LL-49F233α, a Novel Antibiotic Produced by an Unknown Fungus: Biological and Mechanistic Activities

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While screening fermentations of fungal cultures for activity against antibiotic-resistant bacteria, a methanolic extract of the mycelial mass of an unidentified and non-sporulating fungal culture, LL-49F233, was found to exhibit activity against antibiotic-resistant bacteria. The active molecule was identified as a novel antibiotic, LL-49F233α, containing an *N*-methyltetramic acid attached to a bicyclic hydrocarbon skeleton (Figure 1)[1]. This compound was not cytotoxic against a panel of tumor cell lines and was inactive in a screen detecting DNA damage in *Escherichia coli*. Microbial metabolites containing a tetramic acid moiety have been known to have diverse biological activities[2~10]. Equisetin (a potent antibacterial and leukemogenic agent produced by a *Fusarium equiseti*)[4], MBP049 (a proline hydroxylase inhibitor produced by a *Ophiobolus rubellus*)[5], antibiotic PF1052 (an antibacterial agent produced by *Phoma* species)[6] and lydicamycin (a novel antibacterial agent containing tetramic acid and amidinopyrrolidine moieties)[10] are some of the chemically related compounds. Streptolydigin and tirandamycin A are other typical members of the naturally occurring class of 3-dienoyl tetramic acids that possess potent antibacterial activity, particularly against anaerobes, and have been shown to inhibit bacterial RNA polymerase[3,9]. Some tetramic acid derivatives are also reported to be DNA gyrase inhibitors[11]. Since antibiotic LL-49F233α was structurally different from all known tetramic acid-containing

Fig. 1. Chemical structures of LL-49F233α and related compounds.

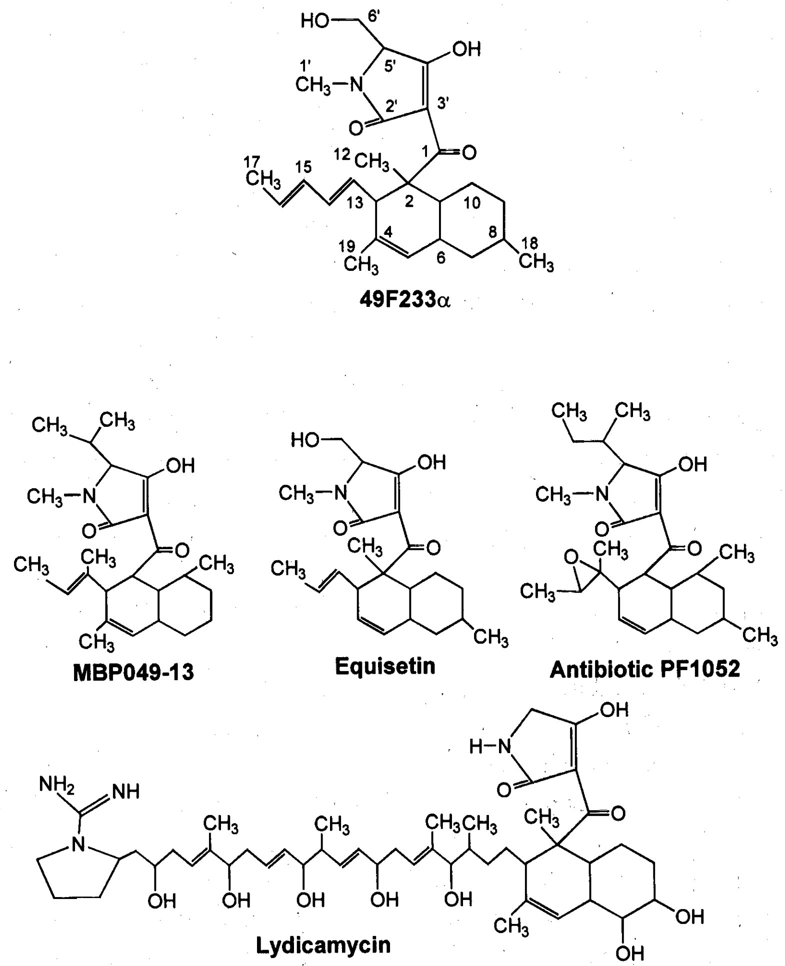

Table 1. *In-vitro* antibacterial activity (MIC range in mg/liter) of LL-49F233α.

