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Effects of Elfamycins on Elongation Factor Tu from *Escherichia coli* and *Staphylococcus aureus*

C. C. HALL,* J. D. WATKINS, AND N. H. GEORGOPAPADAKOU
Roche Research Center, Nutley, New Jersey 07110

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Six kirromycin analogs (elfamycins) were compared on the basis of their inhibition of *Escherichia coli* poly(U)-directed poly(Phe) synthesis and stimulation of elongation factor Tu (EF-Tu)-associated GTPase activity. The elfamycins tested were kirromycin, aurodox, efrotomycin, phenelfamycin A, unphenelfamycin, and L-681,217. The last three lack the pyridone ring present in the other elfamycins. All the elfamycins inhibited poly(U)-dependent poly(Phe) synthesis and stimulated EF-Tu-associated GTPase activity, suggesting that the pyridone ring is not essential for activity. The six elfamycins were also examined in a poly(U)-directed, poly(Phe)-synthesizing system derived from *Staphylococcus aureus* and had 50% inhibitory concentrations of ≥1 mM. When *S. aureus* ribosomes and *E. coli* elongation factors were combined in a hybrid poly(Phe)-synthesizing system, aurodox produced essentially complete inhibition of poly(Phe) synthesis with a 50% inhibitory concentration of 0.13 μM. This suggests that the observed high MICs of kirromycin and its congeners in *S. aureus* reflect a kirromycin-resistant EF-Tu rather than permeability constraints.

Kirromycin and its analogs are inhibitors of bacterial protein synthesis at the elongation stage. Their target is elongation factor Tu (EF-Tu) (13); hence the name elfamycins. The mechanism of inhibition has been shown to involve formation of a nondissociable ribosome • EF-Tu • kirromycin complex (18, 19). The effects of elfamycins are consistent with the stabilization of an EF-Tu conformation which closely resembles that of the ribosome-bound, GTP-hydrolyzing form of EF-Tu which occurs during the EF-Tu-mediated binding of aminoacyl-tRNA to the ribosomal A site (4). Elfamycins promote EF-Tu-mediated binding of [^{14}C]Phe-tRNA to ribosomes in the absence of GTP, which is normally required for this process (18). These antibiotics are characterized by a unique ability to stimulate the GTPase activity of EF-Tu, even in the absence of ribosomes or aminoacyl-tRNA (13).

The antibacterial spectrum of elfamycins is limited (13). Typically, it includes *Streptococcus* species (but not staphylococci), *Clostridium* species, and *Neisseria gonorrhoeae*. Membrane permeability is known to be a critical factor for these antibiotics, as they show little or no activity against wild-type *Escherichia coli* and *Proteus vulgaris* but are active against permeability mutants of *E. coli* and L forms of *P. vulgaris* (7, 12). They also inhibit protein synthesis in *E. coli* cell extracts with 50% inhibitory concentrations as low as 0.1 μM (2, 18).

In the present study, structure-activity relationships of six kirromycin-type compounds in *E. coli* were established by using the poly(U)-directed poly(Phe) synthesis and EF-Tu-associated GTPase assays developed previously (18). The activities of these agents were further examined in a poly(Phe) synthesis assay system derived from a kirromycin-resistant organism, *S. aureus*.

MATERIALS AND METHODS

Chemicals. L-Phenyl[2,3-^{3}H]alanine (specific activity, 40 Ci/mmol) was obtained from the Amersham Corp. (Arlington Heights, Ill.); Omnifluor and [γ-^{32}P]GTP (specific activity, 20 to 40 Ci/mmol) were from New England Nuclear Corp. (Boston, Mass.); GTP, poly(U), and *E. coli* tRNA^{Phe} were from Sigma Chemical Co. (St. Louis, Mo.); isopropyl acetate (reagent grade) was from Eastman Kodak Co. (Rochester, N.Y.); Filtron X was supplied by National Diagnostics (Somerville, N.J.); and nitrocellulose filters (HAWP, 0.45-μm pore size, 25 mm diameter) were from Millipore Corp. (Bedford, Mass.).

Antibiotics. The elfamycins used in this study (Fig. 1) were obtained as follows: kirromycin was from Jill Barber (University of Manchester, United Kingdom); aurodox and L-681,217 (9) were from Roche Laboratories (Nutley, N.J.); efrotomycin was from Merck & Co., Inc. (Rahway, N.J.); phenelfamycin A and unphenelfamycin were from Abbott Laboratories (North Chicago, Ill.).

