# NARCICLASINE: AN ANTITUMOUR ALKALOID WHICH BLOCKS PEPTIDE BOND FORMATION BY EUKARYOTIC RIBOSOMES

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## 1. Introduction

The antitumour activity of crude preparations of bulbs from species of Narcissus [1] is due to the alkaloid narciclasine, of known chemical structure [2,3], which exerts an antimitotic effect during metaphase [4]. Studies in our laboratory have shown that narciclasine immediately halts protein synthesis in Ehrlich ascites tumour and *Saccharomyces cerevisiae* cells without affecting RNA synthesis (A. Jimenez, personal communication). The results presented in this communication show that narciclasine inhibits protein synthesis in rabbit reticulocyte and yeast cell-free systems by blocking peptide bond formation at the ribosome level.

## 2. Materials and methods

Reticulocyte ribosomes and elongation factors EF 1 and EF 2 were prepared from anaemic rabbits as previously described [5–7]. Yeast polysomes and a crude supernatant fraction containing the elongation factors were prepared as described [8].

Poly U-directed [$^{14}$C]phenylalanine incorporation using either rabbit reticulocyte or *E. coli* ribosomes was assayed following standard methods described elsewhere [7,9]. Polypeptide synthesis by yeast cell-free systems was studied in 50 $\mu$l reaction mixtures containing 50 mM Tris–HCl buffer, pH 7.4, 12 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 80 $\mu$g GTP, 4 mM creatine phosphate, 40 $\mu$g/ml creatine phosphokinase, 40 $\mu$g/ml yeast tRNA, 15 $\mu$g of 19 non-radioactive aminoacids (phenylalanine was omitted), 0.015 mM [$^{14}$C]phenylalanine (190 mCi/mmol), 5 $\mu$l of a crude supernatant fraction and 28 pmol yeast polysomes. Polypeptide synthesis was stopped by the addition of 2 ml of 10% trichloroacetic acid. After heating for 10 min at 90°C the mixtures were filtered through GF/C glass fibre Whatman filters, the filters were dried, and radioactivity estimated in a scintillation spectrometer.

Peptide bond formation was studied by estimation of the release of nascent peptides on yeast polysomes by [$^{3}$H]puromycin and the puromycin reaction with either Ac-[$^{3}$H]Phe-tRNA or UACCA-[$^{3}$H]Leu-Ac bound to rabbit reticulocyte ribosomes. The release of nascent peptides was studied in 50 $\mu$l reaction mixtures of 50 mM Tris–HCl buffer, pH 7.4, 12.5 mM $MgCl_2$, 80 mM KCl, 0.5 mM GTP, 7 $\mu$l of a crude supernatant fraction and 43 pmol yeast polysomes incubated for 10 min at 30°C to allow translocation of the peptidyl-tRNA from the A ribosomal site to the P-site; The required concentration of narciclasine and 2 pmol [$^{3}$H]puromycin (3.7 Ci/mmol) were then added, the incubation continued for 1 min at 30°C, the reaction stopped by addition of 2 ml 10% trichloroacetic acid and the radioactivity retained on glass fibre filters estimated. The reaction of the complex Ac-[$^{3}$H]Phe-tRNA (7.8 Ci/mmol)-rabbit reticulocyte ribosome-poly U with puromycin was carried out as described [9,10]. The reaction of puromycin UACCA-[$^{3}$H]Leu-Ac (52 Ci/mmol) bound to rabbit reticulocyte ribosomes (the 'fragment reaction' assay) was performed as previously described [11].

Non-enzymic binding of Ac-[$^{3}$H]Phe-tRNA (7.8 Ci/mmol), enzymic binding of [$^{14}$C]Phe-tRNA (476 mCi/mmol) and sparsomycin-induced binding of


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UACCA-[$^3$H]Leu-Ac 52 Ci/mmol to rabbit reticulocyte ribosomes was carried out as previously described [11,12].

Binding of [$^3$H]anisomycin and [$^{14}$C]trichodermin to ribosomes was carried out in 60 μl mixtures which contained 50 mM Tris–HCl buffer, pH 7.4, 12.5 mM magnesium chloride, 80 mM KCl, either 11.5 mg/ml yeast ribosomes of 9 mg/ml rabbit reticulocyte ribosomes and either $10^{-6}$ M [$^{14}$C]trichodermin (15.4 mCi/mmol). The ultracentrifugation method described previously [13] was used.

[$^3$H]anisomycin and [$^{14}$C]trichodermin were prepared by Dr M. Barbacid following methods described elsewhere [13,14]. Narciclasine was prepared [2] and given to us by Dr Piozzi. Sparsomycin and trichodermin were supplied to us as described elsewhere [10,12]. [$^{14}$C]Phe-tRNA, Ac-[$^3$H]Phe-tRNA and UACCA-[$^3$H]Leu-Ac were prepared as described [11,12].

