Antibiotic GE37468 A: A New Inhibitor of Bacterial Protein Synthesis

I. Isolation and Characterization

SERGIO STELLA*, NICOLETTA MONTANINI, FRANCIS LE MONNIER, PIETRO FERRARI, LUIGI COLOMBO, PAOLO LANDINI†, ISMAELA CICILIATO, BETH P. GOLDSTEIN, ENRICO SELVA and MAURIZIO DENARO

Marion Merrell Dow Research Institute, Lepetit Research Center, 21040 Gerenzano (VA) Italy
†University of Massachussets, Department of Molecular Genetics and Microbiology, 55, Lake Avenue North, Worcester, MA 01655, U.S.A.

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GE37468 A is a new thiazolyl peptide antibiotic obtained by fermentation of *Streptomyces* sp. strain ATCC 55365. It inhibits bacterial protein synthesis by acting on elongation factor Tu and is structurally and functionally related to the GE2270 class of EF-Tu inhibitors. It is active *in vitro* against Gram-positive bacteria and *Bacteroides fragilis*, and protects mice against *Staphylococcus aureus* infection.

Antibiotic GE37468 A was discovered in the course of a screening program for inhibitors of bacterial protein synthesis acting on Elongation Factor Tu (EF-Tu). The antibiotic was obtained by fermenting a *Streptomyces* sp. strain isolated from a soil sample collected at Lamole (FI) in Italy. The antibiotic resulted similar in structure and mode of action to GE2270 A[1~6] and amythiamicins[7~10], a group of antibiotics which are active against MRSA strains[10,11]. In this paper we report its discovery, isolation, physico-chemical and biological properties.

Materials and Methods

Cultural and Growth Characteristics of the Producing Strain

Colonial and morphological characters were determined with standard methods[12,13]. Colour determination was made according to MAERZ and PAUL[14]. Growth on sole sources of carbon was determined after incubation at 28°C for 4 days[12].

Chemotaxonomic Characteristics of the Producing Strain

Freeze-dried biomass was examined to determine the major chemotaxonomic characteristics. The enantiomeric form of cell-wall amino acids (2,6-diaminopimelic acid) was determined by TLC following the method of STANECK and ROBERTS.[15] Fatty acid composition was assessed by GS-MS analysis of the methyl esters (FAMEs) prepared using the method of JANTZEN[16].

Fermentation of the Producing Strain

A culture of *Streptomyces* sp. strain ATCC 55365, grown on an oatmeal agar slant for four days, was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of seed medium (dextrose 2%, yeast extract 0.2%, soybean meal 0.8%, calcium carbonate 0.4%, sodium chloride 0.1%, pH 7.0). After incubation at 28°C for 96 hours on a rotary shaker (200 rpm), the biomass was transferred to a jar fermenter containing 4 liters of the same medium. This culture was grown at 28°C for 24 hours with stirring (900 rpm) and aeration (about 0.5 standard liter of air per volume per minute) and then transferred to a fermenter containing 200 liters of production medium (sorbitol 2%, yeast extract 0.2%, soybean meal 0.8%, calcium carbonate 0.4%, sodium chloride 0.1%, pH 7.0) and incubated for 72 hours at 28°C. Antibiotic production was followed by HPLC or agar diffusion assay using *B. subtilis* ATCC 6633 grown on Davis minimal medium[17]. Inhibition zones were measured after overnight incubation at 37°C.

Isolation of Antibiotic GE37468 A

The antibiotic was found mainly in the mycelium (200 liters of broth) from which it was extracted with 100 liters of acetone. After removing the solvent under reduced pressure, the aqueous residue was extracted with 7 liters of *n*-butanol. A solid (13.3 g) containing GE37468 A was precipitated by addition of petroleum ether to the concentrated organic phase. This material was suspended in 3 liters of $HCOONH_4$ buffer (16 mM, pH 6.0) and extracted three times with an equal volume of ethyl acetate. The aqueous phase was adjusted to pH 4.5 with 1 N HCl and extracted with 3 liters of ethyl acetate. The organic phases were pooled, concentrated under reduced pressure and added to petroleum ether to yield 3.4 g of crude GE37468 A. This crude preparation was first purified by gel filtration on Sephadex LH-20 (Pharmacia) swollen and eluted with CH_3OH. The fractions containing the antibiotic were pooled and

