Antimicrobial Activity of Viridiofungins

J. C. ONISHI, J. A. MILLIGAN, A. BASILIO, J. BERGSTROM, J. CUROTTO, L. HUANG, M. MEINZ, M. NALLIN-OMSTEAD, F. PELAEZ, D. REW, M. SALVATORE, J. THOMPSON, F. VICENTE and M. B. KURTZ

Merck Research Laboratories, R80Y-225, P.O. Box 2000, Rahway, NJ, 07065-0900, U.S.A.

(Received for publication October 7, 1996)

A family of aminoacyl alkyl citrate compounds called viridiofungins, are novel squalene synthase inhibitors. The compounds have broad spectrum fungicidal activity but lack antibacterial activity. Although the compounds inhibit squalene synthase, the first committed step in ergosterol biosynthesis, results presented in this paper show that inhibition of fungal growth is not related to inhibition of ergosterol synthesis.

The preceding paper described the isolation and structure elucidation of novel amino alkyl citrate compounds called viridiofungins[1]. The compounds which inhibit squalene synthase have broad spectrum antifungal activity but lack antibacterial activity. This paper describes the results showing that the antifungal mode of action of these compounds is unrelated to inhibition of ergosterol synthesis despite the fact that viridiofungins inhibit squalene synthase.

Methods

Fermentation

Culture MF 5628 (*Trichoderma viride*) produced viridiofungin. The compound was produced in a two stage fermentation process consisting of growth in seed medium A[2] followed by subsequent product fermentation in appropriate fermentation media. Frozen vegetative mycelia (FVM) of the culture were prepared and used to inoculate flasks of seed medium A. Substantial vegetative fungal growth was obtained from seed flasks after 45~48 hours incubation at 25°C on gyratory shaker (220 rpm). Production flasks were inoculated by aseptic transfer of 2 mls of seed growth and were incubated at 25°C in a medium consisting of yellow cornmeal, 50.0 g/liter; yeast extract, 1.0 g/liter; and sucrose, 80.0 g/liter; dispensed 45 ml/nonbaffled 250-ml Erlenmeyer flask. Flasks were agitated at 220 rpm on a gyratory shaker. Production flasks were harvested after 7 days by MEK extraction of the mycelial growth. Content of viridiofungin in the MEK extracts was determined by HPLC analysis and shown to be 450 μg/ml.

Antimicrobial Activity

Antifungal activity of viridiofungins were determined using microdilution methods with standard U-bottom 96-well plates. Exponential phase yeast cells suspended in Difco yeast nitrogen base supplemented with 0.5% glucose (SM) to 1×10^4 cell per ml were added to microtiter dishes containing 2-fold dilutions of compound. *Aspergillus* spores harvested from a well-sporulated slant in 0.4% Tween 80 and diluted into SM at 1×10^3 spore/ml were added to inhibitor-containing microtiter dishes. After 20 hours at 29°C, the microtiter dishes were examined to determine the lowest concentration necessary to prevent visible growth, the minimal inhibitory concentration (MIC). Aliquots of 0.1 ml were diluted and plated to determine the effect of the compound on the viability of the cells. The lowest concentration to reduce the number of viable cells by 95% compared to the inoculum was defined as the minimum fungicidal concentration (MFC). The effect of the compound on bacteria was assessed using agar diffusion assays[3,4].

Squalene Synthase Assay

Squalene synthase assays were performed using microsomes prepared from *Saccharomyces cerevisiae* MY 1976 and *Candida albicans* MY 1055 cells. The enzyme was assayed using a procedure modified from that of BERGSTROM *et al.*[2] Assay mixtures consisted of 150 mM HEPES, 11 mM KF, 3 mM dithiothreitol, 5.5 mM $MgCl_2$, 0.1 μg per ml of the squalene epoxidase inhibitor terbinafine and microsomes at 1.2 to 2.5 μg protein per ml. After 10 minutes preincubation at room temperature, the enzyme reaction was initiated with 3 μM of ^{3}H-farnesylpyrophosphate and 1 mM of NADPH. The assay mixtures were incubated for 20 minutes at 30°C. The reactions were stopped by the addition of 100 μl of 95% EtOH, vortexed, and 100 μl of a suspension of 1 g per ml of Bio-Rad AG 1X8 resin (400 mesh, Chloride form) was then added, and mixed. 800 μl of heptane was added to each tube strip and the strips were capped and vortexed

for 10 minutes. 400 μl of heptane layer was then removed into a minivial and mixed with 2.5 ml of scintillation fluid and the radioactivity was determined by liquid scintillation counting. IC_{50} values were determined by plotting the log of the concentration of the test compound versus the percentage inhibition. The IC_{50} is the concentration of inhibitor that gave 50% inhibition as determined from these plots.

