# INHIBITORS OF MAMMALIAN PROTEIN SYNTHESIS

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The number of antibiotics active on eukaryotic ribosomes has been considerably increased in the last few years. Some of them are active only in eukaryotic ribosomes whereas some others are active on prokaryotic as well as eukaryotic ribosomes (Table 1). There is ample evidence suggesting a similar mechanism for the elongation cycle by prokaryotic and eukaryotic ribosomes. This evidence is mainly based on studies showing that (a) the functions of the eukaryotic ribosomal subunits are similar to those of their prokaryotic counterpart and (b) antibiotics active on prokaryotic and eukaryotic ribosomes act in both cases on the equivalent subunits. Therefore we can tentatively extrapolate and generalize results obtained with some antibiotics concerning their site of action as indicated in Table 2. Similarly we might generalize data concerning the mode of action of these antibiotics. However, most of the data concerning the specific reactions inhibited by antibiotics on eukaryotic ribosomes were obtained using different experimental systems and ribosomes from an enormous variety of biological sources. Consequently we have studied systematically the effect of these antibiotics on similar systems for the reactions of the elongation cycle using in all cases human tonsil ribosomes. For the purpose of comparative studies we have also studied in some cases the effects of some antibiotics on cell-free systems from other types of eukaryotic cells. The results obtained are presented in this contribution.

## Materials and methods

Human tonsil ribosomes and elongation factors (EF 1 and EF 2) were prepared as previously described (1, 2). The separation of EF 1 and EF 2 was carried out in Sephadex G-200 chromatography after ammonium sulphate fractionation. EF 1 was further purified by Sepharose 4 B column and hydroxyapatite treatment. EF 2 from the Sephadex G-200 was purified by DEAE cellulose and phosphocellulose chromatography (3).

Yeast ribosomes were obtained as described elsewhere (4). Baker's yeast tRNA (Boehringer) was charged with [$^{14}$C]Phenylalanine (513 mCi/mmol) (Radiochemical Centre) using a crude synthetase fraction from yeast prepared from the S 100 by means of a Sephadex G-25 column pooling the active fractions.

[14C]Phe-tRNA was separated after phenol treatment and ethanol precipitation and an aliquot was acetylated as described (5). [$^{3}$H]Phe-tRNA and [$^{3}$H]Leu-tRNA (prepared from *E. coli* tRNA (Sigma) and either [$^{3}$H]Phenylalanine (18 Ci/mmol) or [$^{3}$H]Leucine (52 Ci/mmol)) were acetylated by the same method indicated above. The N-Ac-[$^{14}$C]Phe-tRNA was further purified by BD-cellulose chromatography.

Table 1. Inhibitors of protein synthesis active on eukaryotic systems

| Acting on ribosomal systems of the eukaryotic type | Acting on ribosomal systems of the prokaryotic and the eukaryotic types |
|---|---|
| * Adrenochrome | Abrin |
| Anisomycin | Actinobolin |
| Emetine | Amicetin |
| Enomycin | Aurintricarboxylic acid |
| Glutarimide group: | Blasticidin S |
| Actiphenol | Bottromycin $A_2$ |
| Cycloheximide | Chartreusin |
| Strptimidone | Diphtheria toxin |
| Streptovitacin A | Edeine |
| Pederine | Fusidic acid |
| Phenomycin | Gougerotin |
| Tenuazonic acid | **Griseoviridin |
| Trichodermin group: | Nucleocidin |
| Crotocin | Pactamycin |
| Crotocol | Pyrocatechol violet |
| Fusarenon X | Poly-dextran-sulphate |
| Nivalenol | Puromycin |
| Trichodermin | Ricin |
| Trichodermol | Sparsomycin |
| Trichothecin | Tetracycline group: |
| Verrucarin A | Chlortetracycline |
| Verrucarol | Deoxycycline |
| Tylophora alkaloids: | Oxytetracycline |
| Cryptopleurine | Tetracycline |
| Tylocrebrine | |
| Tylophorine | |

* Adrenochrome has not been tested in prokaryotic systems.
** Griseoviridin is not active in any of the intact cells of the eukaryotic type which have been tested but it is active on eukaryotic cell-free systems.

CACCA-Ac-[$^3$H]Leu-Ac was prepared from N-Ac-[$^3$H]Leu-tRNA by digestion with $T_1$ RNAse and separated by paper electrophoresis (6).

Enzymic binding of [$^{14}$C]Phe-tRNA to the ribosome A site took place at low $Mg^{++}$ concentrations using the EF 1 preparation (7). The complex formed was separated by centrifugation when required, the inhibitors to be tested were added and translocation induced by addition of EF 2 and GTP (Fig. 2). The extent of

translocation was measured by the reaction with puromycin of N-Ac-[$^{14}$C]Phe-tRNA bound to the P-site. Inhibitors of peptide bond formation were tested in these reactions in controls in which inhibitors were not added prior to translocation (Fig. 2).

