ASPICULAMYCIN, A NEW CYTOSINE NUCLEOSIDE ANTIBIOTIC

IV. ANTIMYCOPLASMA ACTIVITY OF ASPICULAMYCIN *IN VITRO* AND *IN VIVO*

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Aspiculamycin, a new cytosine nucleoside antibiotic produced by *Streptomyces toyocaensis* var. *aspiculamyceticus*, showed strong activity against various strains of *Mycoplasma in vitro* and *in vivo*. The minimal inhibitory concentration of the antibiotic ranged between 50 to 0.05 mcg/ml by agar dilution, broth dilution or microtiter method. No influences of inoculum size and pH of the medium on the activity were observed. A number of strains of *Mycoplasma gallisepticum* showing resistance to macrolide antibiotics were all susceptible to the antibiotic. L-Forms derived from *Staphylococcus aureus* and *Proteus vulgaris* were insensitive to the antibiotic. After administration of 0.04% (w/w) of aspiculamycin in the basal diet for seven days, the lung and trachea of mice infected intranasally with *Mycoplasma pulmonis* were free of pathogen. In the experimental mice arthritis induced with *M. pulmonis*, the *Mycoplasma* could not be detected in joints of the mice after treatment with aspiculamycin at a dose of 0.05% (w/w) in the diet for ten days.

Aspiculamycin is a new cytosine nucleoside antibiotic produced by *Streptomyces toyocaensis* var. *aspiculamyceticus* with versatile biological activities, such as anthelmintic and acaricidal activities in addition to weak antibacterial activity, as previously described.[1]

The present paper deals with the activity of aspiculamycin against *Mycoplasma in vitro* and *in vivo*.

Materials and Methods

In Vitro Studies

Mycoplasma strains used were as follows: *M. mycoides* var. *mycoides* (PG-1), *M. agalactiae* (PG-2), *M. mycoides* var. *capri* (PG-3), *M. arthritidis* (PG-6), *M. bovigenitalium* (PG-11). *M. pulmonis* (PG-22), *M. gallisepticum* (PG-31), *M. canis* (PG-14), *M. felis* (ATCC 23391), *M. hominis* type 1 (PG-21), *M. neurolyticum* (PG-28), *M. suipneumoniae* (M 244-67-1) and *Acholeplasma laidlawii* (PG-10). These strains were supplied by Dr. M. OGATA of the Department of Veterinary Microbiology, University of Tokyo, Tokyo. Other strains used were the isolates from lung and trachea of rats, mice and chickens in this laboratory. Especially, *M. pulmonis* MRL-4 strain isolated from mouse lung suffering from the chronic respiratory disease was used for *in vivo* evaluation.[7]

Solid medium used was the same as the agar plate medium described by CHANOCK *et al.*,[2] except that the antimicrobial drugs were eliminated. Liquid medium, glucose PPLO broth (Difco Co., Ltd.), was used for most of the strains and modified GOODWIN's liquid medium for *M. suipneumoniae* (*M. hyopneumoniae*).

The minimal inhibitory concentration of aspiculamycin was determined by agar dilution, broth dilution or microtiter method.

Agar dilution method: One loopful of 10^8 CFU*/ml of 3 days culture of each strain was streaked on each plate after drying its surface thoroughly. This inoculated plate was incubated in a sealed polyethylene bag under appropriate moisture at 37°C for 5 days. The growth of colonies were observed under a dissecting microscope (Nikon Type SMZ).

Broth dilution method: The broth dilution method was carried out for 5 strains of glucose-utilizing *Mycoplasma* in glucose PPLO broth containing bromocresol purple (BCP) as an indicator. After inoculation of 0.2 ml of 3 days culture of each strain to 10 ml of the broth, the tubes were incubated at 37°C for 5 days. The end point of the minimal inhibitory concentration was read by color change from red to yellow. The minimal mycoplasmacidal concentration was determined by subculture of the tubes without visible growth onto antibiotic-free agar medium.

Microtiter method: The microtiter method was carried out against 8 strains of *Mycoplasma*. One drop (0.025 ml) of glucose PPLO broth containing 0.003 % of BCP was placed into each cup of the microtiter U-plate and one drop of the antibiotic solution (800 mcg/ml) was added. Then, a serial two-fold dilution was performed from cup to cup. Three drops of a 100-fold dilution with glucose PPLO broth containing BCP of 5 days culture of each strain was inoculated and the plate was incubated at 37°C for 3 days. The minimal inhibitory concentration was determined by the color change of the broth.

