SCREENING ASSAYS FOR PROTEIN SYNTHESIS INHIBITORS

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Protein synthesis has been an attractive target for antibacterials, and several such agents are currently in clinical use. Most known protein synthesis inhibitors (tetracyclines, aminoglycosides, macrolides, *etc.*) are natural products. The molecular and mechanistic details of the individual reactions involved in protein synthesis have not been fully elucidated, and rationally designed inhibitors have yet to emerge.

In the present study, three protein synthesis assays were compared in *Escherichia coli* for use in inhibitor screens. The first assay measures amino acid incorporation by permeabilized *E. coli*. In this assay, cells utilize natural mRNA, and protein synthesis is initiated by natural mechanisms. The poly(*U*)-directed poly(Phe) synthesis assay[1] and the poly(*A*)-directed poly(Lys) synthesis assay[2~4] are cell-free systems using partially-purified protein synthesis factors, ribosomes, and synthetic mRNA. Several known inhibitors of protein synthesis that act by a variety of mechanisms were tested in the three assays.

Aurodox was obtained from Roche Laboratories (Nutley, NJ), norfloxacin and efrotomycin from Merck Sharp and Dohme Research Laboratories, and kirromycin was a gift of JILL BARBER of the University of Manchester.

Protein biosynthesis in permeabilized *E. coli* ATCC 25922 (American Type Culture Collection) was measured by modifications of an earlier procedure[5]. Cells were grown in 50 ml of Antibiotic Medium 3 (Difco) to an OD_{660} of 0.7, collected by centrifugation, washed with 1.0 ml of 20 mM HEPES buffer (pH 8.0), and resuspended in 0.5 ml of 20 mM HEPES buffer (pH 8.0), 5 mM ethylene glycol bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, and 2 M sucrose. After incubation at 25°C for 5 minutes, the cell suspension was diluted with 2 volumes of 20 mM HEPES (pH 8.0), and used immediately for assays. The assay mixture contained in a total volume of 20 μl: 5 μl of the resuspended cells, all 20 amino acids (5 μM each) except phenylalanine, 50 μM [^{14}C]Phe (Amersham; sp. act., 4 Ci/mmol), ATP, CTP, GTP and UTP (0.5 mM each), 10 mM pyruvate kinase, 80 mM KCl, 20 mM magnesium acetate, 0.4 mM $MnCl_2$, and 40 mM HEPES (pH 8.0). After incubation at 30°C for 30 minutes, 10 μl of 10% TCA was added, and the mixture spotted onto Whatman 3MM disks. The disks were dried, soaked twice for 30 minutes each in 5% TCA (0°C), once for 30 minutes in 95% ethanol (0°C), dried, and counted in Aquasol.

DNA or RNA synthesis in permeabilized *E. coli* were measured as described previously[5].

The *E. coli* poly(Phe) synthesis assay was conducted essentially as described previously[1,6,7]. Precharged [^{3}H]Phe-tRNA (65 pmoles), 70 S ribosomes (0.134 OD_{260} units), and a factor mixture (0.046 OD_{280} units), were incubated with 1 mM GTP and 175 μg poly(*U*) in buffer A-10 (50 mM Tris-HCl (pH 7.6), 80 mM KCl, 80 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM dithiothreitol) in a total volume of 200 μl. The reaction was initiated with GTP and poly(*U*), and terminated after 5 minutes at 37°C with 5 ml of 5% TCA. The mixture was heated for 10 minutes at 100°C, cooled, and filtered on 0.45 μm nitrocellulose membranes (Millipore). Filters were washed twice with 5 ml of 5% TCA, dissolved in Filtron X, and counted.

The *E. coli* poly(Lys) synthesis assay was identical to the poly(Phe) assay, except that poly(*A*) and pre-charged [^{3}H]Lys-tRNA were substituted for poly(*U*) and [^{3}H]Phe-tRNA, Mg^{2+} was raised to 15 mM, and the TCA used to stop the reaction contained 0.5 mg/ml sodium tungstate and 0.1 mg/ml lysine[8].

Protein synthesis in permeabilized cells retained at least 80% of the control activity in the presence of 2% ethanol or DMSO (Fig. 1). Similarly, more than 80% of the poly(Phe) synthesis activity remained in the presence of 2% ethanol or DMSO. The poly(Lys) synthesis assay was slightly more sensitive to these solvents, being inhibited by more than 20% in the presence of 1% ethanol or DMSO.