Nonenzymic binding of purified N-Ac-[$^{14}$C]Phe-tRNA was studied at 15 mM $Mg^{++}$ concentration. Under these conditions the N-Ac-[$^{14}$C]Phe-tRNA binds to the A and P-sites to the same extent. All the substrate bound to the A-site translocated to the P-site in the presence of EF 2 and GTP and reacted with puromycin after translocation.

Peptide bond formation was also studied in the fragment reaction assay using as substrates CACCA-[$^{3}$H]Leu-Ac-N and puromycin and measuring formation of N-Ac-[$^{3}$H]Leu-puromycin (4, 7, 8).

Sources of the protein synthesis inhibitors used in this work were as follows: actinobolin and griseoviridin (Parke Davis), amicetin, chartreusin and cycloheximide (Upjohn), adrenochrome and gougerotin (Calbiochem), anisomycin (Pfizer), aurintricarboxylic acid (ATA) (May and Baker), blasticidin S and bottromycin $A_2$ (Institute of Applied Microbiology, Tokyo, Japan), emetine (Wellcome), fusidic acid and trichodermin (Leo), puromycin (Serva and Nutritional Biochemicals), sparsomycin (National Cancer Institute, Bethesda, USA) and tenuazonic acid (Merck Sharp and Dohme). Edeine $A_1$ was a gift from Dr. Z. Kurylo-Borowska (Rockefeller University, New York, USA). Pederine was given to us by Prof. M. Pavan (Institute of Entomology, University of Pavia, Italia). Diphtheria toxin was a gift from Dr. E. Bermek (Max-Planck Institute for Experimentel Medicine, Göttingen, Germany).

Methods previously described have been used for the preparation of ribosomes from *Euglena gracilis* (9) and *Phaseolus vulgaris* (10).

[$^{3}$H]anisomycin (285 mCi/mmol) and [$^{3}$H]gougerotin (80 mCi/mmol) were obtained by tritium exchange labelling in aqueous solutions and purified as described elsewhere (11). [$^{3}$H]anisomycin and [$^{3}$H]gougerotin binding to human tonsil ribosomes was studied following basically the sedimentation method, essentially as described (12) in 50 mM Tris-HCl, pH 7.4, 11 mM $MgCl_2$, 60 mM KCl and 7 mM 2-mercaptoethanol.

*Effects of the inhibitors on the steps of the elongation cycle.*

Our studies on enzymic binding of [$^{14}$C]Phe-tRNA and non-enzymic binding of N-Ac-[$^{14}$C]Phe-tRNA have shown that adrenochrome and pyrocatechol violet are strong inhibitors of substrate binding (Table 1), and confirm previous reports (13, review) showing that ATA, chartreusin, edeine $A_1$ and tetrycycline are also inhibitors of substrate binding.

In both systems studied we have shown that pederine is a good inhibitor of translocation (Table 2). Diphtheria toxin has been shown previously to act catalytically modifying EF 2 forming ADP-ribosyl-EF 2 which is able to bind to the ribosome (14) but does not induce translocation. Contrary to a number of previous reports (13, review) fusidic acid does not block translocation under the experimental conditions of Table 2. The tylophora alkaloids, cryptopleurine, tylocrebrine and tylophorine are also inhibitors of translocation as already reported for tylocrebrine (15).

Table 2. Inhibitors of protein synthesis acting on eukaryotic ribosomes.
Site of action

| 40S ribosome subunit | 60S ribosome subunit |
|---|---|
| Aurintricarboxylic acid | Actinobolin |
| * Edeine $A_1$ | Amecetin |
| * Pactamycin | Anisomycin |
| * Poly-dextran-sulphate | Blasticidin S |
| * Tetracycline group: | Bottromycin $A_2$ |
| Chlortetracycline | ** Fusidic acid |
| Deoxycycline | Glutarimide group: |
| Oxytetracycline | Actiphenol |
| Tetracycline | Cycloheximide |
| | Streptimidone |
| | Streptovitacin A |
| | Gougerotin |
| | Griseoviridin |
| | Puromycin |
| | Sparsomycin |
| | Tenuazonic acid |
| | Trichodermin group: |
| | Crotocin |
| | Crotocol |
| | Fusarenon |
| | Nivalenol |
| | Trichodermin |
| | Trichodermol |
| | Trichothecin |
| | Verrucarin A |
| | Verucarol |
| | Tylophora alkaloids: |
| | Cryptopleurin |
| | Tylocrebrine |
| | Tylophorine |

* Edeine $A_1$, pactamycin, poly-dextran-sulphate and the tetracyclines bind to both the smaller and the larger subunits but the interaction with the smaller subunit appears to be more relevant for the mode of action of these inhibitors.