Effect of Viridiofungin A on Fungal Macromolecular Synthesis

The effect of viridiofungins on macromolecular synthesis was evaluated using whole cell labeling methods. The MIC of the compounds under the growth conditions used to radiolabel the macromolecules was determined. The organisms were grown to exponential phase in SM supplemented with 1 μM adenine and leucine and adjusted to 1×10^7 colony forming units/ml. The effect of viridiofungins on fungal sterol synthesis was determined by adding the compound to exponential phase cells 10 minutes prior to the addition of [1-^{14}C]-acetate (0.4 μCi/ml). After 30 minutes, labeling was terminated and the fungal cell wall permeabilized prior to lipid extraction by heating the cells in 0.5 N HCl for 30 minutes at 80°C[5]. Cell pellets were washed with saline and resuspended in 2 ml of 5% KOH in methanol. The cell pellet was saponified for 18 hours at room temperature. After the addition of 0.5 volume water, the nonsaponifiable lipids (NSL) were recovered by extracting the alkaline sample with petroleum ether. The NSL lipid extract contained squalene, ergosterol and lanosterol. Fatty acids were recovered by acidifying the remaining alkaline layer and extracting with petroleum ether. The extracts were dried under vacuum, dissolved in chloroform-methanol and counted. The lipid components of both extracts were separated by thin layer chromatography on silica gel plates developed in petroleum ether - diethyl ether - glacial acetic acid (70 : 30 : 1). Labeled lipid components were detected by autoradiography and identified by comparing the mobility to standards. The effect of the compound on RNA and protein synthesis was evaluated by preincubating the cells with viridiofungin A for 10 minutes followed by the addition of 0.4 μCi/ml of [8-^{14}C]-adenine or 4 μCi/ml [^{3}H]-leucine. The amount of adenine and leucine incorporated into RNA and protein, respectively, was determined after 30 minutes by adding one volume of 10% trichloroacetic acid to labeled cells and collecting the TCA insoluble pellet on glass fiber filters (Whatman GF/A). The labeling condition used in these experiments did not result in detectable adenine incorporation into DNA. Incorporation of adenine into DNA was determined using the methods of HENRY[6].

Results

Effect of Viridiofungin on Squalene Synthase

The amino alkyl citrates are inhibitors of squalene synthase, but weak inhibitors compared to the the bicyclic citrate based compounds, zaragozic acids and squalestatin[2,7]. The latter compounds were pM inhibitors while results presented in Table 1 show that the viridiofungins inhibited squalene synthase in the μM concentrations. The compounds varied in potency over a 30-fold range against the *S. cerevisiase* squalene synthase. The tryptophan analogue, viridiofungin C, was 35-fold more active compared to the tyrosine analogue, viridiofungin A, and 5-fold more active compared to the phenylalanine analogue, viridiofungin B. In contrast, the compounds were equipotent against *C. albicans* squalene synthase.

Antimicrobial Activity of Viridiofungin A, B and C

In standard agar diffusion assays used to determine the breadth of spectrum of the amino alkyl citirates, viridiofungins did not inhibit the growth of either Gram positive or negative bacteria (data not shown). The compounds did, however, have broad spectrum antifungal activity. The most active compound in the series, viridiofungin A, had MICs against yeast and filamentous fungi ranging from less than 1 μg/ml to greater than 32 μg/ml as shown in Table 2. At concentrations comparable to the MIC, viridiofungin A had fungicidal activity against the yeast *C. albicans* MY 1055. There was no correlation between the anti-*Saccharomyces* activity of the viridiofungins and the potency as squalene synthase inhibitors. The anti-*Saccharomyces* activity of the most potent *Saccharomyces* squalene synthase inhibitor, viridiofungin C, was not significantly greater compared to the weakest squalene synthase inhibitor, viridiofungin A. Viridiofungin B had the weakest antifungal activity. Similarly, the anti-*Candida* activity of

Table 1. Effect of viridiofungin A, B and C on squalene synthase activity.

Compound	Inhibition of squalene synthase (IC_{50} μM)	
	S. cerevisiae MY 1976	*C. albicans* MY 1055
Zaragozic acid A	0.0001	0.00017
Viridiofungin A	12.0	11.8
Viridiofungin B	1.7	11.3
Viridiofungin C	0.35	11.4

Table 2. Antifungal activity of viridiofungin A, B and C.

