INHIBITORS OF THE REACTION BETWEEN PUROMYCIN AND POLYLYSYL-RNA* IN THE PRESENCE OF RIBOSOMES

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Puromycin specifically inhibits protein synthesis in cellular and in cell-free systems and has been shown to cause the premature release of polypeptide chains; in this process a peptide bond is formed between the free amino group of puromycin (Fig. 1) and the t-RNA-bound carboxy terminus of the growing polypeptide chain which is then released from the ribosome (Smith et al., 1965 and original references therein). This reaction can be considered as a model for peptide bond formation on the ribosome, in which puromycin substitutes for the normal substrate amino acyl-RNA (Fig. 1). Rychlik (1966) has

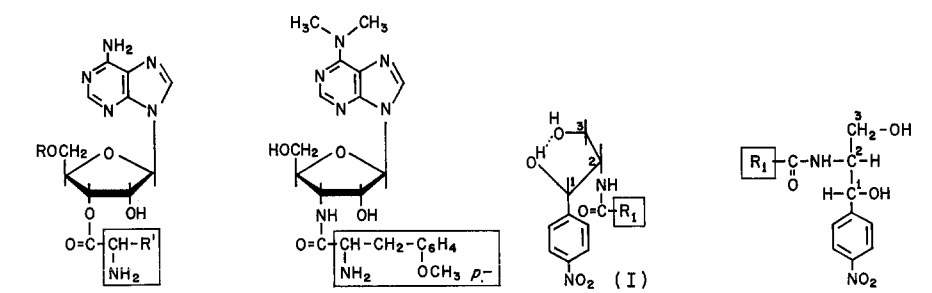

Fig. 1. Structures of amino acyl RNA (partial), of puromycin, and of chloramphenicol and its amino acyl-analogs. R represents the remainder of t-RNA and R' the side chain of an amino acid. The structure of the side chain R_1 for chloramphenicol and its analogs is given in Table II. Structure I gives the assumed conformation of chloramphenicol and its analogs in solution (Coutsogeorgopoulos, 1966).

* Abbreviations used: RNA, ribonucleic acid; t-RNA, transfer RNA; poly A, polyadenylic acid; PCA, perchloric acid; H_2N-CA, "chloramphenicol base", D-threo-1-p-nitrophenyl-2-amino-1,3-propanediol. For further abbreviations see Table II.

described a system, composed of polylysyl-RNA, washed ribosomes from E. coli and polyA, in which the puromycin reaction takes place. Chloramphenicol has been found to inhibit the puromycin reaction in this system (Rychlik, 1966) as well as in an analogous system using polyphenylalanyl-RNA (Traut and Monro, 1964).

Synthetic derivatives of chloramphenicol, in which the dichloracetyl moiety has been replaced by one of several amino acyl-groups (Fig. 1), inhibited the poly U or poly UC (1 : 1) directed polyphenylalanine synthesis in a cell-free system from E. coli (Coutsogeorgopoulos, 1966). In this communication we show that the same analogs of chloramphenicol are antagonists of the puromycin reaction and that the $-CHCl_2$ group in the chloramphenicol molecule can be replaced by a suitable $-CH(NH_2)R'$ group (Table II) without loss of activity in inhibiting this reaction. The suggestion is made that chloramphenicol and its amino acyl-derivatives inhibit the puromycin reaction by acting as analogs of amino acyl-RNA. Two other selective inhibitors of protein synthesis, namely amicetin (Bloch and Coutsogeorgopoulos, 1966) and blasticidin S (Yamaguchi et al., 1966) are also shown to inhibit the puromycin reaction in this system.

Materials and Methods: Ribosomes were prepared according to Nirenberg and Matthaei (1961) from E. coli B cells except that they were washed three times with 0.01 M $MgCl_2$-0.01 M Tris-HCl, pH 7.4 - 0.50 M NH_4Cl, stored in ice in the washing buffer, and used directly in the assays without previous dialysis (procedure adapted from the work of M. Gottesman and F. Lipmann; personal communication). $[C^{14}]$-L-polylysyl-RNA was prepared essentially as described by Rychlik (1965) by using the preincubated S-30 fraction of Nirenberg and Matthaei (1961), deacylated soluble-RNA of E. coli B (General Biochemicals) and $[C^{14}]$-L-lysine (uniformly labeled) of 50 μc per μmole (New England Nuclear Corporation). PolyA was purchased from Miles Chemical Co. and puromycin from Nutritional Biochemicals. Chloramphenicol was a gift from Parke-Davis and Co. and blasticidin S was generously provided by Professor Hiroshi Yonehara of the University of Tokyo, Japan. The sample of amicetin used was prepared by the Upjohn Co., batch No. 9924. The amino acyl analogs of chloramphenicol were prepared as described previously (Coutsogeorgopoulos, 1966). The puromycin reaction (formation of polylysyl-puromycins) was followed by determining the decrease in the radioactivity

precipitable by cold PCA, i.e. the radioactivity associated with t-RNA (Rychlik, 1966).