** Fusidic acid has not been shown to interact directly with the larger ribosome subunit but forms the complex EF 2·GDP·larger ribosome subunit·fusidic acid.

Table 3. Non enzymic binding of Ac-[$^{14}$C]Phe-tRNA and enzymic binding of [$^{14}$C]Phe-tRNA to human tonsil ribosomes. Effects of protein synthesis inhibitors

| Additions | | Ac-[$^{14}$C]Phe-tRNA binding (% control) | [$^{14}$C]Phe-tRNA binding (% control) |
|---|---|---|---|
| Adrenochrome | $10^{-4}$M | 25 | 23 |
| Anisomycin | $10^{-4}$M | 58 | 51 |
| | $10^{-5}$M | 99 | 91 |
| ATA | $10^{-4}$M | 1 | 15 |
| Chartreusin | $10^{-3}$M | – | 37 |
| Edeine $A_1$ | $10^{-6}$M | 26 | 14 |
| Emetine | $10^{-4}$M | 53 | 74 |
| Pyrocatechol violet | $10^{-4}$M | – | 13 |
| Tenuazonic acid | $10^{-3}$M | 67 | 61 |
| | $10^{-4}$M | 96 | 92 |
| Tetracycline | $10^{-3}$M | – | 28 |

Assays were carried out under the experimental conditions described above. Figures given in this Table are percentage of control reactions in the absence of inhibitor. Binding of Ac-[$^{14}$C] Phe-tRNA in the control reactions was 1.6 pmoles whereas [$^{14}$C]Phe-tRNA binding was 2.4 pmoles.

Table 4. Translocation of Ac-[$^{14}$C]Phe-tRNA and [$^{14}$C]Phe-tRNA bound to human tonsil ribosomes. Effects of protein synthesis inhibitors

| Additions | | Ac-[$^{14}$C]Phe-tRNA translocation (% control) | [$^{14}$C]Phe-tRNA translocation (% control) |
|---|---|---|---|
| Cryptopleurine | $2.6 \times 10^{-4}$M | 43 | – |
| Diphtheria toxin | 125 $\mu$g/ml | 36 | 11 |
| Fusidic acid | $2 \times 10^{-4}$M | 99 | 97 |
| Pederine | $2 \times 10^{-5}$M | 32 | 30 |
| Tylocrebrine | $2.6 \times 10^{-4}$M | 43 | – |
| Tylophorine | $2.6 \times 10^{-4}$M | 31 | – |

Assays were carried out under the experimental conditions indicated above. Figures given in this Table are percentage of control reactions in the absence of inhibitors. Average of Ac-[$^{14}$C] Phe-tRNA translocated in the controls was 0.8 pmoles (47 % of the total substrate bound) whereas [$^{14}$C]Phe-tRNA translocated in the control was 0.6 pmoles (21 % of the total substrate bound).

Table 5. Peptide bond formation by human tonsil ribosomes
Effects of protein synthesis inhibitors

| Additions | | Ac-[$^{14}$C]Phe-Pur formation (% control) | [$^{14}$C]Phe-Pur formations (% control) | Fragment reaction (% control) |
|---|---|---|---|---|
| Actinobolin | $10^{-3}$M | – | – | 29 |
| | $10^{-4}$M | 94 | – | 75 |
| Anisomycin | $10^{-4}$M | 25 | 16 | 5 |
| Blasticidin S | $10^{-4}$M | – | 23 | 12 |
| Gougerotin | $10^{-3}$M | – | – | 16 |
| | $10^{-4}$M | 97 | – | – |
| Griseoviridin | $5 \times 10^{-4}$M | – | – | 18 |
| Sparsomycin | $10^{-4}$M | 21 | 19 | 7 |
| Tenuazonic acid | $10^{-3}$M | 51 | – | 22 |
| | $10^{-4}$M | 98 | 50 | 73 |
| Trichodermin | $10^{-5}$M | 32 | – | 13 |

Assays were carried out under the experimental conditions described above. Figures given in this Table are percentage of control reactions in the absence of inhibitor. In the control assays 1.75 pmoles Ac-[$^{14}$C]Phe-Pur or 0.68 pmoles [$^{14}$C]Phe-Pur were formed in the puromycin reaction and 1.39 n moles Ac-[$^{14}$C]Phe-puromycin were formed in the fragment reaction assay.