## 3. Results

### 3.1. *Selective inhibitory effect of narciclasine in eukaryotic systems of polypeptide synthesis. Specific effect of the alkaloid in peptide bond formation*

Narciclasine strongly inhibits polypeptide synthesis in yeast polysomes as well as poly U-directed polyphenylalanine synthesis in a ribosomal system from rabbit reticulocytes but has no effect in a similar system from *E. coli* (fig.1). Polypeptide elongation is affected by narciclasine, since it inhibits peptide bond formation in three systems: a) the release of nascent peptides from yeast polysomes by [$^3$H]puromycin, b) the reaction of the complex Ac-[$^3$H]Phe-tRNA-rabbit reticulocyte ribosome-poly U with puromycin and c) the fragment reaction assay with rabbit reticulocyte ribosomes using UACCA-[$^3$H]Leu-Ac as the donor substrate (fig.2). The most sensitive assay was the fragment reaction which was inhibited by 70% in the presence of $10^{-7}$ M narciclasine.

### 3.2. *Effect of narciclasine on substrate binding to the peptidyl transferase centre*

The inhibitory effect of narciclasine on the enzymic binding of [$^{14}$C]Phe-tRNA and the non-enzymic binding of Ac-[$^3$H]Phe-tRNA to the ribosome

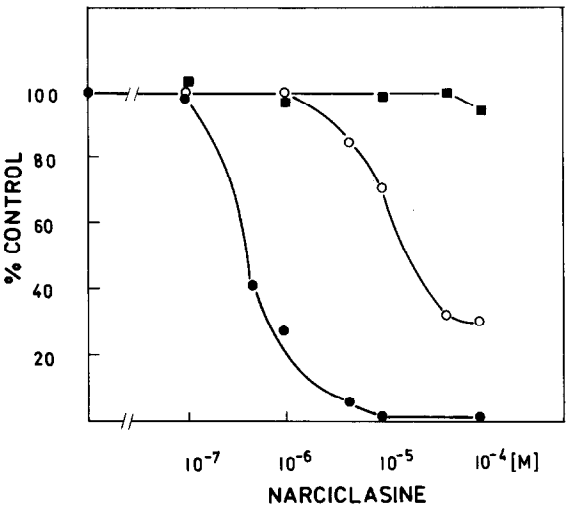


Fig.1. Effects of narciclasine on [$^{14}$C]phenylalanine incorporation in yeast polysomes (●——●), rabbit reticulocyte ribosomes (○——○) and *E. coli* ribosomes (■——■); [$^{14}$C]phenylalanine incorporation in these systems in the controls was 1.87, 4.21 and 4.46 pmol respectively. The results are expressed as the precentage of control incorporation of [$^{14}$C]phenylalanine observed in the presence of narciclasine. Conditions as described in Materials and methods.

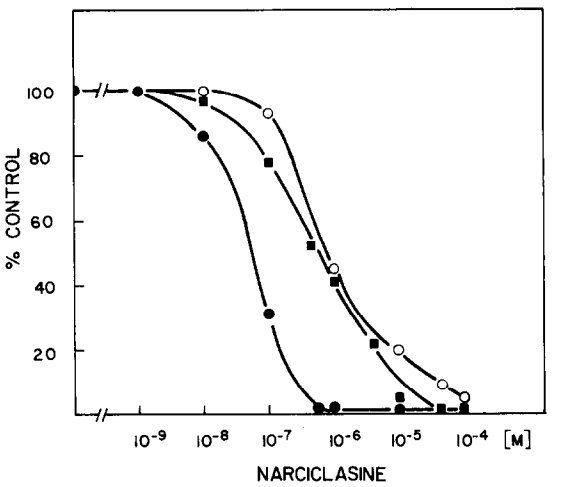


Fig.2. Effects of narciclasine on peptidyl-[$^3$H]puromycin formation by yeast polysomes (■——■), Ac-[$^3$H]Phe-puromycin synthesis in the puromycin reaction assay by rabbit reticulocyte ribosomes (○——○) and Ac-[$^3$H]Leu-puromycin synthesis in the fragment reaction assay by rabbit reticulocyte ribosomes (●——●); 1.11, 0.198 and 0.022 pmol puromycin reacted in the controls in these assays respectively. The results are expressed as the percentage of the product formed in the presence on narciclasine compared to the control reaction in the absence of the alkaloid. Conditions as described in Materials and methods.