TABLE I

Inhibition of the Puromycin Reaction by Several Inhibitors

Conditions	Radioactivity in the cold PCA precipitate: Without puromycin cpm	Radioactivity in the cold PCA precipitate: With 10^{-4}M puromycin cpm	Radioactivity released by puromycin cpm	% Inhibition of the puromycin reaction
Complete	10,500	5,450	5,050	-
minus poly A	10,060	9,252	808	-
plus H_2N-CA	10,341	6,049	4,292	15
plus chloramphenicol	10,408	9,903	505	90
" p-mtx-phen-CA	10,346	9,942	404	92
" glycyl-CA	10,393	9,535	858	83
" leucyl-CA	10,378	8,358	2,020	60
" phen-CA	10,417	8,195	2,222	56
plus amicetin	10,309	9,047	1,262	75
plus blasticidin S	10,398	9,186	1,212	76

The complete system contained in 0.25 ml: 25μmoles of Tris-HCl buffer (pH 7.2), 25μmoles of ammonium chloride, 2.5μmoles of magnesium acetate, 40μg of poly A, 0.28 mg (protein) of washed ribosomes (20μliter) and 2.8 ODU at 260 mμ of [C^{14}]-poly-lysyl-RNA, corresponding to 11,200 cpm per 0.1 ml of incubation mixture. Incubation time 15 min at 37°. The reagents were added in the order cited. Puromycin and the inhibitors were added sequentially after the addition of poly A and before the addition of the ribosomes. At the end of the incubation period two 0.1 ml aliquots were applied on 3 MM Whatman paper discs and the radioactivity of the cold PCA precipitable material was determined as described previously (Bloch and Coutsogeorgopoulos, 1966). The cold PCA precipitable radioactivity decreased as follows (in cpm) after incubation with 10^{-4}M puromycin at 37°, in the absence of inhibitors: (0 min) 11,130; (5 min) 8,707; (15 min) 5,450; (40 min) 3,259. Concentrations of inhibitors: H_2N-CA, chloramphenicol and analogs 10^{-3}M; amicetin and blasticidin S 10^{-4}M.

Results: Under our conditions the puromycin reaction depends on the presence of poly A and is inhibited by chloramphenicol and its amino acyl-analogs, and by amicetin and blasticidin S (Table I). These inhibitors, in the absence of puromycin, do not cause any appreciable decrease of radioactive polylysine attached to t-RNA, indicating that free polylysine chains are not released. The "chloramphenicol base" (H_2N-CA) does not inhibit the puromycin reaction to an appreciable extent and the same result was found when H_2N-CA (10^{-3}M) was used together with 10^{-3}M of L-p-methoxyphenylalanine, glycine, L-leucine or L-phenylalanine. This demonstrates that the inhibition caused by the

amino acyl-analogs of chloramphenicol is not due to amino acids liberated from the analogs during the incubation.

TABLE II

Relation of Structure to the Degree of Inhibition of the Puromycin Reaction in the Chloramphenicol Series

R_1 group in structure I of Fig. 1	Name	% Inhibition at 10^{-4}M
$-CHCl$ $\quad\vert$ $\quad Cl$	Chloramphenicol (abbr. chloramph.)	81
$-CHCH_2-C_6H_4-OCH_3$ p- $\quad\vert$ $\quad NH_2$	L-p-methoxyphenylalanyl-chloramphenicol analog (abbr. p-mtx-phen-CA)	80
$-CH_2$ $\quad\vert$ $\quad NH_2$	Glycyl-chloramphenicol analog (abbr. gly-CA)	77
$-CHCH_2C_6H_5$ $\quad\vert$ $\quad NH_2$	L-phenylalanyl-chloramphenicol analog (abbr. phen-CA)	43
$-CHCH_2CH(CH_3)_2$ $\quad\vert$ $\quad NH_2$	L-leucyl-chloramphenicol analog (abbr. leu-CA)	31

The conditions for the puromycin reaction in the presence of the inhibitors are given in Table IV. Concentration of puromycin 5×10^{-6}M; concentration of inhibitors 10^{-4}M.

In the chloramphenicol series the structure of the side chain R_1 (Fig. 1, I) strongly influences the degree of inhibition (Table II). Tables III and IV show that there is a degree of competition between the inhibitors studied and the substrate puromycin. At a constant level of puromycin the inhibition is increased with increasing concentrations of the inhibitor (Table III). Conversely, at constant levels of the inhibitors the inhibition is decreased with increasing concentrations of the substrate puromycin (Table IV). Table III also shows that amicetin is five times, whereas blasticidin S is ten times, more potent than chloramphenicol.