In the different experimental systems used we have shown that trichodermin and tenuazonic acid block the peptide bond formation step (Table 3). We have confirmed previous evidence that actinobolin, amicetin, anisomycin, blasticidin S, gougerotin and sparsomycin also block the peptidyl transferase centre (4, 8). In most cases the inhibitors preferentially block peptide bond formation in the fragment reaction since it is a more resolved assay. We have also observed that the antibiotic griseoviridin inhibits peptide bond formation in the fragment reaction assay. This finding is interesting since griseoviridin was considered as an antibacterial antibiotic which has neither fungistatic nor antiamebae nor antiprotozoal activity (16). However in cell-free systems we have observed that griseoviridin is indeed an effective inhibitor of poly (U)-directed polyphenylalanine synthesis (results not shown). Results presented in Table 3 show that griseoviridin blocks peptide bond formation by human tonsil ribosomes similarly as in bacterial systems (17), but to a lesser extent.

*Differential properties of the peptidyl transferase centre.*

For most antibiotics considered in this work there is a similar pattern of inhibition when tested on either human tonsil (7) or yeast (4) ribosomes. However, a number of inhibitors active on the peptidyl transferase centre are more active on human tonsil than on yeast ribosomes. This was indeed observed when the effect of the sesquiterpene antibiotics trichodermin and trichodermol was tested under

identical conditions in the fragment reaction assay catalyzed by either human tonsil or yeast ribosomes (Fig. 1).

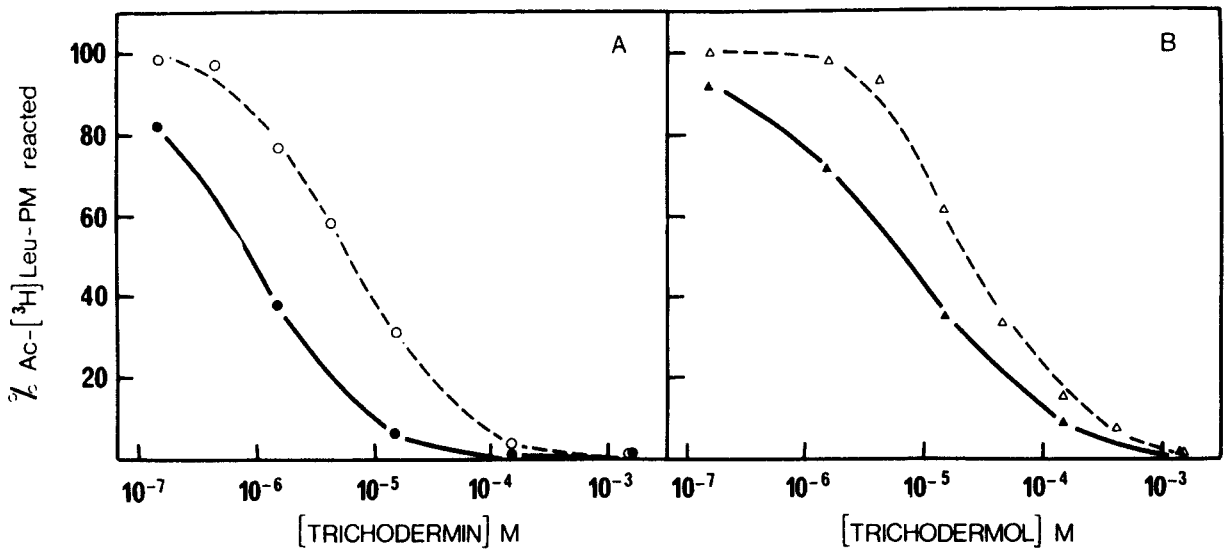


Fig. 1: Effects of sesquiterpene antibiotics on the fragment reaction by human tonsil and yeast ribosomes. *A*: Effects of trichodermin on the fragment reaction by yeast (O - - - O) and human tonsil (●——●) ribosomes. *B*: Effects of trichodermol on the fragment reaction by yeast (△ - - - △) and human tonsil (▲——▲) ribosomes. The experimental systems were as indicated under Materials and Methods.

This preferential activity was indeed more striking in the case of tenuazonic acid since this antibiotic showed no inhibitory effect on poly (U)-directed polyphenylalanine synthesis by yeast ribosomes whereas there was a significant inhibition on the human system (Table 6). This differential sensitivity of human tonsil and yeast ribosomes is due only to the larger ribosome subunit and the smaller subunit is irrelevant for this effect. Tenuazonic acid was tested on the puromycin reaction by hybrid ribosomes (Fig. 2) and the results obtained are presented in Table 7. The extent of inhibition by tenuazonic acid on hybrid ribosomes was similar to that which was observed previously in the same system by human tonsil ribosomes (7), whereas the antibiotic does not affect the puromycin reaction when hybrid ribosomes of 60S subunits from yeast and 40S subunits from human tonsils were used.

Furthermore we have also observed that tenuazonic acid does not inhibit peptide bond in the "fragment reaction" assay by yeast ribosomes, has a reduced effect on the reaction catalyzed by *Euglena gracilis* and *Phaseolus vulgaris* ribosomes and is active on pig liver ribosomes (18). Indeed differences between these types of ribosomes were also observed in the peptidyl transferase centre by studying their activity on the fragment reaction after pretreatment with N-ethyl-maleimide (NEM) (Table 8).

