CALICHEAMICINS, A NOVEL FAMILY OF ANTITUMOR ANTIBIOTICS: TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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A novel family of antitumor antibiotics, the calicheamicins, were isolated from the fermentation broth of *Micromonospora echinospora* subsp. *calichensis*. These antibiotics exhibited significant activity against Gram-positive and Gram-negative bacteria *in vitro*. Calicheamicin γ_1^I demonstrated antitumor activity against P388 leukemia and B16 melanoma *in vivo*.

In the course of our search for novel antitumor agents produced by microorganisms, a culture designated LL-E33288 was isolated from a soil sample and was found to produce a new family of antitumor compounds, the calicheamicins. These compounds exhibited significant antimicrobial activity against Gram-positive and Gram-negative bacteria and demonstrated potent *in vivo* activity against murine tumors. This paper describes the taxonomy of the producing culture, fermentation and biological activities of the calicheamicins.

Materials and Methods

Microorganism

Culture LL-E33288 was isolated from a caliche clay soil sample collected in Texas. This culture was deposited with the Northern Regional Research Center's Culture Collection Laboratory under the accession No. NRRL 15839.

Taxonomic Studies

The taxonomic studies were carried out as described by the International Streptomyces Project (ISP)[1] and GORDON *et al.*[2]. For the evaluation of cultural characteristics, the strains were incubated for 14~31 days at 28°C. Cell wall composition was analyzed by the methods of LECHEVALIER and LECHEVALIER[3].

Media and Fermentation

Culture LL-E33288 was stored as a frozen suspension at −70°C in growth medium. A 1.5-ml aliquot of thawed suspension was used to inoculate 100 ml of seed medium (yeast extract 0.5%, beef extract 0.3%, Tryptose 0.5%, dextrin 2.4%, glucose 0.5%, and $CaCO_3$ 0.4%) in a 500-ml Erlenmeyer flask. This seed inoculum was incubated on a rotary shaker (5 cm orbit) at 200 rpm, at 28°C, for 48 hours and was then added to a 3-liter fermentor containing 1 liter of seed medium. Following 48 hours incubation (aeration; 1 liter/minute, 450 rpm), the contents of this fermentor were inoculated into 30 liters of production medium (sucrose 2.0%, molasses 0.5%, peptone 0.2%, $CaCO_3$ 0.25%, $FeSO_4 \cdot 7H_2O$ 0.01%, $MgSO_4 \cdot 7H_2O$ 0.02%, and KI 0.01%) in a 41-liter fermentor. This fermentation was carried out at 28°C for up to 220 hours (aeration; 30 liters/minute, 550 rpm). The pH of the

media used in these studies was adjusted to 6.8~7.0 prior to sterilization. Microbial growth was determined by packed cell volume. Antibiotic production was monitored by the biochemical prophage induction assay (BIA)[4,5], a paper-disk agar diffusion assay using *Bacillus subtilis* strain 308 (*recE*$_4$) and analytical HPLC.

Isolation

The BIA active compounds produced by culture LL-E33288 were associated with the mycelium and were recovered by extracting the whole fermentation broth with ethyl acetate[6].

TLC/Bioautography and HPLC

The calicheamicins were monitored by TLC on E. Merck Silica Gel 60 F_{254} pre-coated aluminum sheets (0.2 mm layer thickness) developed in a solvent system of ethyl acetate - isopropyl alcohol (97 : 3) saturated with 0.1 M KH_2PO_4. HPLC analysis of the antitumor compounds was carried out on a Waters ALC/GPC 200 Series Liquid Chromatograph equipped with a Waters WISP 710B sample processor. Antimicrobial and BIA activities on the TLC plates were detected by bioautography.

Antimicrobial Activity

The *in vitro* antibacterial activity of the calicheamicins against a spectrum of Gram-positive and Gram-negative bacteria was determined by an agar dilution method employing Mueller-Hinton medium. The lowest concentration of antibiotic that inhibited growth of a bacterial strain after 18 hours of incubation at 35°C was recorded as the MIC.

