

## Targeted Screening for Elongation Factor Tu Binding Antibiotics

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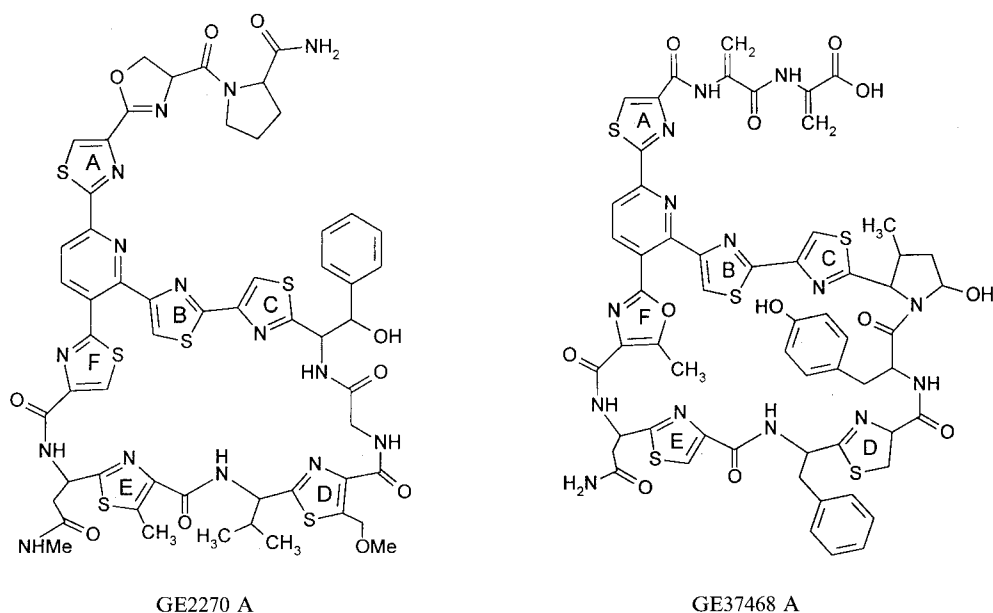
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The development of a screen targeted to antibiotics which bind elongation factor Tu (EF-Tu) is described. The method was based on selection of antimicrobial activities which were antagonized by exogenous EF-Tu. Kirromycin, a known inhibitor of EF-Tu, was positive in this screen. Among 47,000 microorganisms screened, several producers of kirromycin-type antibiotics were detected and the novel antibiotics GE2270 and GE37468 were discovered. These thiopeptide molecules constitute, along with amithiamycin, a novel class of antibiotics acting on EF-Tu.

Filamentous microorganisms and in particular actinomycetes produce a multiplicity of secondary metabolites possessing antibacterial activity. A survey of the literature shows that 6,754 microbial products are known to inhibit bacterial growth. Most of these antibiotics however are toxic to mammalian cells, belong to classes which are already exploited, or have low therapeutic value. The discovery of innovative and valuable antibacterial antibiotics is a challenging enterprise which needs the cooperative contributions of: isolation of microorganisms belonging to different and rare genera; mechanism based assays applied in high throughput mode and rapid

testing against pathogens of relevant clinical importance. Following this approach, we set up a targeted screening for elongation factor Tu (EF-Tu) binding antibiotics. This screening technique was applied to a variety of actinomycetes and filamentous fungi and led to the discovery of antibiotics GE2270 complex<sup>1-3</sup>) and GE37468<sup>4,5</sup>) (Fig. 1). Amythiamicin<sup>6-8</sup>), another functionally and structurally similar antibiotic, was recently described by another group. These antibiotics constitute a novel class of thiopeptide antibiotics acting on EF-Tu<sup>9-11</sup>). This paper describes the development of this screening program.

Fig. 1. Structures of GE2270-type antibiotics.



