In Vivo Evaluation of Three Acid-stable Azalide Compounds, L-701,677, L-708,299 and L-708,365 Compared to Erythromycin, Azithromycin and Clarithromycin

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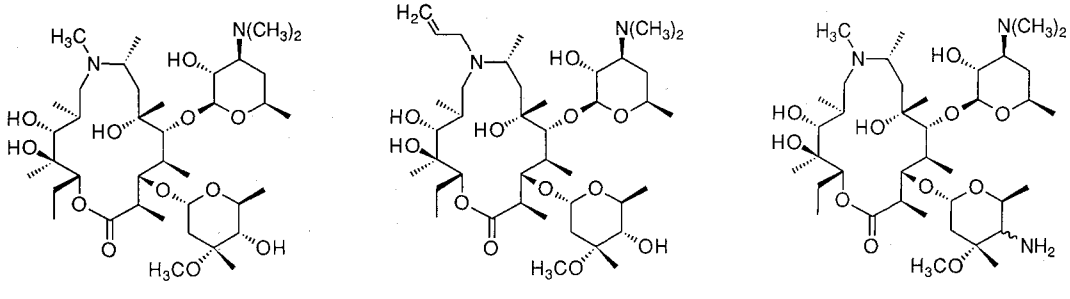
L-701,677, L-708,299 and L-708,365 are novel azalide derivatives of erythromycin that exhibit improved acid stability over erythromycin, azithromycin and clarithromycin. The half-life in aqueous solution at pH=2.1 of these compounds ranged from 0.3 hour for erythromycin to 16.2 hours for L-708,299. The rank order of half-life in acid solution from most to least stable was L-708,299 > L-701,677 > L-708,365 > azithromycin = clarithromycin > erythromycin. In a disseminated *Streptococcus pyogenes* mouse infection model, azithromycin and L-708,365 were slightly more efficacious than clarithromycin, L-701,677 and L-708,299; all 5 compounds being more active than erythromycin. In a *Klebsiella pneumoniae* pulmonary challenge mouse model, azithromycin, L-701,677, L-708,299 and L-708,365 were all equal in efficacy and at least four-fold more active than clarithromycin and erythromycin. Clarithromycin, L-708,365 and interestingly erythromycin, showed greater bacterial clearance than azithromycin, L-701,677 and L-708,299 in a localized infection model that measured clearance of *Staphylococcus aureus* from mouse thigh tissues. Our results indicate that L-701,677, L-708,299 and L-708,365 exhibit improved acid stability and were at least equally efficacious as presently marketed macrolide/azalide antibiotics.

The macrolide class of antibiotics consisting of a macrocyclic lactone ring to which sugars are attached was discovered in the early 1950's. Erythromycin, then called ilotycin, isolated from a strain of *Streptomyces erythraeus* in a soil sample from the Phillipines, was the first macrolide to be discovered and developed as a clinically useful antibiotic[1]. This compound has been used successfully for over 40 years to treat respiratory, cutaneous and genital tract infections caused by a variety of Gram-positive microorganisms, such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*. More recent developments have shown that erythromycin is also active against so called "emerging" respiratory pathogens such as *Legionella pneumophila*, *Mycoplasma* species and *Chlamydia* species. Activity against intracellular pathogens has been attributed to the ability of erythromycin to penetrate and accumulate within human phagocytic cells[2].

Although erythromycin has been prescribed for over four decades, some problems with the drug remain. Primarily, these are the marked variation in the bioavailability of oral preparations and side effects in the gastrointestinal tract. Variable absorption is presumably due to inactivation of the drug by the acid pH of the

Fig. 1. Chemical structures of three novel macrolide compounds.



stomach[3]. Erythromycin base is very unstable under acidic conditions and special formulations are required to protect this antibiotic from the acid environment of the stomach. While erythromycin is low in toxicity and generally well tolerated, the most common side effects are gastrointestinal upset, nausea and vomiting.

Two new macrolide agents, azithromycin (Zithromax, Pfizer) and clarithromycin (Biaxin®, Abbott) have proven to be more acid stable than erythromycin, therefore better absorbed and distributed to tissues[4,5]. Development of these two agents with improved spectrum, bioavailability and efficacy has renewed interest in the macrolide/azalide class of antibiotics. The present study evaluated three new azalide compounds (Fig. 1), L-701,677 (9-deoxo-8a-methyl-8a-aza-8a-homoerythromycin A), L-708,299 (4″-deoxy-4″-amino-9-deoxo-8a-methyl-8a-aza-8a-homoerythromycin A) and L-708,365 (9-deoxo-8a-(2-propenyl)-8a-aza-8a-homoerythromycin A) for acid stability and efficacy in three mouse infection models. Results were compared to those obtained for erythromycin, azithromycin and clarithromycin.