concentrated. Partially purified GE37468 A (1.3 g) was precipitated by addition of diethyl ether and was further purified by medium pressure liquid chromatography (MPLC) on silica gel (150 g, 230~400 mesh) equilibrated in CH_2Cl_2 and eluted with a linear gradient of CH_3OH in CH_2Cl_2 (0 to 10% in 10 minutes, 10 to 50% in 110 minutes, flow rate: 100 ml/minute). The fractions containing GE37468 A were pooled, concentrated and added to petroleum ether to yield 500 mg of pure GE37468 A as a white powder. High purity GE37468 A, suitable for analytical purposes, was obtained by preparative reverse-phase HPLC.

HPLC

Analytical HPLC was performed on a 4.6×250 mm Altex Ultrasphere ODS column (5 μm, Beckman) eluted at a flow rate of 1.5 ml/minute with a linear gradient of eluent A in eluent B (10% to 90% in 20 minutes). Eluents A and B were CH_3CN-16 mM $(NH_4)H_2PO_4$ (pH 6.0 with NH_4OH) in the ratios 80:20 (v/v) and 5:95 (v/v), respectively. UV detection was at 254 nm. Preparative HPLC was performed on a 25×250 mm Lichrosorb RP18 column (7 μm, Merck) eluted at 12 ml/minute with a linear gradient of eluent C in eluent D (45% to 55% in 20 minutes). Eluents C and D were CH_3CN-16 mM $HCOONH_4$ (pH 7.0 with NH_4OH) in the ratios 80:20 and 5:95, respectively. UV detection was at 230 nm.

FAB-MS Studies

The low resolution positive and negative ion FAB-MS spectra were obtained on a Finnigan MAT TSQ700 triple quadrupole mass spectrometer, using a saddle field atom gun (with Xe gas) at 8 kV voltage and 1 mA current. The sample was dissolved in a 1:1 mixture of dimethylsulphoxide (DMSO)-*m*-nitrobenzyl alcohol (*m*-NBA). The high resolution exact mass measurements were obtained on a Kratos MS50 mass spectrometer, using the peak matching technique and operating in positive FAB mode.

MIC Determinations

Minimal inhibitory concentrations were determined by broth microdilution. Inocula were 10^4 CFU/ml, except for *Clostridium perfringens*, *Propionibacterium acnes* and *Bacteroides fragilis* (10^5 CFU/ml). Incubation times were 20~24 hours, except for *Haemophilus influenzae*, *P. acnes*, *C. perfringens* and *B. fragilis* (48 hours). Incubation was at 37°C in air. *H. influenzae* was incubated in 5% CO_2; *P. acnes*, *C. perfringens* and *B. fragilis* in N_3-CO_2-H_2 (80:10:10). The media used were: Iso-Sensitest broth (Oxoid) (staphylococci, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*); Todd Hewitt broth (Difco) (streptococci); Brain Heart Infusion broth (Difco)+1% (v/v) Supplement C (Difco) (*H. influenzae*); Wilkins-Chalgren broth (Difco) (*P. acnes*, *C. perfringens*, *B. fragilis*).

Experimental Septicemia in Mice

Groups of five CD1 mice of both sexes (Charles River, average weight 18~22 g) were infected intraperitoneally with 10^6 CFU/mouse of *Staphylococcus aureus* Smith suspended in 0.5 ml of 5% bacteriological mucin (Difco). The antibiotic was administered intravenously once, immediately after infection, in a solution containing (v/v): 10% dimethylsulphoxide, 10% Cremophor EL, 20% 0.07 M phosphate buffer (pH 8), 60% of a 5% (w/v) glucose infusion solution. The ED_{50} was calculated by the Spearman and Kärber method[18] from the percentages of animals surviving to day 7 at each dose.