Antitumor Activity

The antitumor activity of calicheamicin γ_1^I was determined in male BDF_1 mice against P388 leukemia and B16 melanoma. The P388 leukemia test was initiated by intraperitoneal injection of 10^6 cells per mouse in 0.5 ml of dilute ascitic fluid. For B16 melanoma, a 1-g portion of tumor was homogenized in 10 ml of balanced salt solution, and a 0.5-ml aliquot was implanted intraperitoneally into each mouse. Calicheamicin γ_1^I was administered intraperitoneally days 1, 5, and 9 after initiation of the P388 test and by the same route on days 1 through 9 for B16. The antitumor activity of calicheamicin γ_1^I was expressed as T/C values (median survival time of the treated group/median survival time of the untreated group $\times 100$), with a value of ≥ 125 being considered significant.

Results

Taxonomic Studies of the Producing Culture

Culture LL-E33288 was isolated from a chalky (caliche) soil sample collected in Texas. Ex-

Table 1. Comparison of macromorphology of culture LL-E33288 and *Micromonospora echinospora* subsp. *pallida* NRRL N2996.

ISP agar medium	Spores	Vegetative mycelium[a]	Soluble pigments
LL-E33288			
Yeast - malt (ISP 2)	None	Dark orange-yellow (72)	None
Oatmeal (ISP 3)	None	Colorless → pale orange-yellow (73)	None
Inorganic salts - starch (ISP 4)	Slight border of black spores	Dark orange-yellow (72) to light yellow-brown (76)	Light brown
Glycerol - asparagine (ISP 5)	None	Pale orange-yellow (73) → colorless	None
N2996			
Yeast - malt	None	Beige → medium yellow (87, light)	None
Oatmeal	None	Gray-yellow (90, light)	None
Inorganic salts - starch	None	Gray-yellow (90)	None
Glycerol - asparagine	None	Colorless	None

[a] ISCC, National Bureau of Standard Centroid Color Charts, Publication 440, Washington, D.C., 1976.

Table 2. Comparison of macromorphology of culture LL-E33288 with *Micromonospora echinospora* subsp. *pallida* N2996.

Agar medium		LL-E33288	N2996
Pablum	V:	Beige	Beige
	S:	Slight black	Slight black
	P:	None	None
Yeast - CZAPEK	V:	Beige	Beige
	S:	None	None
	P:	None	Slight soluble brown
CZAPEK	V:	Beige	Orange
	S:	Slight black	Slight black
	P:	None	Slight brown
Yeast - glucose	V:	Tan	Tan
	S:	Moderate black	Slight black
	P:	Slight dark	Slight brown
Nutrient	V:	Colorless to tan	Colorless
	S:	Slight black	Slight black
	P:	None	None
Nutrient - glycerol	V:	Colorless to light beige	Colorless
	S:	None	Slight black
	P:	None	None
BENNETT's dextrin	V:	Colorless to beige	Colorless to beige
	S:	Slight black	Slight black
	P:	Slight rosy-brown	Slight rosy-brown
Glucose - asparagine	V:	Colorless to light orange-beige	Colorless to light orange-beige
	S:	None	Slight black
	P:	None	None

V: Growth of vegetative mycelium, S: spores, P: soluble pigment.

amination of the culture grown at 28°C for 14~31 days on various media revealed that monospores were present either singly or in masses on vegetative hyphae, but no aerial hyphae were observed. The results are summarized in Table 1. A summary of the culture's growth characteristics on various media is presented in Table 2. Electron microscopy examination showed the spores were warty. Whole cell analysis showed that the strain contained the *meso* isomer of diaminopimelic acid (DAP) with the 3-OH derivative of DAP present in large (major) amounts. Additionally, the strain contained xylose plus traces of arabinose in its whole cell sugar hydrolysate, indicating a whole cell sugar pattern of type D. Utilization of carbon sources are summarized in Table 3, and the physiological reactions are recorded in Table 4. From the macromorphological, chemotaxonomic, and physiological studies, it was concluded that culture LL-E33288 and *Micromonospora echinospora* subsp. *pallida* are closely related but differ in growth on salicylate and at 45°C and in decarboxylation of mucate (Tables 1 to 4). For these reasons, culture LL-E33288 is considered a new subspecies of *M. echinospora*, designated

Table 3. Carbohydrate utilization of culture LL-E33288 and *Micromonospora echinospora* subsp. *pallida* N2996.