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## Materials and Methods

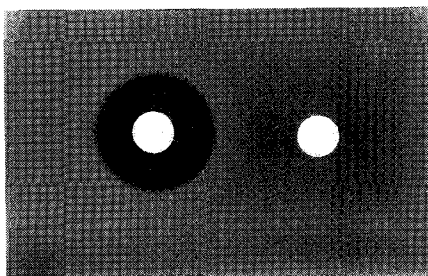
### General

Kirromycin was isolated from strain A8924<sup>12)</sup> and pulvomycin was obtained from *Streptovorticillum neotropis* according to R. J. SMITH *et al.*<sup>13)</sup>. Standard antibiotics were of commercial origin. Some of the microorganisms for screening were freshly isolated from environmental samples and the remainder were derived from the Lepetit Center strain collection.

### EF-Tu Preparation

EF-Tu was purified from cultures of *Escherichia coli* K12 grown aerobically at 37°C. on antibiotic medium 3 (DIFCO Laboratories Detroit, MI). Cultures were harvested at the maximum of exponential growth. Cells were recovered by centrifugation and were stored at -80°C. *E. coli* paste was then suspended in buffer A consisting of 20 mM Tris HCl buffer (pH 7.7), 10 mM MgCl<sub>2</sub>, 5 mM DTT, 100 μM phenylmethylsulfonyl fluoride (PMSF Sigma), 100 μM benzimidazole (Aldrich-Chemie) and 30 μg/ml leupeptin (Calbiochem). Cell disruption was performed with a Dyno-mill KDL-S apparatus (W.A. Bachofen AG Maschinenfabrik, Basel) using 0.1 ~ 0.2 mm glass beads. Cell debris was removed by two subsequent centrifugations at 30,000 *g* for 30 minutes and then at 100,000 *g* for 90 minutes. EF-Tu was precipitated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in the range from 35% to 70% of saturation. The precipitate was then dissolved in buffer A and dialysed against 20 volumes of buffer A. The dialysed preparation was diluted to 4 volumes with buffer A and adsorbed on Mono Q Fast Flow resin (Pharmacia), equilibrated with buffer A and eluted with a linear gradient from 0 to 0.4 M NaCl in buffer A. The purification was monitored by SDS/PAGE 10 ~ 15% polyacrylamide gel gradient (Pharmacia). The fractions containing EF-Tu were pooled and were then purified by affinity with a modified method of R. JACOBSON *et al.*<sup>14)</sup>. After overnight batch adsorption at 4°C on periodate oxidized GDP-Sepharose, the resin was washed with buffer A containing 350 mM NaCl. Elution was performed with buffer A containing 350 mM NaCl and 100 μM GDP (Sigma), yielding EF-Tu in homogeneous form. The eluates were concentrated by filtration with

Fig. 2. Agar diffusion assay.



Inhibition zones of kirromycin against *M. caviae* with (right) and without (left) the presence an excess of EF-Tu.

10,000 Dalton cut-off and were lyophilized. The powder was then dissolved at 5 mg/ml concentration in buffer B consisting of 50 mM Tris-HCl buffer (pH 7.7), 10 mM MgCl<sub>2</sub>, 2.5 mM DTT, 50 μM soybean trypsin inhibitor (Sigma) and 20 μg/ml aprotinin (Richter). This stock solution was stored at -80°C.

### Screening Methods

The assay was based on paper disc diffusion assays against *Staphylococcus aureus* ATCC 6538 and *Moraxella caviae* ATCC 14659. The microorganisms were incubated at 37°C overnight on Iso-sensitest broth (Oxoid) and Todd Hewitt broth (Difco), respectively. The media were supplemented with 17 g/liter Bacto-Agar (Difco). 20 μl of the two fold diluted stock solution of EF-Tu were loaded on 6 mm paper discs. The discs were then lyophilized and stored at -80°C. To economize in the use of the EF-Tu protein, part of the screening was also performed using 3 mm paper discs.