Mechanism of Action of GE37468 A in Intact Bacteria

Bacillus subtilis 566/1 (*thyA*/*thyB*) was grown at 37°C in a rotary bath in DAVIS-MINGIOLI minimal medium[19] with glucose (2%), asparagine (0.1 g/liter), Difco Casamino acids (2 g/liter) and thymidine (2 mg/liter). When the culture reached an optical density (590 nm) of 0.2, it was divided in five parts. To four of these, precursors of DNA (2 mCi/liter ^{3}H-+2 mg/liter unlabeled thymidine), RNA (1 mCi/liter ^{3}H-+10 mg/liter unlabeled uridine), protein (1 mCi/liter) ^{3}H-+2 mg/liter unlabeled tryptophan), or cell wall peptidoglycan (2 mCi/liter) ^{3}H-+2 mg/liter unlabeled *N*-acetylglucosamine) were added. The fifth culture was used to monitor cell density. After 10 minutes of incorporation, each culture was further divided into three parts: untreated control, 1 mg/liter GE37468 A and positive control. The positive controls were 25 mg/liter) nalidixic acid (DNA), 2.5 mg/liter rifampicin (RNA), 50 mg/liter chloramphenicol (protein) and 5 mg/liter teicoplanin (cell wall). At various times, up to 60 minutes, 0.1 ml samples were added to 2 ml ice-cold 5% TCA and filtered on glass fibers. Samples containing tritiated tryptophan were heated for 15 minutes at 75°C before filtering. The filters were placed in 15 ml of Insta-Fluor (Packard) and counted in a liquid scintillation spectrometer.

Activity of GE37468 A in Cell Free Eukaryotic and Prokaryotic Protein Synthesis Systems

L-[^{14}C]phenylalanine (513 mCi/mmol) was from the Radiochemical Centre, Amersham, Bucks., UK. Poly(U) was from Boeringer Mannheim, Germany. Nucleotides and all other reagents were from Sigma Chemical Company, St. Louis, MO, U.S.A.. Crude extracts for polyphenylalanine synthesis were prepared from *E. coli* according to LANDINI *et al.*[6], and from rat liver according to SKOGERSON and ENGELHARDT[20]. Reactions were carried out and the polyphenylalanine synthesis was measured as described previously[6]. Yeast t-RNA was used with rat liver extracts.

Results

Physiological and Morphological Characteristics of the Producing Strain

The strain grew well on most standard media with the optimum growth temperature between 28°C and 37°C. No growth was observed at 15°C or 50°C. There was moderate growth at 20°C. The cultural and physiological characteristics and the carbon source utilization of the strain are reported in Tables 1, 2 and 3. No fragmentation of the mycelium was observed after four days' growth at 28°C in liquid culture (V6 medium). Microscopic examination of the strain on soil extract agar after four days' incubation at 28°C revealed extensively branched vegetative hyphae (≈ 1.1 μm in diameter). No fragmentation was observed. The aerial mycelium contained chains of spores, in both retinaculiaperti (spirals with $1 \sim 2$ loops) and rectiflexible (straight to flexuous) formations.

Chemotaxonomical Characteristics of the Producing Strain

The chemical analysis showed that the cell wall of the producing strain contains LL-2,6-diaminopimelic acid; *meso*-diaminopimelic acid was not detected. The fatty acid profile showed major amounts of saturated, *iso*- and *anteiso*-fatty acids. On the basis of cell-wall composition, macro- and micro-morphological examination, the strain ATCC 55365 was assigned to the genus *Streptomyces*.

Physico-chemical Characteristics of GE37468 A

Pure GE37468 A was obtained as a white powder,

Table 1. Growth characteristics of strain ATCC 55365.