Materials and Methods

Animals

Outbred Viral Antibody Free (VAF) CD-1 female mice (Charles River Laboratories, Wilmington, MA) six weeks old and weighing approximately 20 g were housed conventionally and fasted overnight prior to *Streptococcus pyogenes* disseminated infection. DBA/2 female mice (Taconic Laboratories, Germantown, NY) eight weeks old and 20 g housed in sterile microisolator cages were used in both the *Klebsiella pneumoniae* pulmonary infection model and the *Staphylococcus aureus* thigh infection model. Ten mice per group were used in all experiments. All animal procedures were performed in accordance with the highest standards for the humane handling, care and treatment of research animals and are preapproved by the Merck Institutional Animal Care and Use Committee. The care and use of research animals at Merck meet or exceed all applicable local, national and international laws and regulations.

Antimicrobial Agents

Erythromycin base was purchased from Sigma Chemical Company (St. Louis, MO). Azithromycin dihydrate, clarithromycin, L-701,677, L-708,299 and L-708,365 were synthesized in base form by research medicinal chemists at Merck Research Laboratories (Merck and Co., Inc., Rahway, NJ). All compounds were >95% pure as determined by TLC and NMR. All macrolide powders were dissolved in ethanol and further diluted to 5% ethanol in Sorenson's phosphate buffer pH=8.0. Norfloxacin (Merck and Co., Inc, Rahway, NJ), was dissolved in 0.01 N sodium hydroxide and further diluted in distilled water.

Organisms

S. pyogenes MB 2874, *K. pneumoniae* MB 4005 and *S. aureus* (Smith) MB 2865 were all obtained from the Merck Culture Collection. *Micrococcus luteus* ATCC 9341 was obtained from the American Type Culture Collection (Rockville, MD). All cultures were received in lyophilized form, reconstituted in appropriate broth media and cultured on blood agar plates to check for purity and viability prior to use. *In vitro* antibiotic susceptibility of *S. pyogenes* MB 2874, *K. pneumoniae* MB 4005 and *S. aureus* MB 2865 against the test macrolide compounds was determined by agar dilution methodology according to the National Committee for Clinical Laboratory Standards (NCCLS) Document M7-A2[6]. Minimum Inhibitory Concentration (MIC) values in μg/ml were derived prior to *in vivo* testing.

Acid Stability Studies

The half-life and percent residual activity of the macrolide compounds in acid were determined by diluting stock solutions of 1 mg/ml in 0.1 N HCl, pH=2.1, and incubating at room temperature for six hours. The pH of each compound was measured at t=0, 0.5, 1, 2, 3 and 6 hours. Samples from each timepoint were first neutralized by diluting 1:100 in Sorenson's phosphate buffer (pH=8.0) and the concentration of compound remaining was determined by bioassay utilizing *M. luteus* ATCC 9341.

Streptococcus pyogenes Disseminated Model

A highly virulent *S. pyogenes* strain, MB 2874, was utilized in this model. A 1:10,000 dilution of a seven hour culture prepared in Brain Heart broth supplemented with 10% horse serum and incubated at 35°C was used as the inoculum. CD-1 mice were challenged intraperitoneally (I.P.) with approximately 6×10^3 cells/0.5 ml which was roughly 100× the 14 day LD_{50}. Compounds were tested at levels of 200, 50, 12.5 and 3.125 mg/kg. Oral (P.O.) antibiotic therapy in 0.5 ml was initiated immediately, t=0 (total of 1 dose), after challenge. Mice were surveyed for mortality over 14 days and all moribund mice were euthanized. Effective dose 50% (ED_{50}) values were calculated at 7 and 14 days after challenge using the method of KNUDSEN and CURTIS[7] and test values were compared to values obtained for sham-treated (Sorenson's buffer) mice.