Species	Merck culture number	Minimum inhibitory concentration (μg/ml)		
		Viridiofungin A	Viridiofungin B	Viridiofungin C
Candida albicans	MY 1028	32.0	>32.0	32.0
Candida albicans	MY 1055	4.0	>32.0	8.0
Candida albicans	MY 1750	32.0	>32.0	32.0
Candida guilliermondii	MY 1019	4.0	>32.0	16.0
Candida parapsilosis	MY 1010	2.0	32.0	8.0
Candida pseudotropicalis	MY 2099	2.0	16.0	8.0
Candida tropicalis	MY 1012	32.0	>32.0	16.0
Cryptococcus neoformans	MY 2061	0.25	1.0	2.0
Cryptococcus neoformans	MY 2062	2.0	2.0	4.0
Saccharomyces cerevisiae	MY 1976	16.0	>32.0	32.0
Aspergillus flavus	MF 0383	2.0	8.0	4.0
Aspergillus fumigatus	MF 4839	16.0	>32.0	16.0
Aspergillus fumigatus	MF 5668	32.0	>32.0	32.0
Aspergillus fumigatus	MF 5669	16.0	>32.0	32.0

Table 3. Effect of viridiofungin A, B and C on growth and lipid synthesis in *C. albicans* MY 1055.

Compound	Inhibitor concentration (μg/ml)	MIC units	Label incorporation (% control)	
			NSL	Fatty acid
Zaragozic acid A	16.0	4.0	26.1	70.7
	4.0	1.0	25.5	82.2
	1.0	0.25	86.4	121.7
	0.25	0.0625	100.1	118.1
Viridiofungin A	100.0	1.0	55.5	59.5
	25.0	0.25	82.4	76.2
	6.25	0.0625	80.8	71.9
Viridiofungin B	100.0	Not active	64.9	74.3
	25.0		67.4	69.1
	6.25		82.1	90.0
Viridiofungin C	100.0	1.0	7.2	7.5
	25.0	0.25	42.8	37.8
	6.25	0.0625	95.2	93.5

viridiofungin A, B and C did not correlate with the compounds' potency as squalene synthase inhibitors.

Effect of Viridiofungin on Macromolecular Synthesis

In order to determine whether the antifungal activity of the viridiofungins was accounted for by inhibition of squalene synthase with resulting decreases in ergosterol, the effect of an MIC level of the compounds on cellular ergosterol biosynthesis was assessed in *C. albicans* MY 1055. Results presented in Table 3 show that the pM squalene synthase inhibitor, zaragozic acid A, reduced the incorporation of labeled acetate into the nonsaponifiable lipid extract by more than 75% without significant effect on the incorporation of label into the fatty acid containing extract. In contrast, the viridiofungins, at the MIC, did not specifically reduce the incorporation of labeled acetate into the nonsaponifiable lipid extract. The most potent squalene synthase inhibitor in the viridiofungin class, viridiofungin C, reduced the incorporation of acetate into NSL and fatty acid containing lipid extracts by more than 90%. The most active antifungal in the series, viridiofungin A, also reduced acetate incorporation into both lipid extracts by 50%. There was no evidence that viridiofungin A at the MIC or at subinhibitory concentrations preferentially reduced ergosterol synthesis compared to fatty acid synthesis. The labeled lipids detected in the nonsaponifiable lipid extract following viridiofungin treatment were normal sterols, ergosterol and lanosterol, as well as squalene (data not shown).

The effect of viridiofungin on macromolecular syn-

Table 4. Effect of antifungal agents on macromolecular synthesis in *Ustilago zeae* MF 1996.

Antifungal agent	(μg/ml)	MIC units	Macromolecular synthesis label incorporation (% control)			
			Lipid		Protein	RNA
			NSL	Fatty acid		
Viridiofungin A	20.0	25	131.9	116.9	100.6	74.1
	4.0	5	141.6	127.5	121.2	124.3
	0.8	1	115.6	130.3	115.6	109.6
	0.16	0.2	138.9	122.7	120.9	105.9
Zaragozic acid A	20.0	5	22.0	129.6	88.4	92.8
	4.0	1	55.2	110.5	86.2	97.9
	0.8	0.2	112.8	122.6	85.7	111.6
Amphotericin B	20.0	25	0.5	0.1	0.1	0.0
	4.0	5	0.7	0.2	0.1	0.1
	0.8	1	1.9	0.9	0.1	0.1
	0.16	0.2	13.9	11.5	0.2	0.13

thesis was evaluated in *Ustilago zeae*, an organism 100-fold more sensitive to the compound compared to *Candida*. Following treatment with concentrations of viridiofungin A that affect growth, the cells were labeled with [^{14}C]-acetate, [^{14}C]-adenine and [^{3}H-]-leucine to determine whether the compound inhibited lipid, RNA or protein synthesis. In agreement with the results described above, results presented in Table 4 show that viridiofungin A did not reduce acetate incorporation into the lipid extracts of *U. zeae*. Since the nonsaponifiable lipid extract did not contain different labeled lipids compared to the control, it was concluded that viridiofungin A did not affect ergosterol synthesis. Results also presented in Table 4 show that concentrations of viridiofungin A more than 20-times higher than the MIC did not reduce the incorporation of labeled adenine or leucine into RNA or protein.