Culture medium	Growth	Morphological characteristics
ISP 2	+ + +	Aerial mycelium present—well developed, floccose, white; no diffusible pigment production. Vegetative mycelium: light beige, 11-L-2
Oatmeal - ISP 3	+ + +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: white/opaque
Glycerol - asparagine medium - ISP 5	+ + +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: dark ivory, 10-D-2
Hickey & Tresner	+ + +	Aerial mycelium appeared flat, and velvety; white; brown [15-J-12] diffusible pigment produced. Vegetative mycelium: brown, 14-A-5 Deauville
Bennett's	+ + +	Aerial mycelium poorly developed at intersections of streaks; white; diffusible pigment produced. Vegetative mycelium: cream, 9-G-2
Czapek - glucose	+ + +	aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: white/opaque
Glucose - asparagine	+ + +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: white, 10-B-1 Oyster white
Nutrient	+ + +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: light beige, 11-C-2-Ecru beige
Potato	+ + +	Aerial mycelium, white with velvety appearance; oil droplets also present; brown [16-A-12, Bistra] diffusible pigment produced. Vegetative mycelium: light beige, 11-F-3
Starch - ISP 4	+ + +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: white/opaque
Peptone yeast extract iron - ISP 6	+ + +	Aerial mycelium not produced; brown [16-A-12, Bistra] diffusible pigment produced. Vegetative mycelium: grey, 32-A-1
Tyrosine - ISP 7	+ + +	Aerial mycelium not produced; brown [16-H-2, Bronzesheen] poorly diffused pigment produced. Vegetative mycelium: light brown/grey, 15-A-5 Log Cabin
Calcium malate	+ +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: white/opaque
Skimmed milk	+ + +	Aerial mycelium not produced; no diffusible pigment production; discolouration at growing edge. Vegetative mycelium: brown, 15-A-6 Beaver
Soil extract	+ + +	Barely visible surface mycelium; good aerial mycelium development; floccose, white, with an appearance like glass wool; no diffusible pigment production
Egg albumin	+ + +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: white/opaque
Water	+ + +	Moderate aerial mycelium development, floccose, white; no diffusible pigment production. Vegetative mycelium: white/opaque
Czapek sucrose	+ + +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: white/opaque
Potato glucose	+ + +	Aerial mycelium not produced; no diffusible pigment production. Vegetative mycelium: brown, 13-L-8 Buckthorn
Oatmeal (mod)	+ + +	Weak aerial mycelium development, floccose, white; no diffusible pigment production. Vegetative mycelium: light beige, 11-E-4 Maple
Sabouraud	+ + +	Aerial mcelium not produced; no diffusible pigment production. Vegetative mycelium: light beige, 11-B-3 Champagne

+ +: Moderate growth; + + +: good growth. ISP Numbers refer to the media of SHIRLING and GOTTLIEB (International *Streptomyces* Project codes)[12]. Colour codes (*e.g.* 11-L-2) from MAERZ and PAUL[14].

soluble in organic solvents but sparingly soluble in water. The physico-chemical properties of GE37468 A are reported in Table 4. The antibiotic has a molecular weight of 1309.48 and molecular formula $C_{59}H_{52}N_{14}O_{12}S_5$, as determined by comparison of precise FAB-MS and ^{13}C NMR analyses[21]. The GC-MS analysis of the acid hydrolysis products of GE37468 A showed the presence of cysteine, phenylalanine and tyrosine. The structure of GE37468 A, reported in Fig. 1, was fully elucidated by ^{1}H and ^{13}C NMR and MS spectrometry studies which are reported in an accompanying paper[21].

GE37468 A is a thiazolyl peptide antibiotic which is structurally related to GE2270 A[2~4] and amythiamicins[8,9]. GE37468 A and GE2270 A have chromophoric cores which differ only in the presence of a methyloxazole in GE37468 A at the position in which GE2270 A has a thiazole ring, and the two molecules have very similar UV spectra. The side chain of GE37468 A contains two dehydroalanine units, which are common in other thiazolyl peptide antibiotics[22], while the α-hydroxyproline residue in the macrocycle, which is uncommon in natural products, confers on the molecule an interesting chemical reactivity, described in the accompanying paper[21].

Table 2. Physiological characteristics of ATCC 55365.

Test	Reaction
Calcium malate digestion	Negative
Gelatin liquefaction	Positive
Hydrogen sulphide production	Positive
Milk peptonisation	Negative
Milk coagulation	Negative
Nitrate reduction (aerobic)	Negative
Starch hydrolysis	Positive
Tyrosine reaction (melanin)	Positive

Table 3. Carbohydrate utilisation of ATCC 55365.

Carbon source	Growth	Carbon source	Growth
Arabinose	+++	Mannitol	+++
Cellobiose	+++	Mannose	+++
Cellulose	+[n]	Raffinose	+++
Fructose	+++	Rhamnose	+++
Galactose	+++	Ribose	+++
Glucose	+++	Salicin	+++
Insitol	+++	Sucrose	+++[p]
Lactose	+++	Xylose	+++
Maltose	+++		

+++: Good growth; ++: moderate growth; +: weak growth; −: no growth p: poor aerial mycelium development; n: no aerial mycelium development.