Organism (No. of isolates)	Piperacillin	Vancomycin	LL-49F233α	LL-49F233α (+5% blood)
Micrococcus luteus (1)	>0.06	0.50	2	32
Bacillus cereus (1)	2	1	2	32
Staphylococcus aureus MS (3)	1~4	0.5~1	0.5~1	32
S. aureus MR (4)	>128	0.5~1	0.5~1	32
S. haemolyticus (1)	>128	1	0.50	64
CNS (5)	2~8	0.50~2	0.50~1	32~64
Enterococcus faecalis VS (4)	1~8	0.50~2	1~4	64~>64
E. faecalis VR (1)	8	>128	2~4	64~>64
E. faecium VS (2)	0.12~128	0.5~1	2~4	64~>64
E. faecium VR (1)	>128	>128	4	>64
E. avium VR (1)	>128	>128	2	64
Pseudomonas aeruginosa (1)	4	>128	>64	>64
Morganella morganii (1)	64	>128	>64	>64
Escherichia coli (2)	0.50~2	>128	>64	>64
E. coli imp (1)	>0.06	0.50	2	NT

Method: Agar dilution method in MHA-II. MS, methicillin-sensitive; MR, methicillin-resistant; VS, vancomycin-sensitive; VR, vancomycin-resistant; CNS, coagulase-negative *Staphylococcus*.

compounds, we investigated its microbiological and mechanistic profiles.

In-vitro minimum inhibitory concentrations (MICs) were determined by the agar dilution or microbroth dilution methods[12,13]. Bacterial macromolecular synthesis was studied by measuring the incorporation of appropriate radiolabeled precursors [tritiated thymidine (^{3}H-Tdr), uridine (^{3}H-Udr) and amino acids (^{3}H-AA) for DNA, RNA and protein, respectively] into TCA-precipitable material[13]. Effects on the intracellular potassium level and morphology of a log-phase culture of *E. coli imp* were determined by the method described earlier[14].

LL-49F233α exhibited good activity against Gram-positive bacteria including methicillin-resistant staphylococci and vancomycin-resistant enterococci (Table 1). A two-log increase in cell density resulted in a 2 to 3-fold increase in the MIC, *i.e.* the antibacterial activity was inoculum-dependent. Presence of 5% sheep blood in the medium resulted in a drastic reduction in the antibacterial activity, suggesting strong serum binding of the compound. Although LL-49F233α exhibited good activity against *E. coli imp* (strain with increased outer membrane permeability) it had no activity against wild-type strains of *E. coli* or other Gram-negative bacteria (MIC >64 μg/ml). These data suggest that permeability of this compound across the normal Gram-negative outer membrane may be limited. Additionally, this compound also appeared to diffuse poorly in agar plate assays, as demonstrated by a slope of <1 mm per two-fold change in drug concentration (data not shown).

Inhibition of DNA, RNA, and protein syntheses by LL-49F233α was determined in a logarithmic-phase culture of *E. coli imp*. Control drugs affected the anticipated macromolecular processes (Table 2). Although treatment with 49F233α for 5~20 minutes inhibited incorporation of all three precursors into the respective macromolecules, incorporation of ^{3}H-Udr appeared to be slightly more sensitive than the others. In our previous experiences with membrane active agents, a similar profile, *i.e.*, a slightly preferential inhibition of uptake and incorporation of ^{3}H-Udr into RNA, has been noted. Further studies would be needed to clearly understand the slightly preferential effect of LL-49F233α on ^{3}H-Udr incorporation.

After a two hour exposure of *E. coli imp* to LL-49F233α, lysed cells and cells with irregular morphology were observed microscopically. Similar effects were observed in an osmotically protective medium, but at a two-fold higher concentration. These data are consistent with a disturbance to the cell membrane (Table 3). On the contrary, treatment of the log-phase culture of *E. coli imp* resuspended in sucrose-phosphate buffer containing LL-49F233α released only 10% of the intracellular potassium (Table 4). The membrane-active drug polymyxin B released 55% of the intracellular potassium under the same conditions.

Table 2. Effects of some known antimicrobials and LL-49F233α on the macromolecular synthesis in *E. coli imp*.