Because the permeabilized cell protein synthesis assay uses endogenous mRNA, it was next tested for sensitivity to nucleic acid synthesis inhibitors. Norfloxacin and rifampicin, inhibitors of DNA and RNA synthesis, respectively, inhibited protein synthesis also (Table 1), although their IC_{50}s were higher than for DNA or RNA synthesis. Ethidium bromide, an intercalator of nucleic acids, also inhibited protein synthesis with a higher IC_{50} than

Fig. 1. Effects of organic solvents on protein, poly(Phe), and poly(Lys) synthesis.

○ DMSO, ● ethanol.

(a) Protein synthesis in plasmolyzed cells, (b) poly(*U*)-directed poly(Phe) synthesis, (c) poly(*A*)-directed poly(Lys) synthesis.

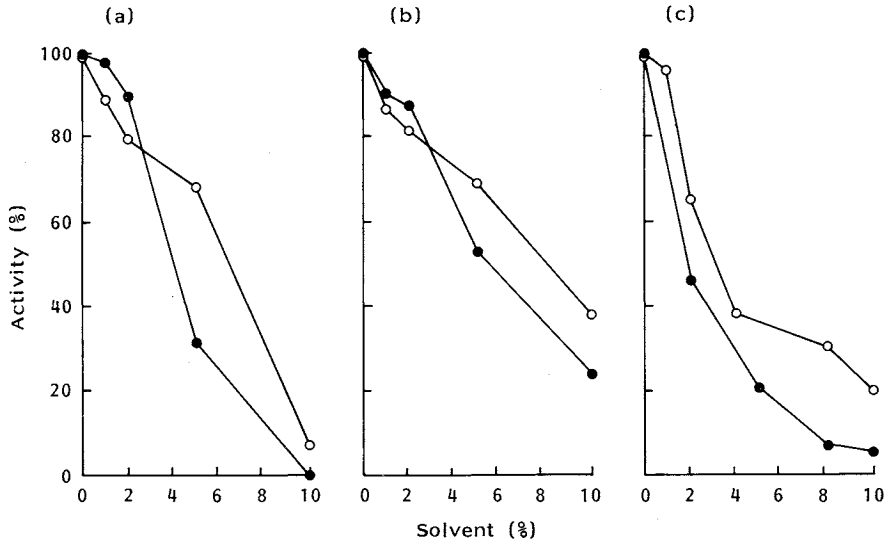

Table 1. Effects of different compounds on DNA, RNA, and protein biosynthesis in permeabilized cells.

Compound	IC_{50} (μM)			MIC (μg/ml)
	DNA[a]	RNA[b]	Protein[b]	
DNA/RNA inhibitors				
Ethidium bromide	50	5	600	100
Norfloxacin	2	100	30	0.1
Rifampicin	>100	1	9	50
Protein inhibitors				
Fusidic acid	ND[c]	>1,000	500	>100
Erythromycin	ND	>100	1	>100

[a] Toluene-treated cells.
[b] Plasmolyzed cells.
[c] ND, not determined.

DNA or RNA synthesis. Other classes of agents, such as sulfonamides, were inhibitors in this assay (data not shown). All classes of protein synthesis inhibitors tested were active in this assay, including those that act at steps other than protein elongation, such as pseudomonic acid (Table 2). Aurodox, which is inactive against intact *E. coli* but active *in vitro* against its target, was also active in this assay.

The poly(Phe) synthesis assay showed a rather narrow scope of sensitivity to protein synthesis inhibitors (Table 2). For example, erythromycin was inactive in this assay, as were chloramphenicol and spectinomycin. This could be due to some mechanistic details of the action of these protein elongation inhibitors, best documented in the case of macrolides[2,3]. The poly(Phe) synthesis assay thus has limited utility as a screening tool, although it has been widely used in biochemical studies[9].

The poly(Lys) synthesis assay was sensitive to a fairly wide range of protein synthesis inhibitors, including macrolides, chloramphenicol, and spectinomycin. Unlike the permeabilized cell system, poly(Lys) synthesis did not respond to RNA or DNA synthesis inhibitors, but unlike poly(Phe)

Table 2. Effects of antibacterials on protein, poly(Phe), and poly(Lys) synthesis.