Klebsiella pneumoniae Pulmonary Challenge Model

K. pneumoniae MB 4005 was used to establish a pulmonary challenge in DBA/2 mice. Mice were lightly anesthetized by brief exposure to CO_2 and then challenged intranasally (I.N.) with a 1:4 dilution of an overnight broth culture (Trypticase soy broth, 35°C), approximately 7.5×10^5 cells/50 μl (100× the 14 day LD_{50}). Macrolide compounds were tested at levels of

100, 25, 6.25 and 1.56 mg/kg. Norfloxacin, used as a positive control drug, was tested at 20, 10, 5 and 2.5 mg/kg. Antibiotic treatment was administered either P.O. or subcutaneously (S.C.) in 0.5 ml at 30 minutes after challenge, then 6 hours later and was continued twice-daily (b.i.d.) for 2 additional days (total of 6 doses). Mice were surveyed for mortality over a period of 14 days; 7 and 14 day ED_{50} values were calculated as described previously.

Staphylococcus aureus Mouse Thigh Infection Model

A mouse virulent *S. aureus* (Smith strain), MB 2865, was grown in Trypticase soy broth (TSB) at 35°C for 10 hours, washed once in fresh TSB, resuspended in half-volume and a 1 : 5 dilution of this culture was used to challenge DBA/2 mice. Mice were injected intramuscularly (I.M.) in the right thigh with approximately 4×10^7 colony forming units (cfu)/0.2 ml ($20 \times$ the 7 day LD_{50}). Antibiotic therapy (0.5 ml, P.O.) at 100 or 25 mg/kg was administered beginning 2 hours after challenge, then 6 hours later and therapy continued b.i.d. for 3 additional days (total of 8 doses). Mice were euthanized 48 hours after the last antibiotic dose. Thighs were dissected, homogenized in phosphate buffered saline supplemented with 10% glycerol utilizing a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and plate counts were determined on selective Mannitol salt agar. Colony counts from antibiotic-treated groups were compared to counts from sham-treated control groups.

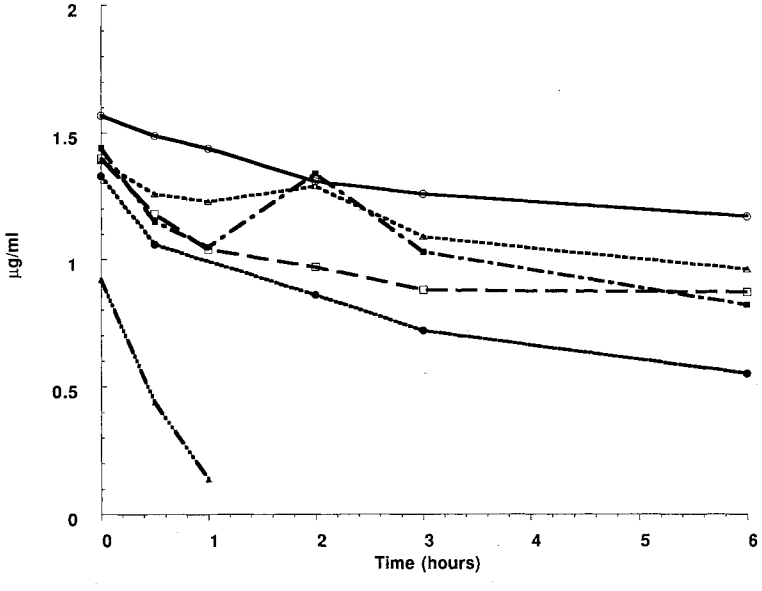
Results

The comparative stability of the macrolide compounds in acid solution (pH = 2.1) is shown in Fig. 2. L-708,299 with a half-life of 16.2 hours was the most stable compound followed closely by L-701,677 ($T_{1/2} = 14.2$ hours) and L-708,365 ($T_{1/2} = 13.2$ hours). Clarithromycin and azithromycin exhibited equivalent acid stability with half-lives of 5.9 hours and 5.7 hours, respectively. Erythromycin with a 0.3 hour half-life was the least acid-stable compound. A direct correlation was also observed between the acid stability of these compounds and the percent residual activity remaining after 6 hours incubation in the acid solution.