Compound	Conc. (μg/ml)	Pretreatment (minutes)	Percent Incorporation of		
			^{3}H-Tdr	^{3}H-Udr	^{3}H-AA
Ciprofloxacin	0.25	5	4.7	107.6	97.3
		10	3.8	97.5	88.6
		20	2.6	93.1	97.4
Rifampin	0.25	5	126.5	5.0	43.0
		10	115.1	1.2	10.0
		20	86.7	7.7	4.7
Chloramphenicol	8.0	5	98.2	131.5	14.2
		10	98.5	105.7	8.9
		20	92.7	93.9	7.6
LL-49F233α	32.0	5	69.5	32.1	37.8
		10	55.5	11.5	22.9
		20	27.4	7.7	13.7
	16.0	5	79.3	51.9	54.5
		10	82.3	23.5	38.2
		20	69.0	10.5	22.5
(4 × MIC)	8.0	5	101.9	75.7	68.3
		10	96.5	38.6	59.7
		20	76.8	23.9	52.4

Experimental condition: Exponential-phase cells were pretreated with the drug for 5～20 minutes and then were pulse labeled for 5 minutes with ^{3}H-Tdr, ^{3}H-Udr, ^{3}H-AA for measuring DNA, RNA, and protein syntheses, respectively.

Table 3. Effect of LL-49F233α on the morphology of *E. coli imp*.

Conc. (μg/ml)	Minimal medium	Minimal medium + 13.7% Sucrose
Untreated	30～40 normal cells	25～30 normal cells, elongated than the cells in unsupplemented medium
128～32	2～6 empty (ghost or lysed) cells	2～4 ghost cells, 1～2 round shaped like spheroplast
16	15～20 very light cells, some ghost, 2～3 swollen and centrally bulged, only a few motile	15～20 total cells, 10 ghosts, 2～4 rounded and 2～3 swollen cells
8	15～20 very light cells, some ghost, 2～3 swollen and centrally bulged	30～40 total cells, 5～8 empty and remaining darker, 2～4 rounded and 2～3 swollen cells, all cells smaller in size (1/2 ×)
4～0.25	30～40 normal cells, 2～3 swollen and centrally bulged	80～100 smaller cells, mostly in 2-units

These data contradicted the radiolabeling and morphological studies performed with actively growing cells, which clearly suggested a strong membrane-damaging effect of the LL-49F233α. It became apparent that LL-49F233α required growing or metabolically active cells to exhibit its antibacterial activity.

A comparison of the effects of reference antimicrobials with LL-49F233α on macromolecular synthesis in a log-phase *E. coli imp* culture resuspended in sucrose-phosphate buffer (0.1 M, pH 7.2) was conducted. Under this condition, cells were in a metabolically compromised state and showed different radiolabeling pattern than the actively growing cells. In the untreated culture pulse-labeled for 5 minutes, the incorporation of ^{3}H-uridine was reduced to almost zero, but the incorporation of ^{3}H-thymidine and ^{3}H-amino acids were only marginally affected. When the cells were pulse-labeled for 5 minutes following a 5 minute drug treat-

Table 4. Effect of LL-9F233α on intracellular potassium of *E. coli imp.*

Sample	K^+ released (%) after	
	5 min	20 min
Untreated	7	4
LL-49F233α (16 μg/ml)	10	10
Polymyxin B (8 μg/ml)	55	53
DMSO (0.6%)	3	4

Medium: 0.1 M sucrose +0.005 M sodium phosphate buffer, pH 7.0.

ment, polymyxin B (8 μg/ml) inhibited incorporation of precursors into DNA (86%) and protein (84%); ciprofloxacin (0.25 μg/ml) specifically inhibited incorporation of precursor into DNA (65%), but LL-49F233α (16 μg/ml) only marginally inhibited incorporation of precursors into DNA (13%) and protein (10%). Since the incorporation of ^{3}H-uridine was very small, the effects of the drugs could not be assessed. In growing cells, however, LL-49F233α (16 μg/ml, 5 minute treatment) was found to inhibit DNA, RNA, and protein synthesis by 21, 48 and 45%, respectively (Table 2). These data suggest that the ^{3}H-uridine incorporation into RNA and the antibacterial activity of LL-49F233α are not favored in the sucrose-phosphate buffer, this may be the primary reason why LL-49F233α failed to show significant effects on macromolecular synthesis and intracellular potassium levels under these conditions.

LL-49F233α is an interesting antibiotic with activity against methicillin-resistant staphylococci and vancomycin-resistant enterococci, but it has poor activity against wild-type Gram-negative bacteria. Structural modification of this new antibiotic to improve the antibacterial activity and spectrum is in progress. Since compounds containing a tetramic acid moiety have been shown to exhibit good activity against anaerobic bacteria, LL-49F233α and its new analogs will also be evaluated against such pathogens.

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