Table 4. Physico-chemical properties of GE37468 A.

Molecular formula	$C_{59}H_{52}O_{12}N_{14}S_5$
Molecular weight	1309.48
Melting point	180~189°C
$[\alpha]_D^{20}$	+105° (*c* 0.04, MeOH/DMSO 85/15)

UV bands (λ_{max}: nm) and absorptivities (ε_{mol}: 1 cm, 1 M) of GE37468 A in solutions of methanol-acetonitrile-water, 7:7:1 at different pH values.

Solvent	250 (sh, 43000), 305 (34400), 340 (sh, 18300).
Solvent+HCl	250 (sh, 43000), 307 (33200), 340 (sh, 17700)
Solvent+KOH	250 (sh, 51700), 302 (36200), 340 (sh, 19700)

Assignments of the main absorption bands (cm^{-1}) of the IR spectrum of GE37468 A in nujol mull.

3600÷3100	νNH & νOH
1705 (sh)	Carboxylic νC=O
1653	νC=O (amide I)
1635, 1616, 1582	Ethylenic and aromatic νC=C
1537	Heterocyclic νC=C & νC=N
1514	δNH (amide II)
1269, 1200	Aromatic δCH
1024, 1007	νC–O & νC–O–C
920	Heterocyclic γCH
808	Para-substituted aromatic γCH
758, 703	Mono-substituted aromatic γCH

Fig. 1. Structure of GE37468 A.

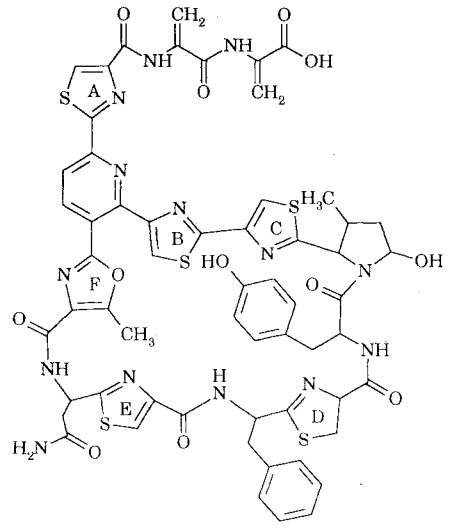


Table 5. *In vitro* antibacterial activity of GE37468 A.

Species	MIC (mg/liter)	
	GE37468 A	GE2270 A
S. aureus Smith	0.016	0.016
S. epidermidis ATCC 12228	0.06	0.06
S. haemolyticus L602	0.13	0.06
Str. pyogenes L49	4	0.25
Str. pneumoniae L44	0.5	0.06
E. faecalis ATCC 7080	0.016	0.008
P. acnes ATCC 6919	0.002	0.002
C. perfringens L290	0.004	0.004
B. fragilis ATCC 25285	4	2
H. influenzae ATCC 19418	>128	64
E. coli L47	>128	>128
P. aeruginosa ATCC 10145	>128	>128

