INHIBITION OF THE PUROMYCIN REACTION WITH BENZAMIDINE AND RELATED COMPOUNDS

Aruna BHUTA and Jiří ŽEMLIČKA*

Michigan Cancer Foundation and Department of Oncology, Wayne State University School of Medicine, Detroit, MI 48201, USA

Received 3 March 1982

1. Introduction

The similarity between the mode of action of proteolytic enzymes and ribosomal peptidyltransferase-catalyzed peptide bond formation is of considerable current interest [1,2]. Peptidyltransferase can function essentially in three possible ways:

(i) Via a double displacement mechanism involving an acyl-enzyme (ribosome) intermediate as found in serine proteases [3];

(ii) By a direct transfer of peptidyl residue to aminoacyl function without forming a covalent ribosomal intermediate or catalytic participation of the ribosome [4]; or

(iii) Via a concerted cyclic mechanism which includes both transfer of peptidyl moiety and proton [5] from a suitably oriented functional group on the ribosome.

One approach to an evaluation of possibility (i) is to study a response of some known inhibitors of proteolytic enzymes in a suitable model system of peptidyltransferase-catalyzed peptide bond formation. Thus, it was reported [6] that phenylboronic acids, a group of chymotrypsin inhibitors [7], inhibited the fragment reaction and also, to a lesser extent, the reaction of *N*-Ac-Phe-tRNA with puromycin. Unlike proteases, which are fairly specific for distinct amino acids, ribosomal peptidyltransferase is capable of catalyzing peptide-bond formation with aminoacyl tRNAs derived from all 20 amino acids. It is then likely that peptidyltransferase can incorporate the active site features of proteolytic enzymes other than simply those of chymotrypsin. Therefore, it was of interest to study a group of aromatic amidines, which are known to inhibit trypsin [8] as possible inhibitors of peptidyltransferase.

2. Materials and methods

All amidine derivatives, L-arginine and sodium benzoate, used here, were commercial products of the highest available purity. β-Phenylethylboronic acid was prepared as in [9,10]. The pH of all inhibitor solutions was adjusted to 7.4 with HCl or NaOH prior to the assay. Inhibition of the puromycin reaction was performed as in [11]. A typical reaction mixture contained in 0.1 ml: 0.05 M Tris–HCl (pH 7.4), 0.1 M NH_4Cl, 0.01 M $MgCl_2$, 4.0 A_{260} units of NH_4-washed 70 S ribosomes from *E. coli* MRE 600 cells, 10 g poly(U), 0.20 A_{260} units of *N*-Ac-[^{14}C]Phe-tRNA (~5200 cpm, spec. act. 0.84 nmol [^{14}C]phenylalanine/mg tRNA), puromycin 1×10^{-5} M and inhibitor at desired concentrations. Following the incubation at 37°C for 30 min, the reaction was stopped by addition of 0.1 ml 0.1 M $Be(NO_3)_2$ in 0.3 M acetate buffer (pH 5.5) saturated with $MgSO_4$ and the products were extracted with 1.5 ml ethyl acetate. Ethyl acetate phase (1 ml) was transferred to a scintillation vial and the radioactivity was determined in 10 ml Scinti Verse™ scintillation mixture (Fisher Scientific Co., Fair Lawn NJ) in a Packard Tri-Carb liquid scintillation spectrometer at 73% counting efficiency. Percent inhibition represents the difference in ethyl acetate extracted *N*-acetyl-L-[^{14}C]phenylalanylpuromycin counts in the absence and in the presence of inhibitors. Further experimental details are provided in the figure legends.