Organisms. *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 were obtained from the American Type Culture Collection (Rockville, Md.). Both organisms were grown at 37°C in Luria broth to mid-log phase and collected as cell pastes.

***E. coli* poly(Phe) synthesis assay.** Ribosomes were prepared from *E. coli* by the method of Ravel and Shorey (14). A partially purified mixture of protein synthesis factors necessary for protein elongation was prepared by the method of Traub et al. (16). This factor mixture was also used to charge *E. coli* tRNA^{Phe} with [^{3}H]phenylalanine (specific activity, 4 Ci/mmol). [^{3}H]Phe-tRNA was subsequently isolated by phenol extraction and ethanol precipitation. The *E. coli* poly(Phe) assay mixture contained, in 200 μl of A-10 buffer (50 mM Tris hydrochloride [pH 7.6], 80 mM NH_4Cl, 80 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol) (14), 0.134 optical density units at 260 nm (OD_{260} units) of *E. coli* ribosomes (approximately 3.5 pmol), 65 pmol of [^{3}H]Phe-tRNA, 0.046 OD_{280} units of *E. coli* factor mixture, 200 nmol of GTP, and 175 μg of poly(U). The reaction was started by the addition of GTP and poly(U). After 5 min of incubation at 37°C, the reaction was terminated by the addition of 5 ml of 5% trichloroacetic acid (TCA). The mixture was heated for 5 min at 95°C, cooled to room temperature, and filtered on nitrocellulose filters. The filters were washed two times with 5 ml of 10% TCA and counted in 10 ml of Filtron X.

***E. coli* GTPase assay.** EF-Tu-associated GTPase activity

* Corresponding author.

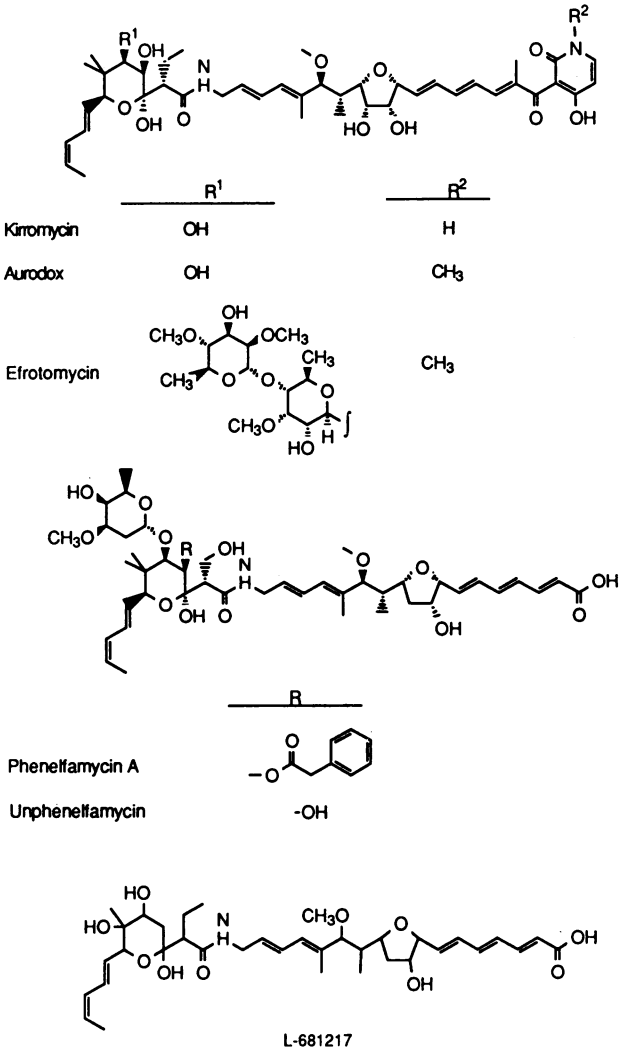

FIG. 1. Structures of kirromycin and related compounds.