Antibacterial Activity of GE37468 A

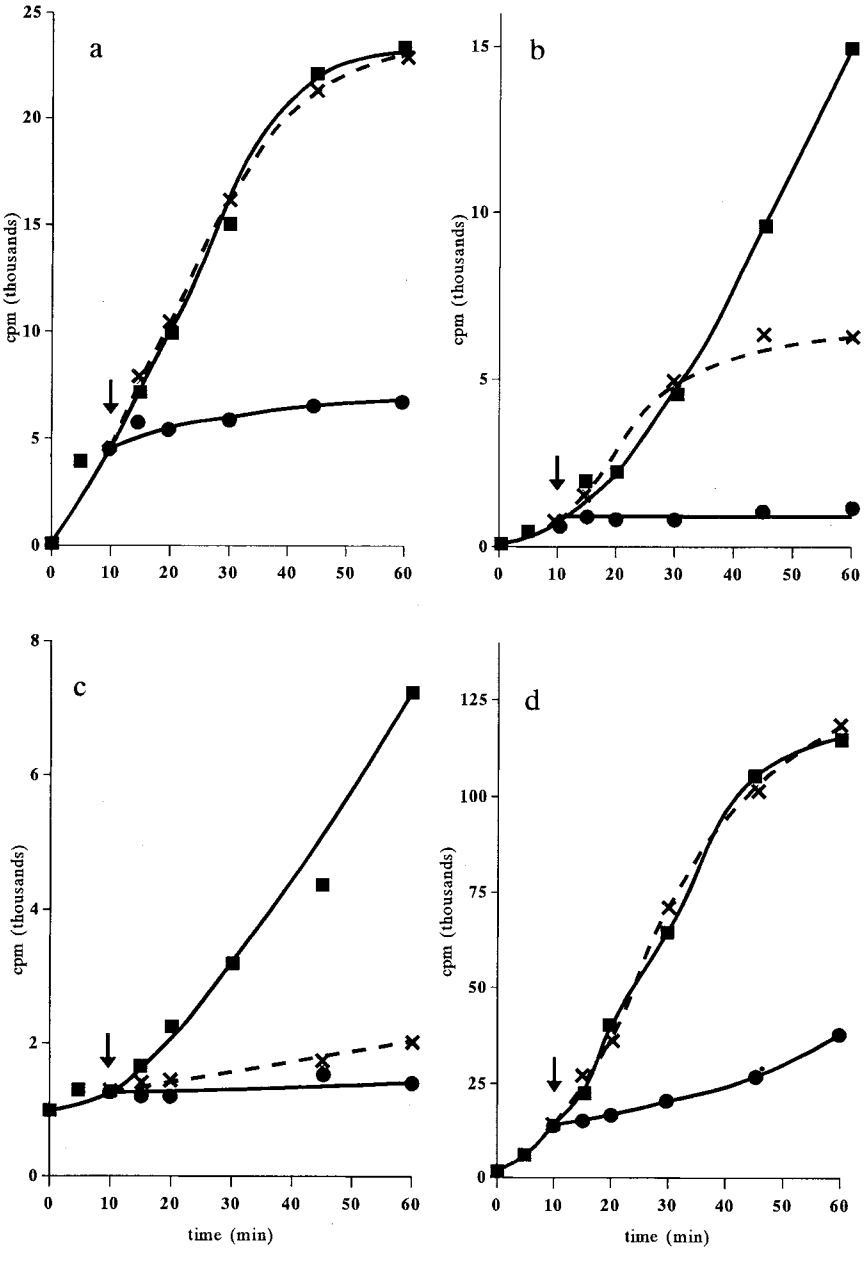
The *in vitro* antibacterial activity of GE37468 A is reported in Table 5, in comparison with that of GE2270 A. GE37468 A showed excellent activity against Gram-positive aerobes, although it was less active than GE2270 A against streptococci. It was also very active against *P. acnes* and *C. perfringens* but, with the exception of *B. fragilis* it had poor activity against Gram-negative bacteria. GE37468 A protected mice infected with *S. aureus* with an iv ED_{50} of 3.2 mg/kg.

Mechanism of Action

GE37468 A inhibited the growth of *B. subtilis* by 80~90% at 1 mg/liter. At this concentration the antibiotic completely blocked protein synthesis immediately upon addition (Fig. 2). DNA and peptidoglycan synthesis were unaffected by the antibiotic while RNA synthesis was inhibited starting at 20 minutes after addition. Delayed inhibition of RNA synthesis after an initial block of protein synthesis (mimicking the stringent response observed upon amino acid starvation) is, in our

Fig. 2. Macromolecular synthesis inhibition by GE37468 A in *B. subtilis* 566/1.

a. NDA, b. RNA, c. protein, d. peptidoglycan. ■, untreated control; ×, GE37468 A, 1 mg/liter; ●, positive control (a. 25 mg/liter nalidixic acid, b. 2.5 mg/liter rifampicin, c. 50 mg/liter chloramphenicol, d. 5 mg/liter teicoplanin). Antibacterial agents were added at the time indicated by the arrow.



experience, a characteristic of the action of EF-Tu inhibitors. GE37468 A also inhibited initiation-independent poly(U)-directed poly(phe) synthesis in a cell-free system from *E. coli* with an IC_{50} of 1 mg/liter (Fig. 3) but had little effect on a eukaryotic (rat liver) system at concentrations up to 50 mg/liter.