	LL-E33288	N2996
Arabinose	+	+
Cellulose	−	−
Fructose	+	+
Glucose	+	+
Inositol	−	−
Mannitol	−	−
Raffinose	±	−
Rhamnose	+	+
Sucrose	+	+
Xylose	+	+

+: Utilized, ±: weakly utilized, −: not utilized.

Table 4. Physiological reactions of culture LL-E33288 and *Micromonospora echinospora* subsp. *pallida* N2996.

	LL-E33288	N2996		LL-E33288	N2996
Hydrolysis of:			Acid from:		
Casein	+	+	Adonitol	−	−
Xanthine	−	−	Arabinose	+	+
Hypoxanthine	−	−	Cellobiose	+	+
Tyrosine	+	+	Dextrin	+	+
Adenine	−	−	Dulcitol	−	−
Gelatin	+	+	Erythritol	−	−
Potato starch	+	+	Fructose	+	+
Esculin	+	+	Galactose	V	−
Production of:			Glucose	+	+
Nitrate reductase	+	+	Glycerol	−	−
Phosphatase	W	+	Inositol	−	−
Urease	−	−	Lactose	−	−
Growth on:			Maltose	+	+
Salicylate	−	+	Mannitol	−	−
5% NaCl	−	−	Mannose	+	+
Lysozyme broth	−	−	Methyl α-D-glucoside	−	−
Decarboxylation of:			Melibiose	−	−
Acetate	+	+	Raffinose	+	+
Benzoate	−	−	Rhamnose	+	+
Citrate	−	−	Salicin	+	+
Lactate	+	+	Sorbitol	−	−
Malate	−	V	Sucrose	+	+
Mucate	−	+	Trehalose	+	+
Oxalate	−	−	Xylose	+	+
Propionate	+	+	Methyl β-D-xyloside	−	−
Pyruvate	+	+	Growth at:		
Succinate	−	−	10°C	−	−
Tartrate	−	−	42°C	+	+
			45°C	+	−

+: Positive, −: negative, V: variable, W: weak.

calichensis.

Fermentation

Culture LL-E33288 was grown in a 41-liter fermentor at 28°C for 200 hours. A typical time course for the production of the antitumor antibiotic is shown in Fig. 1. Calicheamicin production started at approximately 55~60 hours after inoculation and reached maximum titers at approximately 180 hours. Chemical characterization of the calicheamicins revealed the presence of bromine in the molecule[6,7]. Addition of NaBr to fermentation media stimulated production of the antibiotic. The addition of KI significantly increased the yield of the calicheamicins and changed the components from bromine- to new iodine-containing species (Table 5).

Antimicrobial Activity

The antimicrobial activity of the calicheamicins is shown in Table 6. The individual components of the calicheamicins exhibited very potent activity against Gram-positive bacteria with most MIC values in the pg/ml range. Although the MIC values for Gram-negative bacteria were significantly higher than observed for Gram-positives, the activity against Gram-negatives (MIC ≤ 1 μg/ml) was

Table 5. Effects of halide addition on calicheamicin production.

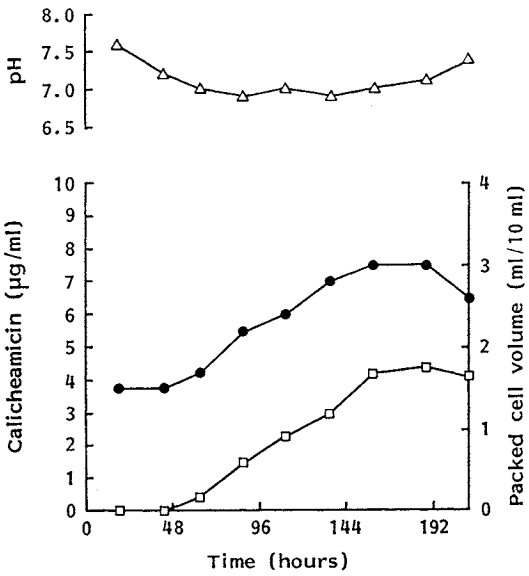
Halide	Calicheamicin (μg/ml)			
	γ_1^{Br}	β_1^{Br}	γ_1^{I}	β_1^{I}
—	<0.05	0.1	<0.05	<0.05
NaBr (0.05%)	2.3	0.3	<0.05	<0.05
KI (0.01%)	<0.05	<0.05	9.8	2.6

Table 6. *In vitro* antimicrobial spectrum of the calicheamicins.

