THE MODE OF ANTIFUNGAL ACTION OF (*S*)2-AMINO-4-OXO-5-HYDROXYPENTANOIC ACID, RI-331

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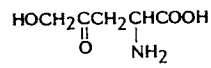
An antifungal amino acid antibiotic, (*S*)2-amino-4-oxo-5-hydroxypentanoic acid (RI-331) isolated from *Streptomyces* sp., inhibited the biosynthesis of protein to a greater extent than that of RNA or DNA in growing *Saccharomyces cerevisiae* cells. Polypeptide biosynthesis in a cell-free system from the yeast was refractory to the antibiotic, suggesting the possibility that the biosynthesis of one or more amino acids might be inhibited. Intracellular amino acid pools, particularly those of methionine, isoleucine and threonine were significantly reduced when yeast cells were incubated in the presence of RI-331. Consistent with this, the growth-inhibitory activity of RI-331 was markedly reversed by the addition of these amino acids into the growth medium, and an even greater effect was exerted by homoserine which works as a common metabolic precursor for these amino acids in yeasts. It looks likely therefore that the inhibition of biosyntheses of some or all of these amino acids by RI-331 is primarily responsible for overall inhibition of protein biosynthesis in yeasts, ultimately leading to cytostasis. This possible mechanism of RI-331 action appears to explain favorably the selective toxicity of the antibiotic against yeasts, since mammalians lack enzymatic systems for synthesizing methionine, isoleucine and threonine which are required as essential amino acids for growth.

In previous papers[1,2] we reported some antifungal properties of an amino acid antibiotic, (*S*)2-amino-4-oxo-5-hydroxypentanoic acid coded as RI-331 (Fig. 1) which was isolated from a *Streptomyces* sp. under our screening program for new antifungal agents. This antibiotic is active against *Candida albicans*, *Cryptococcus neoformans* and other yeasts, and highly tolerated by experimental animals.

In an attempt to understand the selective toxicity of the antibiotic toward such fungi, we studied the mechanism of its antifungal action using the susceptible yeast *Saccharomyces cerevisiae* as the test organism.

Fig. 1. Chemical structure of RI-331.



Materials and Methods

Yeast Strains, Media and Cultivation

S. cerevisiae D273-11A (*adel hisl*) was used throughout the present studies. Cultures were grown aerobically at 27°C in Difco yeast nitrogen base supplemented with glucose 2% and adenine sulfate 0.02% (YNB-GA) or in Difco yeast nitrogen base without amino acids (YNB w/o AA) supplemented with glucose 2%, histidine 100 μg/ml and adenine sulfate 0.02% (YNB w/o AA-GAH).

Growth Studies

A 24-hour culture was diluted with fresh medium to give a cell suspension of approx 10^4 cells/ml, incubated until the OD at 550 nm (OD_{550}) reached 0.2, and then aliquots (10 ml) were dispensed into

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sterile tubes containing graded concentrations of RI-331 with a volume of 0.1 ml. The incubation was maintained at 27°C for up to 18 hours on a shaker. Samples were withdrawn from each tube to determine OD_{550} and cfu. The cfu were counted by plating dilutions of culture samples on PYG (Polypepton 1%, yeast extract 0.5% and glucose 2%) agar plates. After 48 hours incubation at 27°C, numbers of growing colonies were counted.

When the inhibition-reversal effect of amino acids was tested, test tubes containing 1 ml-aliquots of YNB w/o AA-GHA received graded concentrations of RI-331, along with an appropriate concentration of the amino acid under test. All tubes were inoculated with a small volume of *S. cerevisiae* culture to give a final cell density of 10^3 cells/ml. They were incubated at 27°C on a shaker and, after 48 hours incubation, OD_{550} was measured spectrophotometrically to determine IC_{50}.