*Binding of tenuazonic acid to eukaryotic ribosomes*

It was not possible to measure binding of tenuazonic acid to ribosomes since the antibiotic is not available radioactively labelled. However, we have prepared [$^3$H]anisomycin and [$^3$H]gougerotin by the tritium exchange labelling procedure

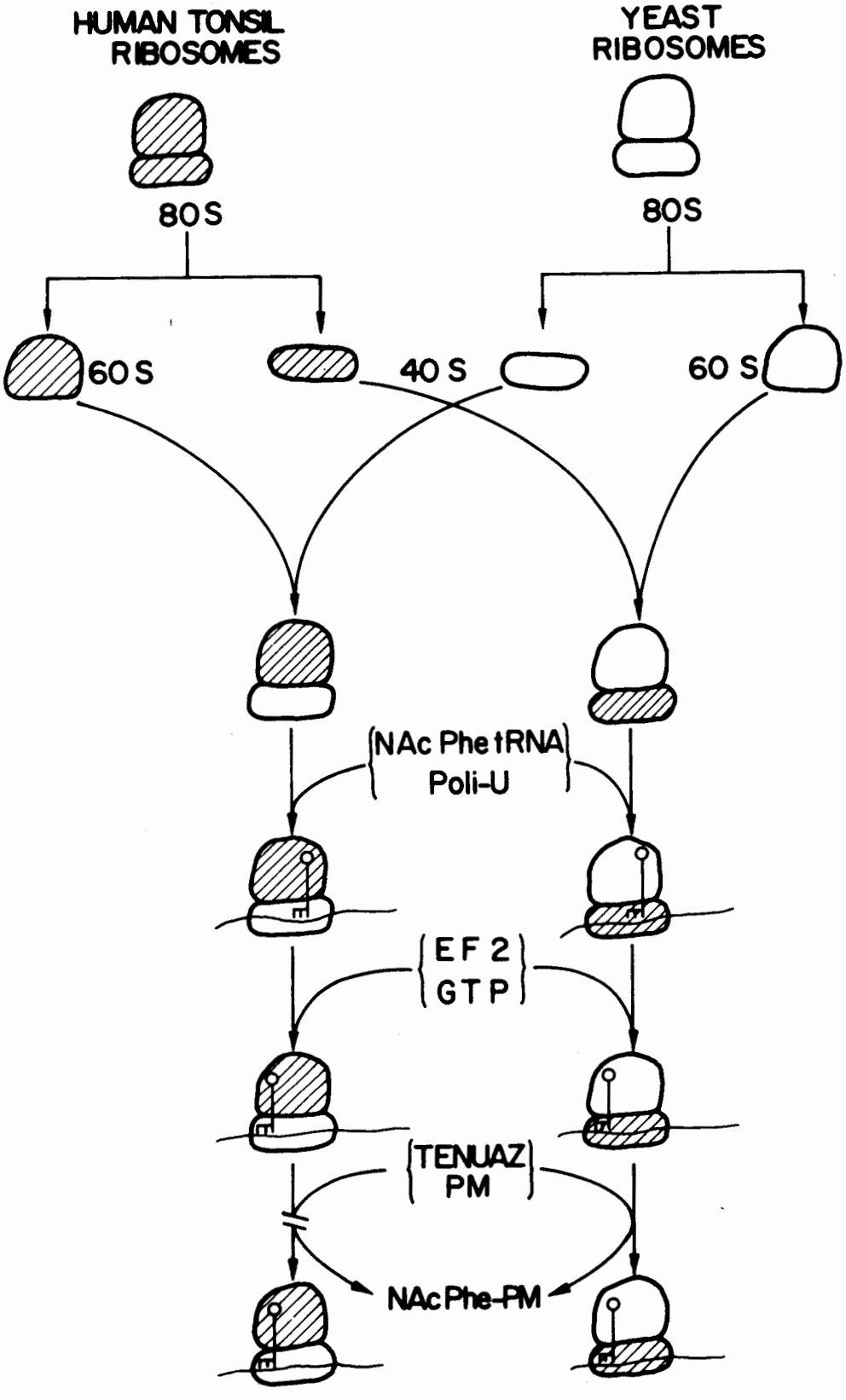


Fig. 2: General scheme followed to study the puromycin reaction by hybrid ribosomes.