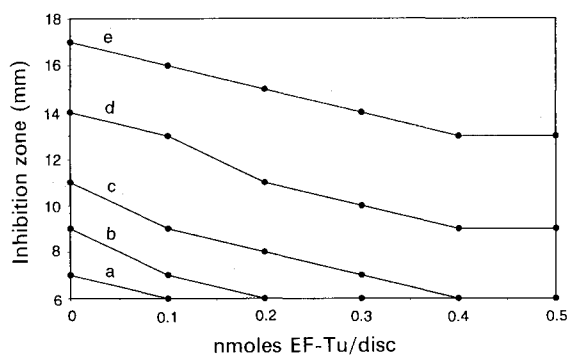
Culture broths were applied by loading 20 μl of broth to EF-Tu discs and to normal paper discs used as reference control. The discs were maintained at room temperature for some minutes prior to placing on the agar plate. Positive samples were also tested with additional control discs prepared using a solution of 2.5 mg/ml of bovine albumin (Sigma) in buffer B.

## Results

### Screening Technique

The screen was based on the selection of antimicrobial activities antagonized by the presence of exogenous EF-Tu. The fermentation broths were tested with a pair of agar diffusion assays, performed by using paper discs with and without the EF-Tu protein. In the presence of EF-Tu, the positive broths showed inhibition zones reduced in size, or completely reverted. The assumption

Fig. 3. Antagonism on kirromycin activity by EF-Tu.



Inhibition zones of kirromycin against *M. caviae* in agar diffusion assay using 6mm paper discs in presence of increasing amounts of EF-Tu; 6mm zones indicate no inhibition. Nanomoles of kirromycin loaded on a paper disc: curve a: 0.05; curve b: 0.1; curve c: 0.3; curve d: 0.6; curve e: 1.2.

Table 1. Sensitivity of the screen to standard antibiotics.

Antibiotic	Concentration ( $\mu\text{g/ml}$ )	Inhibition zone (mm) <sup>a</sup>	
		Control disc	EF-Tu disc
Kirromycin	25	14	No activity
Erythromycin	20	11	11
Kanamycin	20	11	11
Spectinomycin	100	13	13
Chloramphenicol	30	12	11
Tetracycline	100	13	13
Rifampicin	20	13	13
Penicillin G	5	17	17

<sup>a</sup> Agar diffusion assay against *M. caviae* ATCC 14659; 6 mm paper disc.

was that exogenous EF-Tu was acting as a decoy, which binds the specific inhibitor and prevents its entry into the bacterial cell. Kirromycin, a known antibiotic with high affinity for EF-Tu<sup>15)</sup>, was detectable with this method (Fig. 2). Fig. 3 shows that reversion of the antibacterial activity was complete when the EF-Tu protein was present in small stoichiometric excess compared to kirromycin. Selectivity for kirromycin was high, as shown in Table 1. We performed the assays using both *M. caviae* ATCC 14659 and *S. aureus* ATCC 6538. Kirromycin-type antibiotics are very active against *M. caviae* and inactive against *S. aureus*<sup>16,17)</sup>. The occurrence of this uncommon pattern of antibacterial activity and antagonism by EF-Tu thus indicated the presence of a kirromycin-type antibiotic in the broth. The identification of the antibiotic was then performed on the basis of UV absorptions<sup>16)</sup> and MS fragmentation<sup>18)</sup>. The antibiotic pulvomycin<sup>12,19)</sup> acts on EF-Tu and is active against Gram-positive bacteria. However, it was poorly antagonized by EF-Tu and eluded this screen.

We searched novel microbial metabolites active against *S. aureus* and antagonized by EF-Tu and we found the antibiotics GE2270 complex and GE37468. These antibiotics have thiazolyl peptide structures<sup>23)</sup> and are highly active against Gram-positive pathogens, including *Enterococci* resistant to glycopeptides<sup>24,25)</sup>. An equimolecular quantity of EF-Tu completely antagonized their activity. Moreover, EF-Tu saturated with kirromycin, still maintained the capacity to antagonize antibiotic GE2270 A (data not shown). This indicated distinct sites of binding on EF-Tu for GE2270 and kirromycin. The results from the screening were consistent with the mode of action of antibiotic GE2270 as was verified in additional experiments<sup>9,10)</sup>.

Table 2. EF-Tu binding thiopeptides discovered during the screening program.

Antibiotic	Occurrence	Producers
GE2270 complex	2	<i>Planobispora rosea</i>
		<i>Actinomadura pusilla</i>
GE37468	1	<i>Streptomyces</i> sp.

