YEAST RIBOSOMAL SENSITIVITY AND RESISTANCE TO THE *AMARYLLIDACEAE* ALKALOIDS

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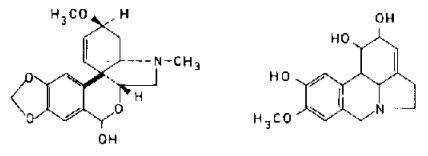
1. Introduction

Yeast ribosomal resistance to trichodermin causes a complex pleiotropic phenotype characterized by the different response of the peptidyl transferase center of the mutant 60S ribosomal subunits to its specific inhibitors as indicated by: (a) a higher level of resistance to other sesquiterpene antibiotics such as fusarenon X and trichothecin [1,2]; (b) a lower level of resistance to the chemically and biologicaly related sesquiterpene antibiotic verrucarin A [2]; (c) cross-resistance to the antibiotic anisomycin [1,2] and the compound narciclasine [3] and (d) a supersensitivity to sparsomycin without alteration in the sensitivity to blasticidin S and gougerotin [4].

We have now extended those studies to the alkaloids haemanthamine, lycorine, pretazettine and pseudolycorine which are chemically related to narciclasine (fig.1) and also obtained from the bulbs of plants of the *Amaryllidaceae* family [5–8]. All these compounds inhibit the peptide bond formation step of protein synthesis catalyzed by eukaryotic ribosomes ([3,9] and results presented in this paper). Since those alkaloids are closely related in their chemical structure, biological activity and biological metabolism [5–8] they might be expected to share both a common mode of action and binding site. However the results presented in this paper show that a yeast mutant strain resistant to narciclasine and ribosomes from this mutant [3] display cross-resistance to pretazettine and haemanthamine but not to pseudolycorine and lycorine. These results strongly suggest that haemanthamine, narciclasine and pretazettine bind to the same area of the yeast ribosomal peptidyl transferase center while lycorine and pseudolycorine do not interfere with that site of this center.



2. Material and methods

The origin of our yeast *(Saccharomyces cerevisiae)* wild type strain sensitive to narciclasine (Y166) and the mutant (TR_1) resistant to narciclasine is given elsewhere [2,3]. Liquid culture (YEP) and minimal media have been described [3,10]. Minimal inhibitory concentration was considered the amount of drug that under the standard conditions used (table 1) did not allow any visually appreciable growth of yeast after 24 h of incubation. Preparation of yeast extracts, high salt washed ribosomes, polysomes and the partially purified supernatant fraction containing elongation factors EF 1 and EF 2 were carried out as described elsewhere [2]. Poly (U)- and endogenous mRNA-directed [^{14}C]phenylalanine incorporation into 5%

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TCA precipitable polypeptides was studied in ribosomal and polysomal systems, respectively [2]. Peptide bond formation was studied in the puromycin and the fragment reaction assays as reported [3,11].

Dr A. Battersby (University Chemical Laboratory, Cambridge, England) provided us with haemanthamine and lycorine; Dr C. Fuganti (Instituto di Chimica, Milano, Italy) with haemanthamine, lycorine, narciclasine and pseudolycorine; Dr E. Furusawa (School of Medicine, University of Hawaii, Honolulu) with lycorine, pretazettine and pseudolycorine; Dr H. Irie (Faculty of Pharmaceutical Sciences, Kyoto University, Japan) with haemanthamine and tazettine; Dr F. Piozzi (Instituto di Chimica Organica, Facoltá di Scienze, Universitá di Palermo, Italy) with narciclasine and Dr W. C. Wildman (Department of Chemistry, Iowa State University, USA) with haemanthamine and tazettine.

3. Results

3.1. *The effects of the alkaloids on yeast cell growth*

Table 1 shows the minimal inhibitory concentrations of several *Amaryllidaceae* alkaloids for Y166 and TR_1 strains. Pretazettine does not inhibit cell growth of either Y166 or TR_1 strains in YEP medium, pH 7.5 [10] at concentrations up to 2×10^{-3} M. This lack of activity could be due either to a permeability barrier of the cells to the charged form of pretazettine at the pH 7.5 of the medium or to a conversion of the alkaloid to the inactive compound tazettine which takes place at alkaline pH [7]. However pretazettine did inhibit Y166 cell growth in the minimal medium (table 1). From the results presented in table 1 it is clear that TR_1 cells are as sensitive to lycorine and pseudolycorine as Y166 cells. On the other hand there is cross-resistance to narciclasine and pretazettine in TR_1 strain (table 1). Haemanthamine, another alkaloid used in this study, was not active in either YEP or minimal media at concentrations up to 4×10^{-3} M (table 1).