Incorporation Studies with Growing Cells

Cultures of yeast cells were grown at 27°C in YNB-GA overnight, diluted to 50-fold with fresh medium, and allowed to regrow until OD_{550} of the culture reached the value of 0.2. Then cells were harvested by centrifugation, washed once with Difco YNB supplemented with 2% glucose (YNB-G) and resuspended in the original volume of fresh medium. Ten-ml aliquots of the experimental cell suspension thus prepared were dispensed into tubes which received the indicated concentration of RI-331, along with [^{3}H]adenine (for the study of RNA and DNA biosyntheses) or [^{14}C]glutamine (Gln) or [^{14}C]asparagine (Asn) (for protein biosynthesis). All the tubes were incubated at 27°C and, after 30 minutes, samples were withdrawn for fractionation and subsequent radioactive assay. Samples taken from an incubation mixture containing 1 μCi/ml of [^{3}H]adenine were mixed with an equal volume of ice-cold 10% TCA, and DNA and RNA were fractionated according to the method as described previously[3].

The rate of protein biosynthesis was determined by measuring the incorporation of labeled amino acids. When the yeast cell suspension grown in YNB-AG reached OD_{550} 0.2, 0.1 μCi/ml of [^{14}C]Gln or [^{14}C]Asn was introduced into the culture. Samples (0.5 ml) were withdrawn, mixed with TCA to a final concentration of 5%, and then heated at 90°C. The resulting TCA insoluble materials were collected on glass fiber (Whatman GF/C) discs, washed three times with cold 5% TCA and finally once with ethanol.

The radioactivity of the specimens was determined by a liquid-scintillation counter.

Protein Biosynthesis in a Yeast Cell-free System

The cytosol fraction and ribosomes were prepared from mechanically disrupted yeast cells by centrifugal fractionation as the $105{,}000 \times g$ supernatant (S105) and the $105{,}000 \times g$ precipitate (P105), respectively.

The reaction mixture contained, in a total volume of 0.1 ml, the following constituents: Tris-acetic acid 50 mM, pH 7.0; NH_4Cl 100 mM; $Mg(CH_3COO)_2$ 10 mM; ATP 3 mM; GTP 1 mM; phosphoenolpyruvate (PEP) 5 mM; 100 μg/ml pyruvate kinase; 100 μg/ml bakers' yeast tRNA; amino acids mixture 0.05 mM lacking leucine; [*U*-^{3}H]leucine (Leu) 1 μCi/ml; 2.0 A_{260} U of P105 and 100 μg protein of S105. The incubation was carried out at 30°C for 30 minutes, terminated by the addition of 1 ml of 5% TCA and heated at 90°C for 20 minutes, before the resulting TCA-insoluble material was trapped on GF/C disc and counted in a liquid scintillation counter.

Analysis of Cellular Amino Acid Pools

S. cerevisiae cells suspended in 100 ml of YNB w/o AA-GHA (OD_{550} 0.1) were incubated with or without RI-331 (15 μg/ml) for 1 hour, harvested by centrifugation, resuspended in cold 5% TCA, and kept for 2 hours in an ice bath. After centrifugation the supernatant was removed, TCA was extracted with ether, and the resulting aqueous solution was subjected to hydrolysis with 6 N HCl. Amino acid analysis of samples taken before and after hydrolysis were performed by HPLC using an Hitachi type 835 autoanalyzer. RI-331 was also quantitatively detected by this method.

Amino Acid Analysis of Cellular Proteins

The cellular protein fraction removed as hot TCA insoluble material after treating cells at 90°C for 20 minutes was hydrolyzed with 6 N HCl and subjected to amino acid autoanalysis.

Chemicals

YNB and YNB w/o AA were purchased from Difco Laboratories, Detroit, Michigan. ATP, GTP,

PEP, pyruvate kinase and yeast tRNA were from Sigma Chemical Co., Ltd., St. Louis, MO. [*U*-^{14}C]Asn (40.2 mCi/mmol), [*U*-^{3}H]Leu (127 Ci/mmol), [*U*-^{14}C]Gln (41.3 mCi/mmol) and [2-^{3}H]adenine (23 Ci/mmol) were from Amersham Japan, Tokyo.

Results

Effect on Growth

The effect of RI-331 on growth of *S. cerevisiae* in YNB-GA was determined turbidimetrically (Fig. 2) and on the basis of viability (Fig. 3). At concentrations above 15 μg/ml, the antibiotic almost completely inhibited the yeast growth within 2 hours as monitored by optical density measurement. However, no significant loss in viable counts was observed at even higher drug concentrations during the 18 hours-experimental period. These data suggest that RI-331 exerts essentially a static action toward the susceptible *S. cerevisiae* strain.