3. Results and discussion

The results of inhibition of the puromycin reaction with benzamidine and some related compounds are summarized in fig.1. It is clear that carboxamidine

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

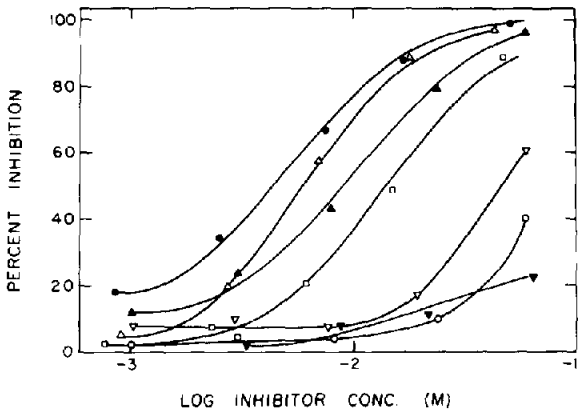


Fig.1. The inhibition of peptidyltransferase-catalyzed *N*-Ac-Phe-puromycin formation by amidine derivatives and sodium benzoate. For assay conditions see section 2: *m*-nitrobenzamidine · HCl (●); *p*-aminobenzamidine · 2 HCl (△); benzamidine · HCl (▲); *N*-phenylguanidine carbonate (□); L-arginine (▽); sodium benzoate (○); and acetamidine · HCl (▼).

derivatives containing an aromatic (phenyl) group were the most potent inhibitors. Thus, *m*-nitrobenzamidine appears as the most active compound followed closely by *p*-aminobenzamidine and benzamidine itself. *N*-Phenylguanidine was the weakest inhibitor of the investigated aromatic derivatives containing a carboxamidine group. Removal of the phenyl group led to a substantial decrease of the inhibitory activity. Thus, both arginine and acetamidine did not inhibit puromycin reaction at $<10^{-2}$ M. This agrees with [12] where arginine inhibited the fragment reaction between C(U)–A–C–C–A–(AcLeu) and puromycin at $\sim10^{-2}$ M, but was without effect on the puromycin reaction with intact *N*-Ac-Phe-tRNA. Finally, the importance of carboxamidine moiety, apart from the effect of the aromatic portion, follows from an experiment with sodium benzoate. The latter anion, which is sterically similar to benzamidine but has an opposite charge, was virtually devoid of activity.

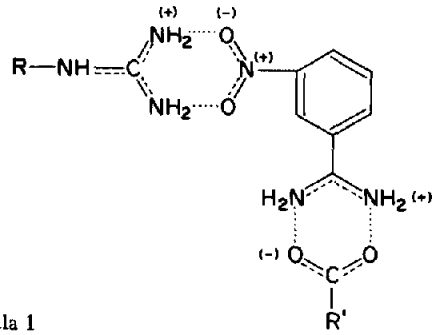
A comparison of the inhibitory potency of aromatic amidines toward peptidyltransferase and trypsin is of additional interest (table 1). Although the inhibition is lower in peptidyltransferase-catalyzed than in trypsin-catalyzed reaction, the pattern is strikingly similar. The small differences between *p*-amino- and *m*-nitrobenzamidine and the fact that the *m*-nitro derivative is more inhibitory than the *p*-amino compound is difficult to reconcile with electronic effects of the respective substituents. The *m*-nitro derivative

Table 1
Inhibition of trypsin and peptidyltransferase-catalyzed reactions with amidine derivatives

Compound	K_i (M × 10^5) (trypsin)[a]	ID_{50} × 10^3 (peptidyltransferase)
Benzamidine	1.84	8.0[b]
p-Aminobenzamidine	0.825	5.8
m-Nitrobenzamidine	–	4.3
N-Phenylguanidine	7.25	12.0
Acetamidine	3.650	[c]

[a] Values taken from [8]
[b] $K_i = 5.5 \times 10^{-3}$ M, as determined from Lineweaver-Burk plot (fig.2)
[c] About 20% inhibition of 0.1 M

was the strongest inhibitor of the fragment reaction among the group of phenylboronic acids studied in [6]. This observation was attributed to the corresponding substituent effect which had also been invoked in the case of chymotrypsin [7]. However, the same explanation is insufficient to account for the observed high inhibitory activity of *m*-nitrobenzamidine because electronic effects would lead to a decrease in the basicity of the carboxamidine group and, presumably, the extent of inhibition. Therefore, such influences as the positioning of the inhibitor between groups of the opposite charge in the binding site, which could offset the adverse influence on the basicity (formula 1), should be considered. Comparison of the inhibitory potency of both groups of inhibitors is also of interest. Thus, phenylboronic acid at 1×10^{-2} M caused ~30% inhibition [6] of the puromycin reaction with *N*-Ac-Phe-tRNA whereas the same level of inhibition with benzamidine was reached at $\sim5 \times 10^{-3}$ M (fig.1). Therefore, the extent of inhibition appears to be similar in both groups of low-M_r inhibitors.



