.88	188
10		5		17.03	9.74	95
10		10		18.70	17.3	73
10		50		22.00	53.0	0
10		100		23.75	96.0	0
50		50		23.00	74.4	49
100		100		24.47	124.	24

on a disc. On the other hand, the same concentration of rapamycin produced a sharply defined clear zone of 17.10 mm in diameter. When both antibiotics were placed on the same disc, a zone of 18.62 mm was obtained. A standard rapamycin dosage-response curve showed that the combined sample gave an "apparent rapamycin" assay of 16.8 μg/ml instead of 10 μg/ml rapamycin which was actually used, thus an enhancement of 68%.

A second experiment revealed that elaiophylin when tested alone had no activity even when tested up to 100 μg/ml, whereas increasing concentrations of rapamycin produced zones of increasing size (Table 2). Various combinations of the two antibiotics showed marked enhancements as high as 219% when rapamycin was combined with high concentrations of elaiophylin. The enhancement decreased to an undetectable level when the rapamycin/elaiophylin ratio was increased.

The activity of nigericin was weak against *C. albicans*

Table 3. Enhancement of activity of rapamycin against *Candida albicans* in agar diffusion assays by nigericin.

Nigericin (μg/ml)	Nigericin Zone Diameter (mm)	Rapamycin (μg/ml)	Rapamycin+Nigericin Zone Diameter (mm)	"Apparent Rapamycin" Assay (μg/ml)	Enhancement Factor (%)
1.56	<6.5				
3.13	<6.5				
6.25	<6.5				
12.5	<6.5				
25	<6.5				
50	8.0				
100	9.8				
0		10	19.3	11.3	--
1.56		10	19.9	15.5	37
3.13		10	20.1	16.5	46
6.25		10	20.3	19.0	68
12.5		10	20.6	20.0	77
25		10	21.2	25.0	121
50		10	21.6	30.0	135
100		10	22.5	42.0	272

Table 4. Lack of enhancement of activity of nigericin by elaiophylin against *Candida albicans* in agar diffusion assays.

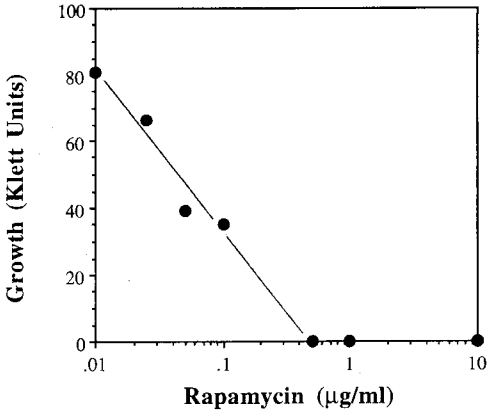
Elaiophylin (μg/ml)	Nigericin (μg/ml)	Elaiophylin+Nigericin Zone Diameter (mm)
	1.56	<6.5
	3.13	<6.5
	6.25	<6.5
	12.5	<6.5
	25	<6.5
	50	8.0
	100	9.8
50	0	<6.5
50	1.56	<6.5
50	3.13	<6.5
50	6.25	<6.5
50	12.5	<6.5
50	25	<6.5
50	50	8.5
50	100	9.4

(Table 3). When 15 μl of a 100 μg/ml solution was used, a fuzzy zone of 9 to 10 mm in diameter was observed. A concentration of 50 μg/ml yielded a zone of <6.5 to 8 mm and lower concentrations (25 μg/ml and below) produced no zones at all. Combining nigericin with rapamycin increased the zone size even at concentrations of nigericin which produced no zones, *i.e.*, 1.56 to 25 μg/ml.

Since elaiophylin enhanced the antifungal activity of rapamycin, we wondered whether it would do the same for nigericin. Surprisingly, this was not the case (Table 4).

Due to the unexpected enhancement of rapamycin's antifungal activity by the antibacterial elaiophylin, we decided to confirm this effect by studying the interaction in liquid culture. We found that elaiophylin failed to inhibit growth of *C. albicans* even at 10 μg/ml. On the other hand, rapamycin decreased the rate of growth at levels as low as 0.02 μg/ml (Fig. 1). Minimal inhibitory concentrations of rapamycin were 0.01 μg/ml in a 24 hour test and 0.1 μg/ml in an 8 day test (Table 5). Addition of elaiophylin reduced the MIC by as much as 50% when added at 0.1 μg/ml and by 50 to 66% when added at 1 μg/ml. Thus the enhancement of activity observed above in agar-diffusion assays could also be seen in liquid culture.

Fig. 1. Inhibition of growth of *Candida albicans* by rapamycin in liquid medium. Growth measured at 4 days.



Discussion

Elaiophylin has the following activities: antibacterial, antihelmintic[16], antitumor, immunosuppression[17] and inhibition of nitric oxide synthesis[15]. However, it does not inhibit Gram-negative bacteria or fungi, including yeasts. Its mode of action is unknown. It is quite interesting that this molecule which has immunosuppresive activity is coproduced with rapamycin, another immunosuppressive agent, and enhances the activity of rapamycin against *C. albicans*. The mode of action of rapamycin in yeast is *via* the inhibition of TOR2 (a phosphatidyl-3-kinase essential for progression from the F1 phase into the S phase of the cell cycle) by rapamycin complexed to its immunophilin FKBP[18]. The means by which elaiophylin enhances rapamycin's activity is unknown.

Nigericin inhibits coccidia, viruses and Gram-positive bacteria and, at higher concentrations, Gram-negative bacteria and fungi (including yeast)[19,20]. Being a polyether, it has ionophoric activity which allows formation of

Table 5. Lowering the MIC of rapamycin against *Candida albicans* in liquid assays by addition of elaiophylin.

Day	Minimum Inhibitory Concentration (MIC) (μg/ml)		
	Rapamycin alone	Rapamycin + elaiophylin at 0.1 μg/ml	Rapamycin + elaiophylin at 1 μg/ml
1	0.010	0.010	0.005
2	0.025	0.025	0.010
3	0.050	0.025	0.025
4	0.050	0.050	0.025
5	0.075	0.050	0.025
6	0.100	0.050	0.050
7	0.100	0.075	0.050
8	0.100	0.075	0.050

lipophilic complexes with monovalent or divalent cations, resulting in a passive, electroneutral cation-proton exchange across cell membranes[20]. It appears to kill bacteria by acidifying the cytoplasm[21]. Its mechanism of action against yeast is not known but loss of K^+ ions is a possibility. How it enhances rapamycin antifungal activity is unclear as is the reason that elaiophylin enhances rapamycin action but not nigericin activity against *C. albicans*.

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