Effect on the Biosyntheses of Protein, RNA and DNA

The effect of RI-331 on the biosyntheses of macromolecules by growing cells of *S. cerevisiae* was explored by determination of the radioactivity taken up from the medium containing the specific precursors. As given in Table 1, RI-331 preferentially inhibited protein biosynthesis in yeast cells. In the presence of 150 μg/ml of RI-331, incorporation of [^{14}C]Asn or [^{14}C]Gln into protein was inhibited by 90% or more, while incorporation of [^{14}C]adenine into RNA or DNA was inhibited by approx 30 to 40%. In order to examine whether RI-331 affects the protein-synthesizing machinery itself, the effect of the antibiotic on cell-free preparations from *S. cerevisiae* capable of synthesizing polypeptides in response to native messengers was studied. The incorporation of [^{3}H]Leu into peptides by this cell-free system was almost completely inhibited by 100 μg/ml of cycloheximide which is known to be a potent and selective inhibitor of protein synthesis in eukaryotes, whereas it was scarcely affected by RI-331 at concentrations up to 1,500 μg/ml (data not shown).

Effect on Intracellular Amino Acid Pools

The free amino acid fraction was prepared from

Fig. 2. Growth curve of *Saccharomyces cerevisiae* cultures in the absence or presence of RI-331.

● Control, △ 15 μg/ml, □ 150 μg/ml.

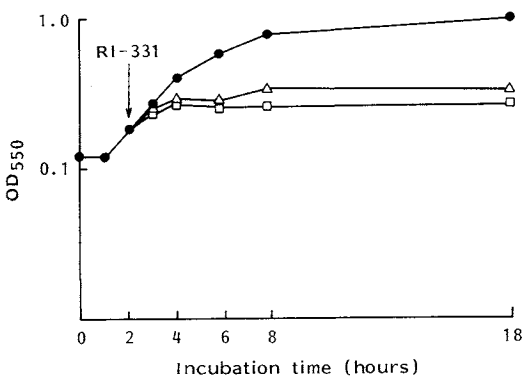
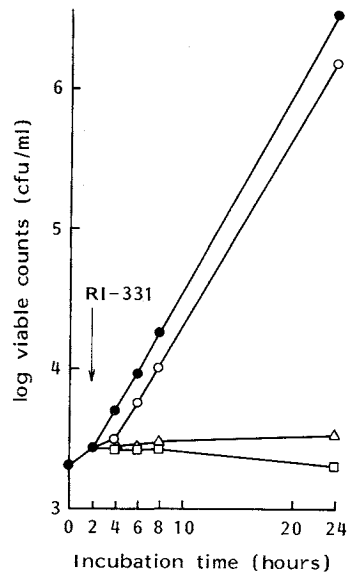


Fig. 3. Time-course of viable counts of *Saccharomyces cerevisiae* cultures exposed to varying concentrations of RI-331.

● Control, ○ 1.5 μg/ml, △ 15 μg/ml, □ 150 μg/ml.



S. cerevisiae cells previously incubated with and without RI-331 (15 μg/ml) and analyzed chromatographically for the relative amounts of each amino acid. When the level of each amino acid in the intracellular pool of control cells was compared with that of RI-331-treated cells, it was found that the antibiotic produced a remarkable change in the size and composition of the amino acid pool. As shown in Table 2, it was characterized by a marked decrease in the level of the aspartate family amino acids (threonine, methionine and isoleucine) that was accompanied by a lesser decrease in the level of histidine and proline, and an increase in the levels of alanine, valine, leucine, phenylalanine, tyrosine, serine and aspartate. These results suggest that the pools of aspartate family amino acids are preferentially exhausted after exposure of cells to RI-331.

Uptake and Intracellular Distribution of RI-331

RI-331 added to the medium appeared to be taken up by growing cells at relatively high rates and to remain unchanged in the cells. Under the present experimental conditions, where a cell suspension of *S. cerevisiae* was exposed to RI-331 at a concentration of 15 μg/ml for 1 hour, and RI-331 was recovered from the cold TCA-soluble fraction of the cells, the intracellular level of the unchanged RI-331 was as high as 40 μM (6 μg/ml) when calculated on the basis of cell volume. On the other hand, this antibiotic was not detectable in the protein fraction from the cells (data not shown).