was assayed by the method of Wolf et al. (18). EF-Tu was purified from *E. coli* ATCC 25922 by the method of Miller and Weissbach (11). The assay mixture contained, in 75 μl of standard buffer (60 mM Tris hydrochloride [pH 7.8], 30 mM KCl, 30 mM NH_4Cl, 10 mM $MgCl_2$, 2 mM dithiothreitol), 10 pmol of *E. coli* EF-Tu, 0.4 OD_{260} units of *E. coli* ribosomes, and 200 pmol of [γ-^{32}P]GTP (specific activity, 1 to 2 Ci/mmol). The reaction was started by the addition of [γ-^{32}P]GTP. After 10 min of incubation at 30°C, the reaction was terminated by the addition of 80 μl of 1 M perchloric acid containing 3 mM KH_2PO_4. The mixture was centrifuged at 400 × *g* for 5 min to remove precipitates, and a 100-μl sample of the supernatant was added to 300 μl of 20 mM sodium molybdate at 4°C. To this mixture, 400 μl of isopropyl acetate at 4°C was added. The mixture was vortexed vigorously for 30 s and centrifuged at 400 × *g* for 1 min to separate the layers. From the upper (organic) layer, a 50-μl sample was removed and spotted on a Whatman 3MM paper filter disk (25-mm diameter). The disk was counted in 10 ml of Omnifluor-toluene (4 g/liter).

***S. aureus* poly(U)-dependent poly(Phe) synthesis.** Cell breakage of *S. aureus* ATCC 25923 was carried out by a previously published procedure (8), and the ribosomes were collected by centrifugation at 140,000 × *g* for 150 min. The

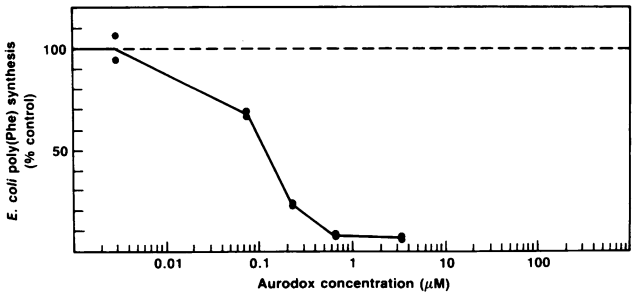

FIG. 2. Inhibition of *E. coli* poly(Phe) synthesis by aurodox. Control activity, 3 pmol of [^{3}H]Phe polymerized.

ribosomes were suspended in 10 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–0.5 M NH_4Cl–5 mM dithiothreitol and were again collected by centrifugation. They were washed once with 10 mM Tris hydrochloride (pH 7.5)–20 mM $MgCl_2$–5 mM dithiothreitol and were suspended in the same buffer for storage at −70°C. It had been reported earlier (10, 21) that for formation of 70S subunits, *S. aureus* ribosomes require 20 mM Mg^{2+} rather than 10 mM Mg^{2+}, which is optimal for *E. coli* 70S ribosomes. A mixture of partially purified *S. aureus* protein synthesis factors was prepared exactly as described above for the *E. coli* system. [^{3}H]Phe-tRNA was prepared in advance by using the *E. coli* factor mixture described above.

The *S. aureus* poly(Phe) synthesis system contained, in a total volume of 200 μl of A-20 buffer (50 mM Tris hydrochloride [pH 7.6], 80 mM NH_4Cl, 80 mM KCl, 20 mM $MgCl_2$, 5 mM dithiothreitol), 0.40 OD_{260} units of *S. aureus* ribosomes, 65 pmol of [^{3}H]Phe-tRNA, 0.118 OD_{280} units of the *S. aureus* factor mixture, 200 nmol of GTP, and 175 μg of poly(U). The incubation was started by the addition of GTP and poly(U) together and was terminated after 30 min at 37°C by the addition of 5 ml of 5% TCA. The TCA precipitate was counted as described above for the *E. coli* poly(Phe) synthesis assay.

Hybrid *E. coli-S. aureus* poly(U)-dependent poly(Phe) synthesis. The hybrid *E. coli-S. aureus* poly(U)-dependent poly(Phe) synthesis assay system was composed of *E. coli* factors and *S. aureus* ribosomes. It contained, in a total volume of 200 μl of A-20 buffer, 0.40 OD_{260} units of *S. aureus* ribosomes, 65 pmol of [^{3}H]Phe-tRNA, 0.046 OD_{280} units of the *E. coli* factor mixture, 200 nmol of GTP, and 175 μg of poly(U). The incubation was started by addition of GTP and poly(U) and was terminated after 30 min by the addition of 5 ml of 5% TCA. The TCA precipitate was counted as described above for the *E. coli* poly(Phe) synthesis assay.

Preincubation of aurodox with *S. aureus* elongation factors. Aurodox inactivation by the partially purified elongation

TABLE 1. Effects of kirromycin analogs on poly(Phe) synthesis and EF-Tu-dependent GTPase activity (*E. coli*)

Compound	EC_{50}[a] (μM)	
	GTPase	Poly(Phe)
Kirromycin	0.23	0.23
Aurodox	0.17	0.11
Efrotomycin	0.13	0.13
Phenelfamycin A	0.6	0.4
Unphenelfamycin	20	8
L-681,217	6.0	0.4

[a] Concentration producing half-maximal effect.