Table 1
Minimal inhibitory concentrations of the alkaloids for Y166 and TR_1 cells

Drug	Minimum inhibitory concentration (M)	
	Y166	TR_1
Haemanthamine[a]	$>4 \times 10^{-3}$	$>4 \times 10^{-3}$
Haemanthamine[b]	$>4 \times 10^{-3}$	$>4 \times 10^{-3}$
Lycorine[a]	2×10^{-4}	2×10^{-4}
Narciclasine[a]	2×10^{-4}	$> 10^{-3}$
Pretazettine[a]	$>2 \times 10^{-3}$	$>2 \times 10^{-3}$
Pretazettine[b]	2×10^{-3}	$> 10^{-2}$
Pseudolycorine[a]	4×10^{-3}	4×10^{-3}

Yeast growing logarithmically was diluted with either YEP, pH 7.5 (a) or minimal (b) media at 10^6 cells/ml. 1 ml aliquots of these suspensions were added to sterile tubes containing several dilutions of the required drug. Incubations were at 37°C in YEP medium and at 30°C in minimal medium. After 24 h incubation cell growth was determined by simple observation of the cultures.

3.2. *The effects of the alkaloids on in vitro polypeptide synthesis by extracts from yeast strains*

Genetic studies have shown that there is a single mutation in the TR_1 strain is nuclear [2–4]. Therefore it might be expected that the cross-resistance observed in vivo to several *Amaryllidaceae* alkaloids was due to the alteration induced by the TR_1 mutation in the ribosome. The results presented in table 2 show that the alkaloids inhibit protein synthesis supported by either ribosomes or polysomes from the wild type yeast. The exception is lycorine which, at the concentrations used, does not affect polyphenylalanine synthesis. This situation is not unique, since an ample number of inhibitors of protein synthesis like chloramphenicol [12], erythromycin [12] and trichodermin [13] are very poor inhibitors of poly (U)-directed cell-free systems for polyphenylalanine synthesis. Although haemanthamine does not inhibit growth of intact yeast it is an active inhibitor of protein synthesis in cell-free systems from the wild strain. Table 2 also shows that cell-free polypeptide synthesis by extracts from mutant cells is much less sensitive to haemanthamine and pretazettine than synthesis by extracts from wild type strain. A similar result was previously found for narciclasine ([3] and table 2). However ribosomes and polysomes from Y166 and TR_1 strains have a similar sensitivity to lycorine and pseudolycorine.

Table 2
The effect of alkaloids on yeast ribosomes endogenous RNA-directed polypeptide and poly(U)-directed polyphenylalanine synthesis

Drug	Concentration	% Control			
		Polyphenylalanine synthesis		Endogenous incorporation	
		Y166	TR_1	Y166	TR_1
Haemanthamine	10^{-4} M	64	97	20	90
	10^{-3} M	34	100	11	72
Lycorine	7×10^{-5} M	100	100	72	60
	7×10^{-4} M	100	100	61	50
Narciclasine	10^{-5} M	13	57	8	35
Pretazettine	5×10^{-5} M	44	99	13	89
	2×10^{-4} M	7	92	4	63
Pseudolycorine	10^{-4} M	42	45	60	52
	10^{-3} M	38	32	46	40

The reactions took place for 25 min or 15 min for poly(U)- or endogenous mRNA-directed polypeptide synthesis respectively. Other conditions as described in Materials and methods. Incorporation in the controls in the absence of drug was 175.13 or 16.38 (Y166 extracts) and 220.9 or 17.97 (TR_1 extracts) pmol of [^{14}C]phenylalanine for poly(U)- or endogenous mRNA-directed polypeptide synthesis respectively.

Table 3
Effect of alkaloids on peptide bond formation by yeast polysomes and ribosomes

Drug	Concentration (M)	% Control			
		Peptidyl-PM 'Fragment reaction' formation			
		Y166	TR_1	Y166	TR_1
Haemanthamine	10^{-4}	39	90	64	97
	10^{-3}	13	63	34	100
Lycorine	7×10^{-5}	62	54	36	12
	7×10^{-4}	28	20	6	0
Narciclasine[a]	10^{-7}	–	–	51	97
	10^{-6}	58	99	5	48
	10^{-5}	5	30	–	–
Pretazettine	4×10^{-5}	27	99	44	99
	4×10^{-4}	13	77	7	92
Pseudolycorine	10^{-4}	66	50	34	10
	10^{-3}	32	19	5	0

The reactions were carried out as described in Materials and methods. Reactions in the controls in the absence of inhibitors amounted to 3.86 and 0.19 pmol of peptidyl-[^{3}H]puromycin and Ac-[^{3}H]leucyl-puromycin respectively. [a]Data taken from [3].