Formula 1

Experiment with β-phenylethylboronic acid (I), a strong inhibitor of chymotrypsin [13], has indicated that differences between the interaction of boronic acid derivatives with chymotrypsin and peptidyltransferase may be more substantial than simply the degree of inhibition. Thus, compound I was non-inhibitory up to 2.6×10^{-3} M despite the fact that the K_i values of chymotrypsin inhibition with phenylboronic [7] and β-phenylethylboronic acid [13] are 2×10^{-4} and 4×10^{-5} M, respectively. Interesting differences were also found in the enzyme kinetics of phenylboronic acids and benzamidines in the peptidyltransferase-catalyzed reactions. Thus, the former are competitive inhibitors [6] of puromycin, acting at the A-site of peptidyltransferase, in contrast to benzamidine which is a non-competitive inhibitor (fig.2,3). Consequently, benzamidine must act at a locus different from the A-site. It should be emphasized that benzamidine is a competitive inhibitor of trypsin [8] that is located

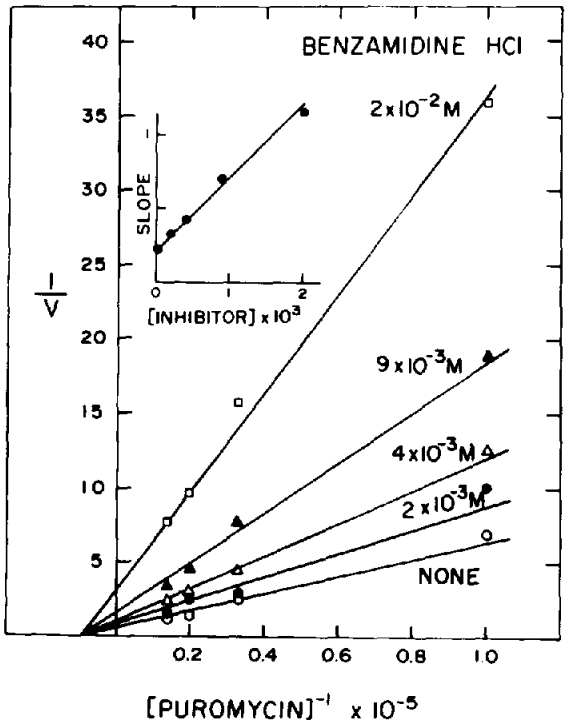


Fig.2. Lineweaver-Burk plot of the effect of benzamidine · HCl on the rate of *N*-Ac-Phe-puromycin formation. The initial velocity was determined as the amount of radioactivity extracted in 1 ml ethyl acetate in the first 5 min of reaction at 32°C. For experimental conditions see section 2. Inset shows the linear relationship between the concentration of benzamidine · HCl and the slope obtained from the plot.