Reversal by Amino Acids

The question arises whether the exhaustion of some amino acid(s) in the intracellular pool, induced by RI-331, is responsible for its antifungal activity. To answer the question, different amino acids were examined for their antagonistic effect on the growth inhibitory activity of RI-331 toward *S. cerevisiae*. The results obtained from the reversal experiment, which was performed in a chemically defined medium supplemented with 200 μg/ml of

Table 1. Effect of RI-331 on protein, RNA and DNA syntheses in growing *Saccharomyces cerevisiae* cells measured by the radioactivity of [^{14}C]Asn or [^{14}C]Gln taken up into protein, and [^{3}H]adenine into RNA and DNA.

RI-331 (μg/ml)	[^{14}C]Asn	[^{14}C]Gln
0	17,264 (100)	16,773 (100)
15	5,796 (34)	5,282 (32)
150	1,407 (8)	1,032 (6)

RI-331 (μg/ml)	Radioactivity (dpm) of [^{3}H]adenine taken up into	
	RNA	DNA
0	10,457 (100)	3,543 (100)
15	9,775 (93)	2,930 (83)
150	7,014 (67)	1,956 (55)

The number in the brackets represent % incorporation.

Table 2. The change of composition of amino acid pools induced by RI-331 in *Saccharomyces cerevisiae* cells.

Amino acid	Control (C)	+RI-331 (R)	R/C	Amino acid	Control (C)	+RI-331 (R)	R/C
Gly	1.499	1.139	0.76	Pro	1.075	0.381	0.35
Ala	5.665	12.237	2.16	His	65.274	33.158	0.51
Val	1.153	12.949	11.23	Ser	1.619	14.804	9.14
Leu	0.207	0.597	2.88	Thr	11.217	1.257	0.11
Ile	0.315	0.015	0.05	Glu	52.523	8.552	0.16
Met	0.064	0.007	0.11	Asp	4.579	11.758	2.57
Cys	0.025	0.017	0.68	Lys	2.885	3.604	1.25
Phe	0.096	0.151	1.57	Arg	42.149	40.592	0.96
Tyr	0.088	0.939	10.67				

The values of (C) and (R) represent the amounts of intracellular amino acids expressed as nmol 2×10^8 cells without and with RI-331 (15 μg/ml)-treatment, respectively.

Table 3. The antagonistic effect of several amino acids on the growth inhibitory activity of RI-331 against *Saccharomyces cerevisiae*.

Amino acid added	IC_{50} of RI-331 (μg/ml)	Degree of reversion	Amino acid added	IC_{50} of RI-331 (μg/ml)	Degree of reversion
None (control)	1.7	1.0	Ser	4.8	2.8
Gly	2.5	1.5	Thr	9.9	5.8
Ala	2.4	1.4	Homoserine	50.0	29.4
Val	2.9	1.7	Asp	2.0	1.2
Leu	2.1	1.2	Asn	6.6	3.9
Ile	6.3	3.7	Glu	2.4	1.4
Met	12.5	7.4	Gln	3.7	2.2
Phe	2.8	1.6	Arg	1.9	1.1
Pro	2.0	1.2	Lys	1.9	1.1

The value of IC_{50} of RI-331 was determined on the basis of the optical density of cultures.

testing antagonist, are shown in Table 3. The antagonistic activity was expressed by the increase of the value of IC_{50} of RI-331 as compared with that measured without addition of antagonist. Among 18 amino acids or metabolites tested, homoserine was most potently antagonistic; it induced a 29-fold increase in the IC_{50} value. Also, but to lesser extents threonine, methionine and isoleucine significantly reversed the RI-331 action. The antagonistic effects of homoserin and these aspartate family amino acids were dose-dependent. They were scarcely antagonistic at concentrations lower than 50 μg/ml, whereas other amino acids were at best only slightly antagonistic at a concentration of 200 μg/ml.