Compound	Protein synthesis[a]	Poly(Phe) synthesis		Poly(Lys) synthesis	
	IC_{50} (μM)	IC_{50} (μM)	% Max. inh.[c]	IC_{50} (μM)	% Max. inh.
Chloramphenicol	0.8	>1,000	20	10	100
Clindamycin	400	ND[b]	ND	4.5	100
Fusidic acid	500	100	100	25	100
Gentamicin	50	0.008	60	ND	ND
Negamycin	1,000	1,000	30	ND	ND
Pseudomonic acid	20	>1,000	<5	ND	ND
Spectinomycin	40	>1,000	<5	2.2	100
Tetracycline	60	3	100	14	100
Thiostrepton	10	0.012	100	0.24	100
Macrolides					
Erythromycin	0.8	>1,000	<5	0.026	100
Midecamycin	ND	ND	ND	0.050	100
Spiramycin	ND	ND	ND	0.040	100
Tylosin	ND	ND	ND	0.073	100
Elfamycins					
Aurodox	2	0.11	100	1.6	100
Efrotomycin	50	0.10	100	ND	ND
Kirromycin	60	0.20	100	ND	ND

[a] Plasmolyzed cells.
[b] ND, not determined.
[c] Maximum inhibition.

synthesis it was sensitive to macrolides, chloramphenicol, and spectinomycin. However, the poly(Lys) synthesis assay was not sensitive to protein synthesis inhibitors that affect processes other than elongation, such as pseudomonic acid, an inhibitor of Ileu-tRNA charging[10].

In summary, the permeabilized cell assay is the most general screening tool for protein synthesis inhibitors. It is relatively inexpensive and simple and thus suitable for primary screening. However, it must be followed up by protein synthesis-specific tests, such as the poly(Phe) and poly(Lys) synthesis assays, in order to ensure that leads are indeed protein synthesis inhibitors.

References

1) RAVEL, J. M. & R. L. SHOREY: GTP-dependent binding of aminoacyl-tRNA to *Escherichia coli* ribosomes. Methods Enzymol. 20: 306~316, 1971
2) BELITSINA, N. V.; G. ZH TNALINA & A. S. SPIRIN: Template-free ribosomal synthesis of polylysine from lysyl-tRNA. FEBS Lett. 131: 289~292, 1981
3) MAO, J. C.-H. & E. E. ROBISHAW: Effects of macrolides on peptide-bond formation and translocation. Biochemistry 10: 2054~2061, 1971
4) PICKING, W. D.; O. W. ODOM, T. TSALKOVA, I. SERDYUK & B. HARDESTY: The conformation of nascent polylysine and polyphenylalanine peptides on ribosomes. J. Biol. Chem. 266: 1534~1542, 1991
5) STAUDENBAUER, W. L.: Novobiocin—a specific inhibitor of semiconservative DNA replication in permeabilized *Escherichia coli* cells. J. Mol. Biol. 96: 201~205, 1975
6) TRAUB, P.; S. MIZUSHIMA, C. V. LOWRY & M. NOMURA: Reconstitution of ribosomes from subribosomal components. Methods Enzymol. 20: 391~407, 1971
7) HALL, C. C.; J. D. WATKINS & N. H. GEORGOPAPADAKOU: Effects of elfamycins on elongation factor Tu from *Escherichia coli* and *Staphylococcus aureus*. Antimicrob. Agents Chemother. 33: 322~325, 1989
8) GOLDMAN, R. C. & S. K. KADAM: Binding of novel macrolide structures to macrolides-lincosamides-streptogramin B-resistant ribosomes inhibits protein synthesis and bacterial growth. Antimicrob. Agents Chemother. 33: 1058~1066, 1989
9) SPIRIN, A. L.: Chapter 2. Ribosome preparation and cell-free protein synthesis. *In* The Ribosome. Structure, Function, and Evolution. *Ed.*, W. E. HILL *et al.*, pp. 56~70, American Society for Microbiology, 1990
10) HUGHES, J. & G. MELLOWS: Inhibition of isoleucyl-transfer ribonucleic acid synthetase in *Escherichia coli* by pseudomonic acid. Biochem. J. 176: 305~318, 1978