The *in vitro* susceptibility results indicated both Gram-positive organisms used in the *in vivo* models to be very susceptible to all macrolide compounds tested. The Minimal Inhibitory Concentration (MIC) for L-701,677, L-708,299, L-708,365, azithromycin, erythromycin and clarithromycin against *S. pyogenes* MB 2874 ranged between 0.01 μg/ml and 0.06 μg/ml (Table 1). Against *S. aureus* MB 2865 the MIC values for erythromycin, clarithromycin, azithromycin, L-701,677 and L-708,365 were ten-fold higher ranging from 0.125 μg/ml to 0.25 μg/ml. The MIC value for L-708,299 against this organism was slightly higher at 1.0 μg/ml. However, L-708,299 was the most active compound against the Gram-negative organism, *K. pneumoniae* MB 4005, with an MIC value of 0.125 μg/ml. L-701,677, L-708,365 and azithromycin were equally active at 1 μg/ml while clarithromycin and erythromycin were the least active compounds with MIC values of 16 μg/ml and 32 μg/ml, respectively.

In the *S. pyogenes* disseminated mouse model (Table 1), at 7 days after challenge, azithromycin was two to

Fig. 2. Stability of macrolide compounds in acid solution, pH = 2.1.



		$T_{1/2}$	%
—○—	L-708299-	16.2	75
—□—	L-701677-	14.2	60
--△--	L-708365-	13.2	68
—●—	Clarithromycin-	5.9	40
—■—	Azithromycin-	5.7	50
—▲—	Erythromycin-	0.3	0

$T_{1/2}$ = Half-life of compound in acid solution.
% = Percent bioactivity remaining after 6 hours in acid solution.

six-fold more active than L-708,365, clarithromycin, L-708,299 and L-701,677. Fourteen days after challenge, the ED_{50} values for all compounds were expectedly higher with azithromycin maintaining its two to six-fold activity over the other compounds. Erythromycin was not active against *S. pyogenes* in this model.

Comparative effective dose values for the macrolide compounds in the *K. pneumoniae* pulmonary challenge model are shown in Table 2. At 7 days after challenge, azithromycin, L-701,677, L-708,365 and L-708,299 were all comparable in efficacy in this model. At 14 days after challenge, azithromycin appeared slightly more active than L-708,299 and was almost 1.5 × more active than L-701,677 and L-708,365.

Azithromycin, L-708,299 and L-701,677 also were administered S.C.; azithromycin with a 14 day ED_{50} value of 30.2 mg/kg was slightly more active than L-708,299 and L-701,677. Erythromycin and clarithromycin which were the least potent compounds *in vitro* were, as expected, not active in this model. None of the macrolide compounds tested was as efficacious as the quinolone antibacterial control drug, norfloxacin.

Fig. 3 displays the efficacy of the macrolide compounds in the mouse thigh infection model. At 100 mg/kg clarithromycin, erythromycin and L-708,365 were the most active compounds in this model with greater than a 3 $\log_{10}$ reduction in *S. aureus* cfu/thigh when compared to sham-treated infected controls. Azithromycin and L-708,299 were slightly more active than L-701,677 with a greater than 2 $\log_{10}$ decrease in *S. aureus* cfu/thigh. At 25 mg/kg only clarithromycin (>3 $\log_{10}$ decrease) and L-708,365 (>1 $\log_{10}$ decrease) exhibited activity in this model.

Table 1. *In vivo* efficacy of macrolides in a *Streptococcus pyogenes* (MB 2874) disseminated mouse model.[a]

Compound	MIC (μg/ml)[b]	ED_{50} (mg/kg)[c] (95% Confidence interval)	
		Day 7	Day 14
L-708,365	0.03	7.8 (3.2~18.7)	15.8 (5.2~47.9)
L-708,299	0.06	16.6 (6.4~43.5)	19.9 (7.1~56.3)
L-701,677	0.015	21.5 (8.9~51.7)	25.0 (11.4~54.7)
Azithromycin	0.03	2.7 (1.0~6.9)	3.9 (1.5~10.2)
Clarithromycin	0.015	10.9 (3.4~35.3)	10.9 (3.4~35.3)
Erythromycin	0.015	>200	>200

[a] Mice were challenged with *S. pyogenes* at t=0 and immediately treated P.O. with test compound (total of 1 dose).
[b] Minimum inhibitory concentration determined by agar dilution method on Mueller Hinton agar containing 5% sheep blood.
[c] ED_{50} = Effective dose 50% calculated on days 7 and 14 in mg/kg that protects 50% of infected mice (10 mice/group).