3.3. *The effects of the alkaloids on the peptide bond formation step of protein synthesis*

The alkaloids under study are closely related in their biological origin, chemical structure and mode of action, Also narciclasine has been shown to inhibit specifically the formation of the peptide bond [9]. Therefore we have studied the effects of the alkaloids on the activity of the peptidyl transferase center of the yeast cytoplasmic ribosomes, in order to characterize their mode of action more precisely. Indeed table 3 shows that the five alkaloids inhibit the formation of Ac-[^{3}H]Leu-puromycin (fragment reaction assay) and peptidyl-[^{3}H]puromycin (puromycin reaction) in cell-free systems from the sensitive strain. Therefore the compounds halt the peptidyl transferase activity of the yeast ribosome, as previously shown with narciclasine [9]. In addition table 3 shows that the resistance towards haemanthamine and pretazettine at the peptide bond formation step was similar to that previously observed with narciclasine. However ribosomes from the mutant are sensitive to lycorine and pseudolycorine. Furthermore it is interesting that in the well resolved 'fragment reaction' assay, ribosomes of the mutant strain are even more sensitive to lycorine and pseudolycorine than the ribosomes from the wild type strain.

4. Discussion

The *Amaryllidaceae* alkaloids studied here have a potent antitumor activity [14,15]. The results presented in this paper show that haemanthamine, lycorine, pretazettine and pseudolycorine inhibit protein synthesis in yeast cell-free systems by blocking the peptide bond formation step, as previously reported with the chemically and biologically related substance narciclasine [9]. Pretazettine (initially known as residual alkaloid A-3) inhibits the reverse transcriptase in cell-free systems at rather high concentrations (4 mg/ml) [16]. Furthermore this alkaloid and pseudolycorine have been reported as preferentially inhibiting protein synthesis in tumor cells [17].

The yeast mutant strain TR_1 has a single mutation with pleiotropic effects on the peptidyl transferase centre of the ribosome. Thus ribosomes from the TR_1 strain are resistant both in vivo and in vitro to some sesquiterpene antibiotics [2], anisomycin and narciclasine with enhancement in their sensitivity towards sparsomycin [2–4]. Furthermore mutant ribosomes are cross resistant in vitro to pretazettine and haemanthamine but sensitive to lycorine and pseudolycorine (tables 2 and 3).

Haemanthamine, pretazettine and narciclasine appear to have a common binding site on the peptidyl transferase centre of the 60S ribosomal subunit. On the other hand lycorine and pseudolycorine apparently bind to a different site on the peptidyl transferase centre. The binding site of lycorine and pseudolycorine might be different from that of narciclasine; an alternative explanation could be that lycorine, pseudolycorine and narciclasine bind to the same or overlapping sites but the conformational modification induced by the TR_1 mutation in the ribosome might impair the binding of narciclasine without affecting the interaction of lycorine and pseudolycorine. Furthermore the binding sites of lycorine and pseudolycorine might be somehow related to the interaction site of sparsomycin since the three compounds show a higher inhibitory effect on peptide bond formation by ribosomes of the TR_1 mutant [4] (table 3). Binding experiments with the radioactive alkaloids might help to elucidate this problem.

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References

[1] Schindler, D., Grant, P. and Davies, J. (1974) Nature 248, 535–536.
[2] Jimenez, A., Sanchez, L. and Vazquez, D. (1975) Biochim. Biophys. Acta 383, 427–434.
[3] Jimenez, A., Sanchez, L. and Vazquez, D. (1975) FEBS Lett. 55, 53–56.
[4] Jimenez, A. and Vazquez, D. (1975) Eur. J. Biochem. 54, 483–492.
[5] Wildman, W. C. (1960) in: The Alkaloids (Manske, R. H., ed.), Vol. VI, pp. 289–413, Academic Press (New York, London).

[6] Piozzi, F., Fuganti, C., Mondelli, R. and Ceriotti, G. (1968) Tetrahedron 24, 1119–1121.
[7] Wildman, W. C. and Bailey, D. T. (1968) J. Org. Chem. 33, 3749–3753.
[8] Fuganti, C., Staunton, J. and Battersby, A. R. (1971) Chem. Commun. pp. 1154–1155.
[9] Carrasco, L., Fresno, M. and Vazquez, D. (1975) FEBS Lett. 52, 236–239.
[10] Grant, P., Sanchez, L. and Jimenez, A. (1974) J. Bacteriol. 124, 1308–1314.
[11] Battaner, E. and Vazquez, D. (1971) Biochim. Biophys. Acta 254, 316–330.
[12] Vazquez, D. (1966) Biochim. Biophys. Acta 114, 289–295.
[13] Carrasco, L., Barbacid, M. and Vazquez, D. (1973) Biochim. Biophys. Acta 312, 368–376.
[14] Ceriotti, G. (1967) Nature 213, 595–596.
[15] Suzuki, N., Furusawa, S. and Furusawa, E. (1974) Proc. Soc. Exptl. Biol. Med. 145, 771–777.
[16] Papas, T. S., Sandhouse, L., Chirigos, M. A. and Furusawa, E. (1973) Biochem. Biophys. Res. Commun. 52, 88–92.
[17] Suzuki, N., Furusawa, S. and Furusawa, E. (1974) Clin. Pharmacol. Therapeut. 15, 220–221.