(19). We have observed that tenuazonic acid does not affect binding of [$^3$H]gougerotin to either human tonsil or yeast ribosomes. However, tenuazonic acid totally inhibits [$^3$H]anisomycin to human tonsil ribosomes but not to yeast ribosomes (Fig. 3). It certainly appears that tenuazonic acid binds to human tonsil ribosomes to the same set of sites as anisomycin but its interaction with yeast

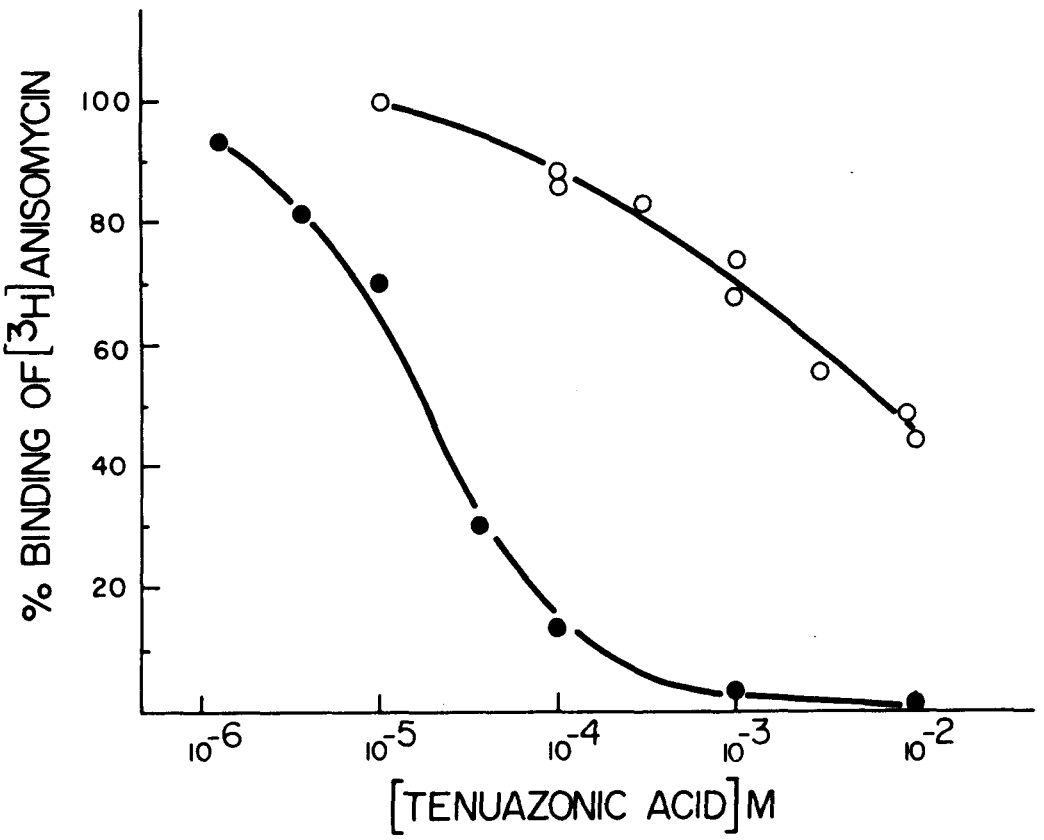


Fig. 3: Effects of tenuazonic acid on [$^3$H]anisomycin binding to ribosomes. (O——O) yeast ribosomes and (●——●) human tonsil ribosomes. Data were taken from an assay following the sedimentation method as described under Materials and Methods. Yeast ribosome concentration was 2.5 x $10^{-6}$ M. Human tonsil ribosome concentration was 3.5 x $10^{-6}$ M. [$^3$H]anisomycin concentration was in all cases $10^{-6}$ M.

ribosomes is negligeable; this explains the lack of effect of tenuazonic acid on yeast ribosomes reported above. Therefore the affinity of tenuazonic acid for the ribosome might be known if tenuazonic acid competes for the binding site of a radioactive antibiotic. We have studied the effects of different concentrations of tenuazonic acid on [$^3$H] anisomycin binding to human tonsil ribosomes and the data obtained were taken to a Klotz plot (Fig. 4). From this plot the value obtained for the dissociation constant for tenuazonic acid is $K_d$ = 2 x $10^{-5}$ M following the relationship given to calculate affinity of an unlabelled compound provided that it competes for binding with a radioactive one of known dissociation constant (20).

Table 6. The effect of tenuazonic acid on synthesis of poly-phenylalanine by human tonsil ribosomes

| Tenuazonic acid (molarity) | [$^{14}$C]Phenylalanine incorporated | | | |
|---|---|---|---|---|
| | Yeast system | | Human tonsils system | |
| | pmoles | % control | pmoles | % control |
| None | 4.53 | 100 | 1.18 | 100 |
| $10^{-5}$ | 4.53 | 100 | 1.11 | 94 |
| $10^{-4}$ | 4.58 | 101 | 1.04 | 88 |
| $5 \times 10^{-4}$ | – | – | 0.67 | 57 |
| $10^{-3}$ | 4.62 | 102 | 0.44 | 37 |

Yeast tRNA was charged with [$^{14}$C]Phenylalanine. Incorporation was studied as described elsewhere using purified EF 1 and EF 2 in the human tonsil system and a crude supernatant fraction in the yeast system.