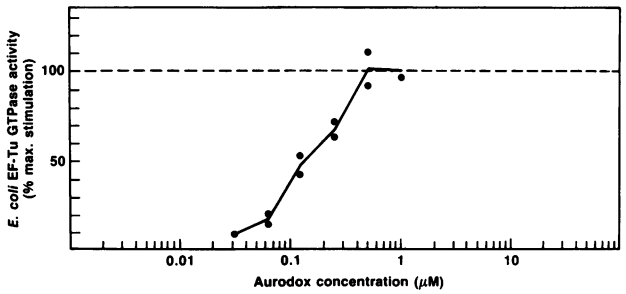

FIG. 3. Stimulation of *E. coli* EF-Tu-associated GTPase activity by aurodox. Maximum stimulation, 24 pmol of [^{32}P]phosphate generated in total assay volume.

factor mixture of *S. aureus* was investigated by incubating 93 μM aurodox in the *S. aureus* poly(Phe) synthesis assay mixture, minus *S. aureus* ribosomes, for 30 min at 37°C. The aurodox activity remaining was assayed by the addition of 4.4 μl of this preincubation mixture to the *E. coli* poly(Phe)-synthesizing system described above to produce a final aurodox concentration of 2 μM, corresponding to complete (≥95%) inhibition of poly(Phe) synthesis in *E. coli*.

RESULTS

In vitro activity of elfamycins in *E. coli*. Aurodox produced complete inhibition of *E. coli* poly(Phe) synthesis, with a 50% inhibitory concentration of 0.11 μM (Fig. 2). Kirromycin, aurodox, efrotomycin, phenelfamycin A, unphenelfamycin, and L-681,217 also produced complete inhibition in the *E. coli* poly(Phe) synthesis assay (Table 1).

Aurodox stimulated EF-Tu-dependent GTPase activity from *E. coli*, with a half-maximal effect at 0.17 μM (Fig. 3). This result was consistent with earlier reports (2). All elfamycins investigated produced a maximal degree of EF-Tu-associated GTPase stimulation comparable to that produced by aurodox. The relative potencies of the elfamycins in the two *E. coli* assays are shown in Table 1.

In vitro activity of elfamycins in *S. aureus*. Aurodox was a far less potent inhibitor of *S. aureus* poly(Phe) synthesis than of the comparable *E. coli* system (Fig. 4 and Table 2).

The mixture of *E. coli* elongation factors used in the *E. coli* poly(Phe) synthesis assay was also able to support the functions of *S. aureus* ribosomes, and the aurodox sensitivity of this hybrid system of *S. aureus* ribosomes and *E. coli* elongation factors was examined. The aurodox inhibition of this hybrid poly(Phe)-synthesizing system (Fig. 4) showed a 50% inhibitory concentration of approximately 0.1 μM, very similar to the inhibition observed with *E. coli* elongation

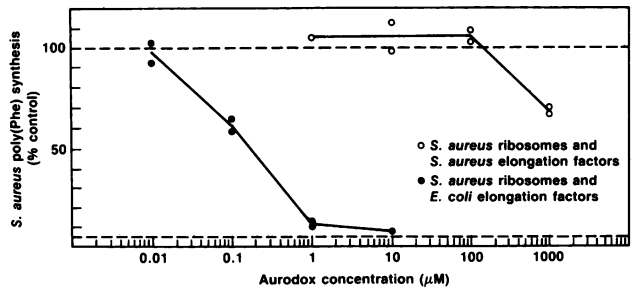

FIG. 4. Inhibition of *S. aureus* poly(Phe) synthesis by aurodox. Control activity, 3 pmol of [^{3}H]Phe polymerized.

TABLE 2. Effects of kirromycin analogs on *S. aureus* poly(Phe) synthesis

Compound	EC_{50}[a] (μM)
Aurodox	>1,000
Efrotomycin	>1,000
Phenelfamycin A	>1,000
Unphenelfamycin	>1,000
L-681,217	>1,000
Thiostrepton	0.05
Tetracycline	0.7
Fusidic acid	25

[a] Concentration producing half-maximal effect.

factors and *E. coli* ribosomes (Fig. 2). This is consistent with a resistance mechanism involving differences in components other than the ribosome, presumably EF-Tu itself. Preincubation of aurodox with *S. aureus* poly(Phe) assay components did not affect subsequent inhibition of *E. coli* poly(Phe) synthesis, suggesting that aurodox is not inactivated by the *S. aureus* poly(Phe) assay components (Table 3).