Organisms (strains tested)	MIC (μg/ml)[a] range			
	β_1^{Br}	β_1^{I}	γ_1^{Br}	γ_1^{I}
Escherichia coli (3)	0.12~0.25	0.25~0.5	0.25~0.5	0.25
Klebsiella pneumoniae (2)	0.12~0.25	0.5	0.5	0.25
Enterobacter sp. (2)	0.25~0.5	0.25~0.5	0.5	0.5
Serratia sp. (2)	0.12	0.25~0.5	0.25~0.5	0.12~0.25
Citrobacter sp. (2)	0.12	0.25~0.5	0.25~0.5	0.12~0.25
Acinetobacter sp. (2)	0.06~0.12	0.25	0.25	0.06~0.12
Pseudomonas aeruginosa (2)	0.25~0.5	0.25~0.5	0.5~1	0.12~0.25
Staphylococcus aureus (7)	≤0.00025	≤0.000031	≤0.000031	≤0.000031
S. epidermidis (2)	≤0.00025	≤0.000031	≤0.000031	≤0.000031
Enterococcus sp. (1)	0.0038	0.031	0.062	0.0078
Bacillus subtilis (1)	≤0.00025	≤0.000031	≤0.000031	≤0.000031

[a] MIC values were determined by the standard agar dilution method in Mueller-Hinton medium.

Fig. 1. Fermentation profile of culture LL-E33288.
□ Calicheamicin, ● packed cell volume, △ pH.



still very good. The antimicrobial activity of the β and γ components had essentially the same MIC values, and only minor differences were noted for the bromine- vs. iodine-containing antibiotics.

Table 7. Antitumor activity of calicheamicin γ_1^{I} against P388 leukemia and B16 melanoma in mice.

Dosage (μg/kg)	MST[a] (days)	T/C (%)	Survivors[b]
P388 leukemia			
20.0	10.0	90	0/5
10.0	14.5	132	0/5
5.0	25.5	232	2/5
2.5	24.5	223	0/5
1.25	19.0	173	0/5
0.6	20.0	182	0/5
0.3	19.0	173	0/5
0.15	17.0	155	0/5
1,600.0 (cisplatin)	29.5	268	3/6
Control	11.0	100	0/5
B16 melanoma			
1.25	39.0	186	0/5
0.6	35.0	167	0/5
0.3	29.5	140	0/5
0.15	24.5	117	0/5
0.075	25.5	121	0/5
400.0 (cisplatin)	31.5	150	0/5
Control	21.0	100	0/5

[a] Median survival time.
[b] 30 days for P388, 60 days for B16.

Antitumor Activity

The antitumor activity of calicheamicin γ_1^I was determined in male BDF_1 mice against P388 leukemia and B16 melanoma. The results are summarized in Table 7. Calicheamicin γ_1^I exhibited maximal antitumor activity against P388 at 5.0 μg/kg (T/C 232) and B16 at 1.25 μg/kg (T/C 186).

Discussion

A new actinomycete strain, *M. echinospora* subsp. *calichensis*, produces a novel family of antibiotics, referred to originally as LL-E33288 and now as the calicheamicins[7]. These compounds possess potent activity against both Gram-positive and Gram-negative bacteria and exert antitumor effects against both P388 leukemia and B16 melanoma in mice. The calicheamicins are related structurally to the esperamicins from *Actinomadura verrucosospora*[8], veractamycins from *A. verrucosospora* subsp. *veractimyces*[9], FR-900405/6 from *Actinomadura pulveracea*[10] and the CL-1724 antibiotics from *Actinomadura* sp. NRRL 15758[11]. However, the calicheamicins are the only representative of this related class of antibiotics reported to be produced by a member of the genus *Micromonospora*. The isolation and chemical characterization of the calicheamicins will be published in a separate communication.

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