Increasing the concentration of the other substrate, polylysyl-RNA, up to eight fold did not influence the percentage inhibition (conditions of Table I, 10^{-4}M puromycin, 10^{-4}M inhibitors, data not shown). This result indicates that there is no competition between the inhibitors and the polylysyl-RNA substrate.

TABLE III

Effect of the Concentration of Inhibitor on the Degree of Inhibition of the Puromycin Reaction

	Concentration of inhibitor (M)					
	10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4}	10^{-3}
Inhibitor	percent inhibition of the puromycin reaction					
chloramph.	-	13	42	51	67	90
p-mtx-phen-CA	-	3	28	35	71	92
gly-CA	-	1	23	29	57	83
phen-CA	-	3	16	19	43	56
leu-CA	-	0	7	15	42	60
amicetin	22	42	55	68	-	-
blasticidin S	15	52	68	80	-	-

Conditions of incubation identical with those of Table I; 10^{-4} M puromycin, 37°, 15 min.

TABLE IV

Effect of the Concentration of the Substrate Puromycin on the Degree of Inhibition

	Concentration of puromycin (M)				
	5×10^{-6}	7×10^{-6}	12×10^{-6}	24×10^{-6}	10^{-4}
Inhibitor	percent inhibition of the puromycin reaction				
chloramph.	81	78	74	66	51
p-mtx-phen-CA	80	77	71	61	35
gly-CA	77	75	68	59	29
phen-CA	43	32	27	25	19
leu-CA	31	29	22	20	15
amicetin	71	68	64	60	52
blasticidin S	82	79	75	68	52

Conditions of incubation identical with those of Table I. The concentrations of inhibitors were as follows: for chloramphenicol and its analogs 10^{-4} M, for amicetin 4×10^{-5} M and for blasticidin S 10^{-5} M. Incubation at 37° for 15 min. The corresponding radioactivity released in the presence of increasing concentrations of puromycin, in the absence of inhibitors, was in cpm (starting with 5×10^{-6} M puromycin): 2,221; 2,561; 3,219; 4,056; 5,050.

Discussion: The replacement of the $-CHCl_2$ group of chloramphenicol by a $-CH(NH_2)$ R' group (Fig. 1) results in the retention of its ability to inhibit the puromycin reaction to various degrees depending on the structure of R' (Table II). A functional similarity is thus revealed between the $-CHCl_2$ and $-CH(NH_2)$ R' groups when they are bound to the rest of the chloramphenicol structure. It is suggested that this structure (shown in Fig. 1 outside the squared group R_1) can act as a "handle" and hold group R_1 in a position in which it is capable of inhibiting the participation of puromycin in peptide bond formation with an efficiency which depends on the structure of the side chain R_1. As shown in Fig. 1 a structural similarity exists between puromycin, amino acyl-RNA and the amino acyl-analogs of chloramphenicol. This similarity is suggested to be the reason for the inhibition of the puromycin reaction by the amino acyl analogs of chloramphenicol, and for the observed competition between these analogs and the substrate puromycin (Table III, IV). On the basis of this structural similarity, and assuming that puromycin participates in peptide bond formation in the same way as the normal substrate amino acyl-RNA, these findings suggest that the amino acyl-derivatives of chloramphenicol act as analogs of amino acyl-RNA, a hypothesis which has been previously advanced to explain the inhibitory activity of the same derivatives in polypeptide synthesis (Coutsogeorgopoulos, 1966). These results also suggest that the amino acyl-analogs of chloramphenicol interfere with polypeptide synthesis by inhibiting the peptide bond forming steps per se, much as chloramphenicol itself (Traut and Monro, 1964; Julian, 1965). The present work further indicates that, in inhibiting the puromycin reaction, the $-CHCl_2$ group in the chloramphenicol molecule plays the role of the $-CH(NH_2)$ R' group in its amino acyl-analogs. On this basis chloramphenicol, too, could be viewed as an analog of amino acyl-RNA.

The pattern of inhibition of the puromycin reaction by amicetin and blasticidin S was found to be similar to that of chloramphenicol and its amino acyl-derivatives (Table III, IV). A similar activity of blasticidin S in an analogous system has recently been reported by Yamaguchi and Tanaka (1966). Gougerotin is a structurally related antibiotic and has been also found to inhibit the puromycin reaction (Clark and Chang, 1965; Casjens and Morris, 1965). Amicetin, blasticidin S and gougerotin are N-glycosides of cytosine

and contain amino acids or peptides linked through their carboxyl group to the rest of the molecule by an amide bond. A comparison between the structures of these N-glycosides and those of the amino acyl-analogs of chloramphenicol (Fig. 1) leads to the following proposal: A generalized requirement for the inhibition of the puromycin reaction, and by inference of the analogous reaction of amino acyl-RNA, appears to be the presence of amino acids or peptides linked through their carboxyl to an amino function of a suitable structure containing either cytosine, as has been noted (Fox et al., 1966), or a p-nitrophenyl group.

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