Discussion

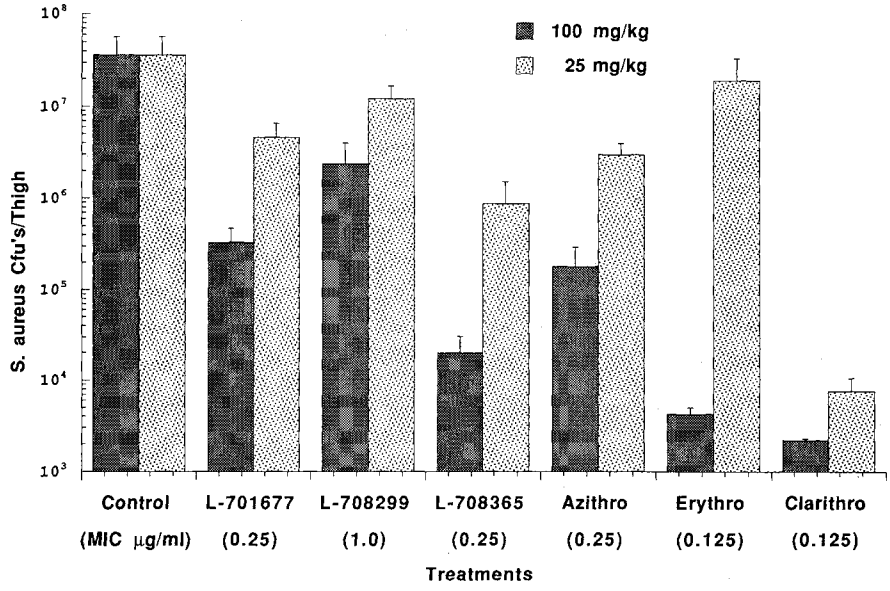
Although erythromycin has been clinically useful in the treatment of skin, respiratory and genital tract infections, it has not been useful against infections caused by Gram-negative organisms such as *Haemophilus influenzae*. Also, due to poor absorption and gastro-

Table 2. Comparative effective dose values (P.O. *vs.* S.C.) for macrolide compounds against a *Klebsiella pneumoniae* (MB 4005) pulmonary infection in DBA/2 mice.

Compound	MIC (μg/ml)[b]	ED_{50} (mg/kg)[a] (95% Confidence interval)			
		P.O.[c]		S.C.[c]	
		7 day	14 day	7 day	14 day
L-708,365	1	18.0 (7.5~43.2)	30.7 (15.5~60.5)	Not tested	Not tested
L-708,299	0.12	19.1 (9.7~37.6)	22.1 (11.2~43.5)	48.2 (21.9~105.5)	48.2 (21.9~105.5)
L-701,677	1	14.9 (6.2~35.9)	30.7 (15.5~60.5)	50.0 (25.0~75.0)	66.8 (38.4~116.4)
Azithromycin	1	14.5 (5.5~37.8)	17.0 (7.2~41.5)	30.2 (17.4~52.6)	30.2 (17.4~52.6)
Clarithromycin	16	>100	>100	>100	>100
Erythromycin	32	>100	>100	>100	>100
Norfloxacin	0.06	7.6 (4.7~12.3)	13.8 (7.9~23.9)	1.95 (1.2~3.3)	7.07 (3.6~13.9)

[a] ED_{50} = Effective dose in mg/kg that protected 50% of infected mice (10 mice/group).
[b] Minimal inhibitory concentration determined by agar dilution method on Mueller Hinton agar.
[c] Mice were challenged with *K. pneumoniae* MB 4005 at t=0 and treated P.O. or S.C. with compound 0.5 hour after challenge, then 6 hours later, twice daily for three days (total of 6 doses).

Fig. 3. Efficacy of macrolide compounds in the mouse thigh infection model.



DBA/2 mice challenged with 0.2 ml *S. aureus* MB2865 in the right thigh. Antibiotic therapy administered P.O. at t=2 hours after challenge, then 6 hours later and continued twice daily for 3 days (total of 8 doses).

intestinal side effects after oral administration, its utility has been limited[8]. Recently marketed macrolides such as azithromycin, containing a methyl substituted nitrogen at position 9a of the lactone ring and clarithromycin with an O-methyl substitution at position 6 of the lactone ring have demonstrated improved gastric acid stability[9,10] and exhibit distinct advantages over erythromycin. Both have longer half-lives, yield higher tissue levels and concentrations of both are increased in polymorphonuclear leukocytes and macrophages[11~17]. These properties along with the greater acid stability lead to a shorter course of therapy with improved patient compliance. These structural modifications also expand the spectrum of activity of these compounds over that of erythromycin. While comparable to erythromycin against most Gram-positive organisms, azithromycin and 14-hydroxy-clarithromycin, the active metabolite of clarithromycin, exhibit improved activity against Gram-negative organisms such as *H. influenzae*, *Moraxella catarrhalis*, *Neisseria gonorrheae*, *Salmonella enteritidis*, *Shigella* species, *Vibrio* species and *Yersinia enterocolitica*[18~20].