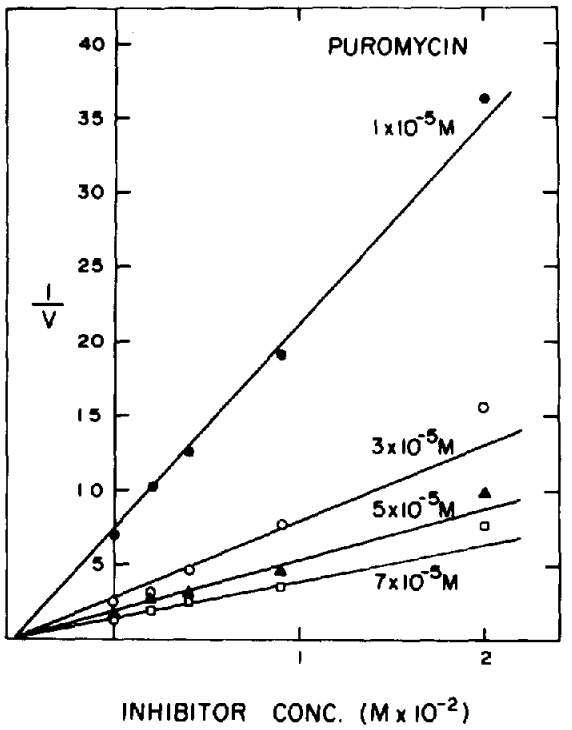


Fig.3. Dixon plot of the effect of benzamidine · HCl on the rate of *N*-Ac-Phe-puromycin formation. For conditions see fig.2 and section 2.

between the anionic and hydrophobic portion of the enzyme active site [14]. A similar interaction of benzamidine can then take place with the requisite portion of the ribosome, presumably the P-site. It is even possible that the A-site of peptidyltransferase resembles more closely chymotrypsin than trypsin whereas the opposite is true for the P-site.

Comparison of benzamidine with a somewhat structurally similar derivative, arginine, is also of interest. The latter, which is apparently a weaker inhibitor than benzamidine, inhibited the fragment reaction [12] competitively with 2 molecules bound to the A-site of peptidyltransferase. However, only one molecule of non-competitive inhibitor benzamidine is involved in binding, as shown by linearity of the slope *vs* inhibitor concentration plot (fig.2, inset).

A generally weaker response of peptidyltransferase-catalyzed peptide bond formation toward low-M_r inhibitors, such as phenylboronic acids [6] or benzamidines relative to that of chymotrypsin- [7] or trypsin-catalyzed [8] reactions, is probably the consequence of a complexity of the ribosome and hence stricter

structural requirements for inhibition. A high affinity of *N*-Ac-Phe-tRNA toward the P-site could also affect the magnitude of inhibition.

Acknowledgements

Our thanks are due to Dr Prakash Bhuta for his preparation of *N*-Ac-[^{14}C]Phe-tRNA. This investigation was supported in part by US Health Service Research grant GM-21093 from the Institute of General Medical Sciences and in part by an institutional grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit.

References

[1] Nierhaus, K. H., Schulze, H. and Cooperman, B. S. (1980) Biochem. Int. 1, 185–192.
[2] Rychlík, I. and Černá, J. (1980) Biochem. Int. 1, 193–200.
[3] Blow, D. M. (1976) Acc. Chem. Res. 9, 145–152.
[4] Krayevsky, A. A. and Kukhanova, M. K. (1979) Prog. Nucleic Acids Mol. Biol. 23, 1–51.
[5] Cheney, V. M. (1974) J. Med. Chem. 17, 590–599.
[6] Černá, J. and Rychlík, I. (1980) FEBS Lett. 119, 343–348.
[7] Philipp, M. and Bender, M. L. (1969) Proc. Natl. Acad. Sci. USA 68, 478–480.
[8] Mares-Guia, M. and Shaw, E. (1965) J. Biol. Chem. 240, 1579–1585.
[9] Yabroff, D. L., Branch, G. E. K. and Bettman, B. (1934) J. Am. Chem. Soc. 56, 1850–1857.
[10] Bean, F. R. and Johnson, J. R. (1932) J. Am. Chem. Soc. 54, 4415–4425.
[11] Murata, M., Bhuta, P., Owens, J. and Žemlička, J. (1980) J. Med. Chem. 23, 781–786.
[12] Palacián, E. and Vazquez, D. (1979) Eur. J. Biochem. 101, 469–473.
[13] Koehler, K. A. and Lienhard, G. E. (1971) Biochemistry 10, 2477–2483.
[14] Krieger, M., Kay, L. M. and Stroud, R. M. (1974) J. Mol. Biol. 83, 209–230.