Discussion

Viridiofungins, a novel class of amino alkyl citrate compounds, are broad spectrum, fungicidal antifungal agents which lack antibacterial activity. The compounds do inhibit squalene synthase but are 4 orders of magnitude less active compared to the zaragozic acids. The antifungal activity of viridiofungin, however, is not accounted for by a reduction in ergosterol synthesis *via* inhibition of squalene synthase. This conclusion is based on the fact that there is no correlation between the potency of the compounds as inhibitors of squalene synthase and antifungal activity. Additionally, at concentrations sufficient to inhibit the growth of *Candida albicans* and *Ustilago zeae*, specific inhibition of ergosterol synthesis, as measured by labeling with [^{14}C]-acetate, is not observed. Thus, despite the highly anionic nature of the compound, viridiofungin does not directly alter the function of the membrane. This conclusion is based on results showing that viridiofungin A does not reduce the incorporation of label nonspecifically into macromolecules even when *Ustilago* cells are exposed to a level of compound 25-times higher than the MIC. In this type of analysis a membrane active antifungal agent, such as amphotericin B, nonspecifically reduces label incorporation into macromolecules. Additionally, the lack of inhibition of incorporation of acetate, adenine and leucine into the lipids, nucleic acid and protein, respectively, argues that viridiofungin A is not a general metabolic poison. Results presented by MANDALA *et al.*[8] show that the mechanism of action of these novel, fungicidal natural products are due to effects on sphingolipid metabolism.

References

1) HARRIS, G.; E. T. JONES, M. M. MEINZ, M. NALLIN-OMSTEAD, G. L. HELMS, G. F. BILLS, D. ZINK & K. E. WILSON: Isolation and structure elucidation of viridiofungins A, B and C. Tetra. Lett. 34: 5235~5238, 1993
2) BERGSTROM, J. D.; M. M. KURTZ, D. J. REW, A. M. AMEND, J. D. KARKAS, R. G. BOSTEDOR, V. S. BANSAL, D. DUFRESNE, F. L. VANMIDDLESWORTH, O. D. HENSENS, J. M. LIESCH, D. L. ZINK, K. E. WILSON, J. ONISHI, J. A. MILLIGAN, G. BILLS, L. KAPLAN, M. NALLIN OMSTEAD, R. G. JENKINS, L. HUANG, M. S. MEINZ, L. QUINN, R. W. BURG, Y. L. KONG, S. MOCHALES, M. MOJENA, I. MARTIN, F. PELAEZ, M. T. DIEZ & A. W. ALBERTS: Zaragozic acids: A family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. Proc. Natl. Acad. Sci. U.S.A. 90: 80~84, 1993
3) KAVANAGH, F.: Analytical microbiology. Vol. I, pp. 1~85, Academic Press, 1963
4) KAVANAGH, F.: Analytical microbiology. Vol. II, pp. 1~145, Academic Press, 1963
5) THOMPSON, E. D. & L. W. PARKS: Effect of altered sterol composition on growth characteristics of *Saccharomyces*

cerevisiae. J. Bacteriol. 120: 779～784, 1974

6) HENRY, S. A.; K. D. ATKINSON, A. I. KOLAT & M. R. CULBERTSON: Growth and metabolism of inositol-starved *Saccharomyces cerevisiae*. J. Bacteriol. 130: 472～484, 1977

7) BAXTER, A.; B. J. FITZERALD, J. M. HUTSON, A. D. MCCARTHY, J. M. MOTTERAM, B. C. ROSS, M. SAPRA, M. A. SNOWDEN, N. S. WATSON & R. J. WILLIAMS: Squalestatin 1, a potent inhibitor of squalene synthase, which lowers serum cholesterol *in vivo*. J. Biol. Chem. 267: 11705～11708, 1992

8) MANDALA, S.; R. A. THORNTON, B. R. FROMMER, S. DREIKORN & M. B. KURTZ: Viridiofungin, novel inhibitors of sphingolipid synthesis. J. Antibiotics 50: 339～343, 1997