As shown in Table 6, a strain of *S. aureus* selected for resistance to GE2270 A was also resistant to GE37648 A. As it is known that resistance to GE2270 A is determined by alteration of EF-Tu[6], this observation provides additional evidence that the antimicrobial activity of GE37468 A is mediated by interaction with EF-Tu. In contrast, other thiazolyl peptide antibiotics, which are also protein synthesis inhibitors[23,24] but act on a different molecular target (thiostrepton, A-10255, nosiheptide, sulfomycin), were as active against the resistant strain as against the parental strain.

Fig. 3. Protein synthesis inhibition by GE37468 A in *E. coli* and rat liver cell free systems.

▲: *E. coli*, ■: rat liver.

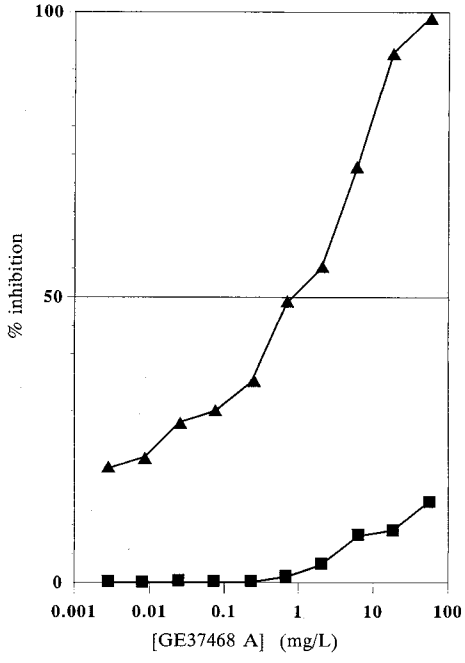


Table 6. Activity of GE37468 A and structurally related antibiotics against isogenic susceptible and GE2270 A-resistant strains of *S. aureus*.

Antibiotic	MIC (μg/ml)	
	L165 (parental)	L165 GE2270-res
GE2270 A	0.016	>128
GE37468	0.016	>128
Thiostrepton	0.03	0.016
A-10255	2	2
Nosiheptide	0.002	0.004
Sulfomycin	0.13	0.13

Discussion and Conclusion

GE37468 A was highly active *in vitro* against Gram-positive bacteria and protected mice against *S. aureus* infection. Comparative studies in cell free *E. coli* and rat liver systems showed that the inhibition is selective for prokaryotic protein synthesis and indicate that, as already observed for GE2270 A, the lack of activity against Gram-negative bacteria is probably due to lack of penetration of the antibiotic. Antibiotic GE37468 A is similar to GE2270 A in its mode of action[5,6] and spectrum of antibacterial activity. The main difference is the reduced activity of GE37468 A against streptococci.

GE37468 A, GE2270 A and amythiamicins also have similar structures; they are thiazolyl peptide antibiotics. These antibiotics are characterized by the presence of peptide macrocycles containing thiazolyl amino acids and a chromophoric core containing a pyridine ring. The biosynthetic pathways of thiostrepton and nosiheptide are known[25,26]. The thiazolyl amino acids originate from one molecule of cysteine and the carboxyl group of the adjacent amino acid and the pyridine originates from two units of dehydroalanine. The peptide macrocycle of GE37468 A, GE2270 A and amythiamicins are of the same size. Assuming a common biosynthetic pathway for thiazolyl peptide antibiotics, the GE37468 A macrocycle could originate from 11 amino acids. The peptide macrocycles of sulfomycin and micrococcin are derived from 13 and 10 amino acids, respectively. Thiostrepton and nosiheptide have a 10 amino acid macrocycle plus an additional macrocycle containing a lactone bond. GE37468 A, GE2270 A and amythiamicins appear to represent a distinct sub-group of this class of antibiotics characterized by the size of their peptide macrocycle.

GE37468 A and GE2270 A are also unique for their mode of action on EF-Tu. Amythiamicins were recently reported to have a similar mechanism of action[10]. EF-Tu is also the molecular target of the kirromycin class of antibiotics[27] and of pulvomycin[28]. However, these antibiotics have quite different structures and spectra of antibacterial activity[1,29].

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