Table 7. The puromycin reaction by hybrid ribosomes. Effects of tenuazonic acid

| Ribosome subunits | | Tenuazonic acic (molarity) | Ac-Phe-puromycin formation | |
|---|---|---|---|---|
| 60S | 40S | | pmoles | % Control |
| Human tonsils | Yeast | – | 1.37 | 100 |
| Human tonsils | Yeast | $10^{-4}$ | 1.37 | 100 |
| Human tonsils | Yeast | $10^{-3}$ | 0.88 | 64 |
| Human tonsils | Yeast | $10^{-2}$ | 0.32 | 23 |
| Yeast | Human tonsils | – | 1.61 | 100 |
| Yeast | Human tonsils | $10^{-4}$ | 1.61 | 100 |
| Yeast | Human tonsils | $10^{-3}$ | 1.66 | 103 |
| Yeast | Human tonsils | $10^{-2}$ | 1.63 | 101 |

An experimental system was used similar to that described in Table 5 but using hybrid ribosomes (see Fig. 2).

An identical value of $K_d$ for tenuazonic acid was obtained for two different concentrations of this antibiotic suggesting that this compound involves the same ribosomal set of sites in human tonsil ribosomes as anisomycin but with an affinity 12–13 times smaller.

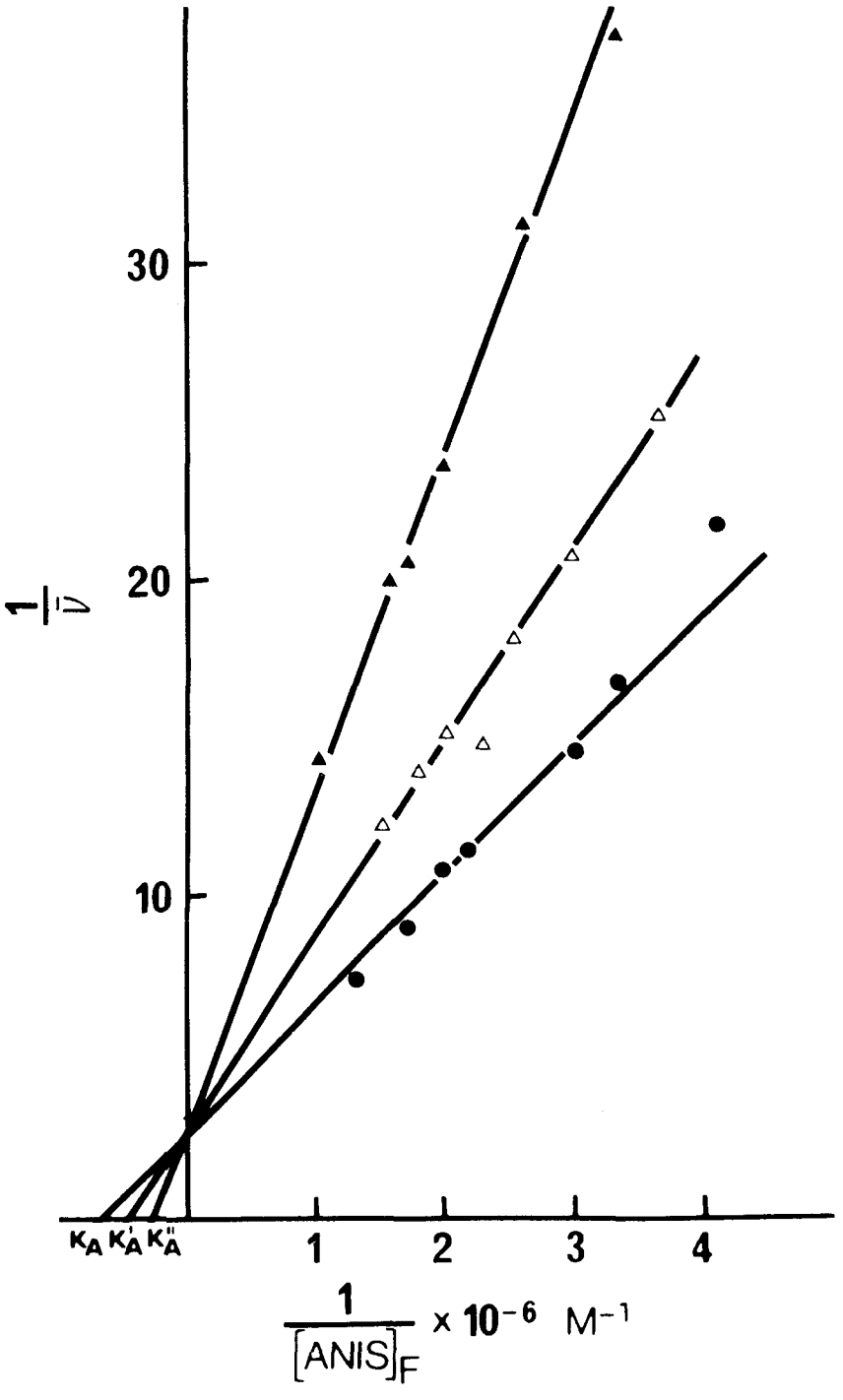


Fig. 4: Calculation of the affinity constant of tenuazonic acid for human tonsil ribosomes. (●——●) Klotz plot for [$^3$H]anisomycin binding in the absence of tenuazonic acid ($K_d$ = 1.7 x $10^{-6}$ M); (△——△) Klotz plot for [$^3$H]anisomycin binding in the presence of $10^{-5}$ M tenuazonic acid; (▲——▲) Klotz plot for [$^3$H]anisomycin binding in the presence of 3 x $10^{-5}$ M tenuazonic acid.

Ribosome concentration was 3 x $10^{-6}$ M. [$^3$H]anisomycin concentration was ranging from 0.5 to 1.5 x $10^{-6}$ M. The experiment was carried out at 0° following the sedimentation method quoted under Materials and Methods.

Table 8. Effects of NEM on the peptidyl transferase centre of ribosomes

| Source of ribosomes | Additions | Fragment reaction (cpm) | % Control |
|---|---|---|---|
| Human tonsils | None | 1710 | 100 |
| | 5 mM NEM | 2957 | 173 |
| Yeast | Non | 1276 | 100 |
| | 5 mM NEM | 1313 | 102 |
| Phaseolus vulgaris | None | 1950 | 100 |
| | 5 mM NEM | 2454 | 126 |
| Escherichia coli | None | 2975 | 100 |
| | 5 mM NEM | 3073 | 103 |

Fragment reaction assay was carried out as indicated under Material and Method using CACCA-[$^3$H]Leu-Ac (6 nmolar; 6000 cpm/tube) as a donor substrate and puromycin (1 mM) as an acceptor substrate.

## Discussion

The results presented in this contribution show that adrenochrome, pyrocatechol violet, ATA, edeine $A_1$, chartreusin and tetracycline block substrate binding to both the A- and the P-sites of human tonsil ribosomes.