L-701,677, L-708,299 and L-708,365 are three novel macrolide compounds which exhibit much greater acid stability than erythromycin, azithromycin and clarithromycin. L-708,299 was the most acid stable compound with a half-life of 16.2 hours at pH 2.1 while erythromycin was the least stable with a half-life of 0.3 hour. L-701,677 ($T_{1/2}=14.2$ hours) and L-708,365 ($T_{1/2}=13.2$ hours) exhibited stability more than twice that of azithromycin ($T_{1/2}=5.7$ hours) and clarithromycin ($T_{1/2}=5.9$ hours). The percent bioactivity demonstrated a positive correlation with the half life of these compounds. Pharmacokinetically, the serum parameters of these compounds were similar to those obtained for azithromycin, while tissue concentrations were lower ($1\sim5\times$) than those observed for azithromycin[21].

In the disseminated *S. pyogenes* mouse infection model azithromycin and L-708,365 were equal in efficacy at 7 days while azithromycin and clarithromycin were most active at 14 days. L-708,299 and L-701,677, with MICs $\leq 0.06\,\mu g/ml$, also exhibited good activity in this model. Erythromycin, which appeared to have very good potency *in vitro* (MIC value of $0.015\,\mu g/ml$), was not efficacious in this model even at doses as high as 200 mg/kg. This inactivity could be attributable to the poor acid stability of erythromycin base at the pH of the stomach. Other formulations such as erythromycin stearate or enteric-coated tablets have been used to protect the antibiotic in the stomach[8]. Also, oral administration of one dose of erythromycin may have been insufficient to achieve peak and sustained serum levels required for efficacy in this model.

Efficacy for azithromycin, L-701,677, L-708,365 and L-708,299 in the *K. pneumoniae* pulmonary challenge model indicated these compounds were equipotent in their activity. However, when administered S.C. the efficacy of these compounds was at least two-fold less than when given orally, indicating possibly better absorption, distribution or bioavailability following oral administration. Clarithromycin was not efficacious in this pulmonary mouse model. We feel this inactivity of clarithromycin is primarily due to the decreased susceptibility of the organism (MIC = $16\,\mu g/ml$) since good activity was seen in the *S. pyogenes* disseminated model

and the *S. aureus* tissue infection model. As expected, erythromycin (MIC=32 μg/ml) also was not active against *K. pneumoniae* in this model for the same reason mentioned above.

In the *S. aureus* tissue infection model, all three compounds (L-701,677, L-708,299 and L-708,365), were equal to or greater than azithromycin in organism clearance from the thigh. In this model, clarithromycin was the most efficacious compound at both concentrations tested. At 100 mg/kg, L-708,365 and surprisingly erythromycin were equal to clarithromycin in organism clearance from the thigh. Interestingly, clarithromycin exhibited better tissue activity than azithromycin in this model. This may be attributable to the ability of clarithromycin to achieve higher tissue levels than azithromycin sooner after P.O. dosing[22]. At 25 mg/kg, only clarithromycin and L-708,365 appeared to have significant activity when compared to controls. The better activity of erythromycin in this *S. aureus* tissue infection model as compared to the *S. pyogenes* septicemia model possibly may be explained by a different dosing regimen (8 doses *vs.* 1 dose), better tissue loading or better tissue pharmacokinetics.

In summary, L-701,677, L-708,299 and L-708,365 are three novel azalide compounds which exhibit improved acid stability over erythromycin and the new generation macrolides (azithromycin and clarithromycin). These three compounds were found to be at least as potent as presently marketed macrolide compounds in three mouse models of bacterial infection. Should their spectrum of activity be determined to be broader than the presently available agents, then further development and evaluation of these original compounds may be warranted to determine their usefulness in treating a variety of skin, soft tissue and respiratory bacterial infections.

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