Elfamycins other than aurodox also inhibited *S. aureus* poly(Phe) synthesis only at 1 mM (Table 2). However, the *S. aureus* poly(Phe)-synthesizing system was sensitive to the nonelfamycin protein elongation inhibitors thiostrepton, tetracycline, and fusidic acid.

DISCUSSION

All kirromycin analogs examined showed complete inhibition in the *E. coli* poly(Phe) synthesis assay, as well as fivefold stimulation of the *E. coli* EF-Tu-associated GTPase activity. The structures of phenelfamycin A, unphenelfamycin, and L-681,217 are noteworthy in that they entirely lack the pyridone moiety. In these biochemical assays only modest effects on elfamycin activity were observed to result from the absence of the pyridone group. The ability of these kirromycin analogs to stimulate EF-Tu-dependent GTPase activity or inhibit poly(Phe) synthesis and the relative potency of these agents in a single biochemical assay have not been previously reported. Our observations are consistent with microbial susceptibility data (9) and recent nuclear magnetic resonance studies on kirromycin binding to EF-Tu (1). Taken together, the data indicate that the pyridone ring of kirromycin is not essential for the interaction with EF-Tu.

Kirromycin analogs show poor activity against intact *S. aureus* (typical MICs, >150 μM). Elfamycins were investigated in an *S. aureus* poly(Phe)-synthesizing, cell-free system to determine the intrinsic sensitivity of *S. aureus* protein

TABLE 3. Effect of aurodox preincubation with *S. aureus* partially purified elongation factor mixture

Condition		*E. coli* poly(Phe) synthesized (% of control)[a]
Preincubation	Incubation	
None	− Aurodox	100
None	+ Aurodox[b]	0.6
− Aurodox[c]	− Aurodox	105
− Aurodox	+ Aurodox	1.2
+ Aurodox	− Aurodox	3.0
+ Aurodox	+ Aurodox	1.2

[a] Control activity, 16 pmol of [^{3}H]Phe polymerized.
[b] Aurodox was added to the *E. coli* poly(Phe) synthesis assay only. The final aurodox concentration was 2 μM for each aurodox addition indicated.
[c] Preincubated *S. aureus* factors were added to the *E. coli* assay.

synthesis machinery to these agents in the absence of permeability barriers. In all cases, kirromycin analogs showed half-maximal inhibitory concentrations of >1 mM in the *S. aureus* poly(Phe) assay. Thus, unlike *E. coli*, *S. aureus* protein synthesis is resistant to kirromycin, and this is sufficient to explain *S. aureus* resistance to elfamycins. Because the mechanism of elfamycin inhibition of poly(Phe) synthesis has been shown to involve the formation of a nondissociable EF-Tu • ribosome complex (18), elfamycin resistance due to alterations in the ribosome permitting the dissociation of this complex is theoretically possible. However, the sensitivity to aurodox of the hybrid system composed of *S. aureus* ribosomes and *E. coli* elongation factors (Fig. 4) argues against this possibility. Resistance to elfamycins in the *S. aureus* poly(Phe) synthesis system was dependent on *S. aureus* factors and is consistent with a resistant EF-Tu. In this context, it should be noted that mutants of *E. coli* with kirromycin-resistant forms of EF-Tu which have single-amino-acid alterations in the EF-Tu sequence have been characterized (5, 15). The reported mutations either reduced the affinity of kirromycin binding to EF-Tu (17) or altered the ribosome • EF-Tu interaction so that the ribosome • EF-Tu complex was no longer irreversibly stabilized by kirromycin (6, 17). It is likely that the elfamycin resistance of *S. aureus* EF-Tu is the result of only a small number of differences between the EF-Tu protein sequences of *S. aureus* and *E. coli*. A wild-type kirromycin-resistant EF-Tu from another gram-positive organism, *Lactobacillus brevis*, has been described by Worner and Wolf (20). Bacterial phylogenetics would predict that *S. aureus* EF-Tu would show more homology to the EF-Tu of *L. brevis* than to the kirromycin-sensitive EF-Tu of *E. coli*.

Since the binding of kirromycin to EF-Tu has been shown to be competitive with EF-Ts binding (3), another possibility is that in *S. aureus*, EF-Tu exists as a very tight complex with EF-Ts, which has a low affinity for kirromycin.

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