Pederine, diphtheria toxin and the tylophora alkaloids: cryptopleurine, tylophorine and tylocrebrine inhibit substrate translocation from the A- to P-site of the ribosome. Fusidic acid also inhibits translocation but only when either limited amounts of EF 2 or a large excess of free ribosomes are added to the system (21).

Sparsomycin, trichodermin, anisomycin and tenuazonic acid are good inhibitors of peptide bond formation in all the different experimental systems used. Inhibition of peptide bond formation by sparsomycin and anisomycin is common to other eukaryotic systems previously described (4, 8). However, trichodermin is a better inhibitor in human tonsil than in yeast ribosomes (22). The selective action in mammalian ribosomes is more remarkable in the case of tenuazonic acid since this antibiotic is a good inhibitor of peptide bond formation by human tonsil and pig liver ribosomes, has reduced activity on *Phaseolus vulgaris* and *Euglena gracilis* ribosomes and practically has no effect on yeast ribosomes (18). This is indeed the first case in which such a selective activity of an antibiotic in mammalian ribosomes has been reported. Parallel interesting differences were observed in the peptidyl transferase centre of these ribosome preparations since N-ethyl-maleimide strongly enhances the activity of human tonsil ribosomes in the fragment reaction but does not have a similar effect on yeast, *Euglena gracilis, Phaseolus vulgaris* and *Escherichia coli* ribosomes (Table 8).

The antibiotics actinobolin, blasticidin S and gougerotin were found in this work to be very poor inhibitors of peptide bond formation in our model systems for the puromycin reaction but were quite active in blocking peptide bond formation in the fragment reaction since it is a more resolved system for the reaction.

Concerning the site of action of tenuazonic acid on the peptidyl transferase centre it appears that the antibiotic act on the same site(s) as anisomycin and the $K_d = 2 \times 10^{-5}$ M was calculated for its interaction with human tonsil ribosomes whereas a $K_d = 1.7 \times 10^{-6}$ M has been calculated for the anisomycin interaction. This should be expected since anisomycin is 10—20 times more active than tenuazonic acid in the puromycin and fragment reaction by human tonsil ribosomes (7). Tenuazonic acid is hardly active on yeast ribosomes and therefore has no significant inhibitory effect of [$^3$H]anisomycin to yeast ribosomes.

Acknowledgement

This work was supported by a grant from the Fundacion March.

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