Discussion

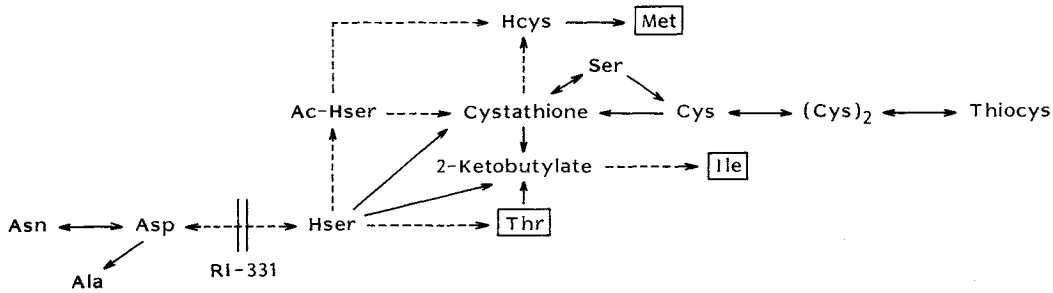
RI-331 is an antifungal antibiotic which is characterized by an amino acid-related structure and low toxicity against experimental animals. These unique properties of RI-331 tempted us to study the mechanisms of action of the antibiotic by which growth of susceptible fungi was inhibited.

In growing *S. cerevisiae* cells RI-331 preferentially inhibited the synthesis of protein with lower levels than inhibition of RNA and DNA syntheses. However, the polypeptide synthesis in a cell-free system prepared from the yeast was not affected by the antibiotic. These results suggest that the primary target of RI-331 action on protein synthesis is not on the protein biosynthesis machinery itself but on some preceding step(s), most probably involving the biosynthesis of certain amino acids. This postulation is supported by the observation that the intracellular levels of some amino acids, especially threonine, methionine and isoleucine, markedly decreased after exposure of the cells to RI-331, and that the addition of these amino acids to the medium reversed the antifungal activity of RI-331. It looks likely therefore that the antifungal activity of RI-331 is mainly due to the inhibition of protein biosynthesis caused by the decreased rate of biosyntheses of threonine, methionine and isoleucine. All of these amino acids belong to the aspartate family and share the common biosynthetic pathway which starts with aspartate and proceeds *via* homoserine as an intermediate. The most potent action of homoserine as the antagonist of RI-331 action led us to the possibility that the exact site of action of the antibiotic might be on the metabolic step(s) involved in biosynthesis of homoserine or its precursor(s). Confirmation of the validity of our speculation awaits further studies using yeast cell-free systems capable of converting aspartate to homoserine. Results of experiments with such cell-free systems will be published in an accompanying paper of this series of studies.

The preferential inhibition by RI-331 of biosyntheses of threonine, methionine and isoleucine favorably explain the selective toxicity of this antibiotic, because mammalians are unable to synthesize any of these amino acids. In other words, such RI-331-susceptible metabolic step(s) occur in fungi and probably other prototrophic organisms but not in mammalians.

A large number of amino acid analogues have been developed as antimetabolite since 1940[4]. Of particular interest in the present context is 2-amino-4-oxo-5-chloropentanoic acid (AOC1-PA)[5] closely

Fig. 4. Biochemical pathway involved in metabolism of the aspartic acid family of amino acids (aspartate, threonine, isoleucine and methionine) in prototrophic microorganisms and an assumed site of action of RI-331.



The marks are shown as follows: ⟶ General, ⇢ defective in mammals, and essential amino acid for mammals are boxed.

Abbreviations: Hser, Homoserine; Hcys, homocysteine; $(Cys)_2$, cystine; Thiocys, thiocysteine.

related in chemical structure to RI-331. The two compounds have the same structure except that the hydroxy group on C-5 in the RI-331 molecule is replaced by a chloride group in AOCl-PA. It was reported that AOCl-PA preferentially interacts with and inhibits homoserine dehydrogenase (HSDH) in *Escherichia coli*[5].

Concerning the antifungal agents available for systemic use, only a few types of action mechanism have been characterized including physico-chemical interaction with membrane sterol (for polyenes), inhibition of DNA and RNA biosyntheses (for flucytosine) and inhibition of sterol biosynthesis (for azoles). The biochemical pathways involved in biosynthesis of those amino acids which are required for mammalians as the essential amino acids, especially the aspartate family of amino acids, should be considered as a new target site of antifungal action with selective toxicity.

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