The protein needed for the screening was obtained by fermenting *E. coli* K12. It was not necessary to increase the yield by using recombinant strains, because EF-Tu is an abundant protein in *E. coli*<sup>20,21)</sup>. About 1.5 mg of purified EF-Tu was recovered from 1 g of wet *E. coli* paste. This yield was adequate to sustain the screening program.

Protease inhibitors were added to minimize the possible proteolytic cleavage of EF-Tu in the assay environment.

#### Selection of Lead Cultures

A total of 47,000 microorganisms were examined. 34,000 of them were actinomycetes, and the remaining part were filamentous fungi. 28 producers of kirromycin-type antibiotics and three producers of GE2270-type antibiotics (Table 2) were identified. All these EF-Tu binding antibiotics were produced by actinomycetes. Antibiotic GE2270 was discovered in broths of *Planobispora rosea*, a rare microorganism<sup>22)</sup> and antibiotic GE37468 from a *Streptomyces* sp.

#### Discussion

Examples of screening approaches based on antagonism by receptor mimetic molecules are reported in the literature for polyene<sup>26,27)</sup> and for glycopeptide<sup>28)</sup> antibiotics. These screens were based on antagonism of the antimicrobial activity by sterols and by synthetic analogues of the peptidoglycan acyl-D-alanyl-D-alanine, respectively. In these cases the receptor-mimetic molecules were compounds of low molecular weights and the screens were aimed at detecting analogues of known antibiotics. We extended this concept using the entire receptor and searching novel classes of antibiotics acting on it. EF-Tu showed the traits of a suitable molecular target for this approach. It is essential for the bacterial growth. The gene encoding EF-Tu is highly conserved in eubacteria and is significantly different from the functionally analogous factor in mammalian cells<sup>29,30)</sup>. The EF-Tu target was thus attractive for screening because a new inhibitor was expected to be effective in many bacteria and of low intrinsic toxicity for mammalian cells. In addition, EF-Tu interacts with a number

of low and high molecular weight ligands and thus may be theoretically inhibited in a variety of modes. Indeed kirromycin and pulvomycin have distinct modes of action on the EF-Tu<sup>15,19</sup>). This screening technique had however some intrinsic limitations in detecting antibiotics which do not bind tightly to the target. In the case of the assay for glycopeptides<sup>28</sup>), acyl-D-alanyl-D-alanine was used in a large molecular excess in respect to the amount of glycopeptides normally produced in the broths. This enabled antagonism of glycopeptides which have relatively low affinity constant for acyl-D-alanyl-D-alanine<sup>31</sup>). Due to the 43,200 molecular weight of EF-Tu, it was feasible to load in a paper disc an amount of EF-Tu sufficient to be in a stoichiometric ratio with kirromycin produced at about 40 µg/ml in the broth. This was effective for antibiotics of the classes of kirromycin and GE2270. Broths containing these antibiotics in quantity exceeding the EF-Tu available in the assay, showed inhibition zones reduced in respect to the control disc with EF-Tu and were positively selected. Pulvomycin was however poorly detected, probably because pulvomycin acts on the EF-Tu·GTP form whereas we used EF-Tu·GDP in the assay<sup>19</sup>).

False positive antibiotics with a non specific binding capacity to proteins showed reduced inhibition zones in an additional assay in the presence of bovine albumin and were discarded. We observed also in some cases antagonism by DTT, one constituent of the buffer used to dissolve EF-Tu. We discriminated this type of activity on the basis of the reduction of inhibition zones after incubation with DTT. We repeatedly isolated naphthomycin<sup>32</sup>), which is known to form an inactive reaction adduct with thioalcohols. Some other cases of coupling with thiol groups are described in the literature, for instance for damavaricin, nisin, leinamycin, cellodigin and showdomycin. The cases observed in the course of our screening are likely attributable to this type of reactivity.

### Conclusion

This screening approach appears generally applicable to a variety of targets. It selects inhibitors active against whole cell microorganisms and possessing high affinity for the molecular target. In the EF-Tu case, indeed, this screen identified antibiotics GE2270 and GE37468, which are very active *in vitro* against bacteria and bind tightly to EF-Tu.

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