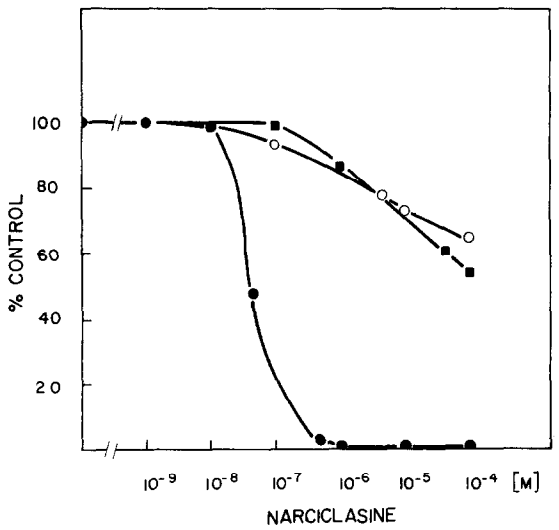


Fig.3. Effect of narciclasine on non-enzymic binding of Ac-[$^3$H]Phe-tRNA (■——■), enzymic binding of [$^{14}$C]Phe-tRNA (○——○) and sparsomycin-induced binding of UACCA-[$^3$H] Leu-Ac (●——●); 0.82, 6.98 and 0.088 pmol substrate were bound in the controls in these assays respectively. The results are presented as the percentage of the substrate bound in the presence of narciclasine compared to the controls in the absence of the alkaloid.

is relatively weak compared to its blocking action of sparsomycin-induced binding of the donor substrate UACCA-[$^3$H]Leu-Ac to rabbit reticulocyte ribosomes (fig.3). This result suggests that narciclasine blocks the fragment reaction assay by preventing the interaction of only the 3′ terminal end of the donor substrate to the peptidyl transferase centre of the 60S ribosome subunit; an action that might explain its reduced inhibitory effects on the binding of Ac-Phe-tRNA to the ribosome.

### 3.3. *Effect of narciclasine on [$^3$H]anisomycin and [$^{14}$C]trichodermin binding to eukaryotic ribosomes*

The antibiotics anisomycin and trichodermin resemble narciclasine in that they also are specific inhibitors of peptide bond formation by eukaryotic ribosomes and affect substrate binding to the ribosome [10,12]. We studied the binding of [$^3$H]anisomycin and [$^{14}$C]trichodermin [11,13,14] to yeast and rabbit reticulocyte ribosomes in the presence of narciclasine (table 1) and found that the alkaloid inhibited binding of [$^3$H]anisomycin to both types of eukaryotic ribosomes. This observation supports the notion that the site of action of anisomycin and narciclasine at the ribosome level is identical or closely connected. On the other hand, narciclasine was a rather poor inhibitor of [$^{14}$C]trichodermin binding to ribosomes. This finding suggests different sites of action of trichodermin and narciclasine at the ribosome level in the peptidyl transferase centre.

## 4. Discussion

Our results indicate that narciclasine interacts with the 60S ribosome subunit. The alkaloid inhibits peptide bond formation by preventing the binding of the 3′ terminal end of the donor substrate to the peptidyl transferase centre. The inhibition by narciclasine of sparsomycin-induced binding of UACCA-[$^3$H]Leu-Ac might be due to an indirect effect of the alkaloid, i.e. prevention of the association of the antibiotic with the ribosome. But it is most unlikely that narciclasine inhibits sparsomycin binding since the alkaloid strongly inhibits anisomycin association with its binding site on the 60S ribosome subunit, while sparsomycin does not inhibit

Table 1
Effect of narciclasine on the binding of [$^3$H]anisomycin and [$^{14}$C]trichodermin to ribosomes

| Additions | [$^3$H]anisomycin binding (cpm) | | [$^{14}$C]trichodermin binding (cpm) | |
|---|---|---|---|---|
| | Yeast ribosomes | Rabbit ribosomes | Yeast ribosomes | Rabbit ribosomes |
| Control | 1186 | 1475 | 2005 | 1479 |
| + $10^{-4}$ M narciclasine | 79 | 117 | 1256 | 1045 |

The binding assay was carried out as described in Materials and methods.

the binding of anisomycin [11]. Indeed all the evidence presented in this communication suggests that narciclasine acts on a moiety of the peptidyl transferase known as the 'anisomycin area' which is somehow independent from the sparsomycin site of action [15; review].

We conclude that the mode of action of narciclasine on mammalian ribosomes is similar to anisomycin and trichodermin. Narciclasine, like anisomycin and trichodermin, is active on the different eukaryotic cells and protein synthesizing systems which have been tested. Our binding experiments suggest that the binding site of narciclasine and anisomycin might be the same. However the binding sites of narciclasine and trichodermin are not precisely the same although both inhibitors interact with the 'anisomycin area' of the peptidyl transferase centre.

## Acknowledgements

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