The sensitivity to aspiculamycin of the L-form derived from *Staphylococcus aureus* and *Proteus vulgaris* by penicillin treatment[4,5] was also determined by the agar dilution method. Effects of the inoculum size and pH of the medium on the activity of aspiculamycin were determined by agar dilution method. Stability of aspiculamycin (free base) in aqueous solution was examined at 37°C, room temperature and 5°C for 5 weeks and the residual antimycoplasma activity was measured weekly by agar dilution method.

The cytotoxic effect of the antibiotic on BHK-21 and L-929 cells was determined by the method of TOPLIN.[3]

Mycoplasmacidal effect of aspiculamycin against *M. gallisepticum* was examined in the liquid medium by inoculation of 10^5 CFU/ml of the cells and inoculation at 37°C for 7 days. Different concentrations of the antibiotic were added one day after inoculation. The CFU were determined daily by subculture of the tubes without visible growth onto antibiotic-free agar plate.

In Vivo Studies

In vivo evaluation of aspiculamycin in mice infected with *M. pulmonis* MRL-4 was performed using the ICR strain of female, 4~5 weeks old, *Mycoplasma*-free mice. Different concentrations of the antibiotic in the diet were administrated with water *ad libitum* and the mice were maintained aseptically in a "Vinyl Isolator."

In the case of intranasal infection, mice infected intranasally with 10^7 CFU of *M. pulmonis* MRL-4 were divided into 6 groups (10 mice in each group). Four groups were bred with the diet containing different concentrations of the antibiotic and 2 groups of the control were non-infected and non-treated. After treatment for 7 days, viable cells of the *Mycoplasma* from the homogenates of lung and trachea were counted.

In the case of the arthritic experiment,[6] mice infected intravenously with 10^7 CFU of *M. pulmonis* MRL-4 were divided into 7 groups and each group was bred with the diet containing different concentrations of the antibiotic for 10 days. After ending administration of aspiculamycin, each group was bred for 20 days further without the antibiotic. Mice were sacrificed and examined whether typical symptoms of arthritis or pneumonia were observed. Isolation

* Colony-forming unit.

of the *Mycoplasma* from blood, lung, trachea, spleen, kidneys and joints of the mice was also performed.

Results and Discussion

In Vitro Activity of Aspiculamycin

The susceptibility of various species of *Mycoplasma* to aspiculamycin is summarized in Table 1. The minimal inhibitory concentrations of the antibiotic against most of the strains were 1.56 or 3.12 mcg/ml by the agar dilution method.

Table 1. Antimycoplasma activity of aspiculamycin.

Mycoplasma species	MIC (mcg/ml)			MCC* (mcg/ml)
	Agar dilution	Microtiter	Broth dilution	
M. mycoides var. *mycoides*	25	6.25	12.5	25
M. agalactiae	3.12			
M. mycoides var. *capri*	3.12	6.25		
M. arthritidis	3.12			
M. bovigenitalium	1.56			
M. pulmonis	1.56	1.56		
M. gallisepticum	1.56	1.56	1.56	6.25
M. canis	1.56		0.78	12.5
M. felis	3.12	1.56	1.56	12.5
M. hominis type 1	1.56			
M. neurolyticum	0.39	0.05		
M. suipneumoniae			6.25	
A. laidlawii	50	12.5	6.25	50

* MCC: minimal mycoplasmacidal concentration

Table 2. Sensitivity of aspiculamycin to *Mycoplasma* isolated from mice and rats.

Strains	Origin	MIC(mcg/ml)
Mycoplasma sp.	Rat	0.39
〃	〃	1.56
〃	〃	1.56
〃	〃	1.56
〃	〃	1.56
〃	〃	0.78
〃	〃	1.56
〃	〃	1.56
〃	〃	1.56
〃	〃	1.56
〃	Mouse	1.56
〃	〃	1.56
〃	〃	1.56
〃	〃	1.56
〃	Rat	3.12
〃	〃	3.12
〃	〃	3.12
〃	〃	3.12

M. mycoides var. *mycoides* and *A. laidlawii* were more resistant to the antibiotic than other strains. *M. neurolyticum* was markedly sensitive to the antibiotic. *M. suipneumoniae* causing swine enzootic pneumonia was also sensitive to aspiculamycin. In general, the minimal inhibitory concentrations obtained by the microtiter method were lower than those by the other two methods.

Mycoplasma isolated from the lungs and trachea of the rats and mice suffering from respiratory diseases were also sensitive to the antibiotic in the range between 3.12 to 0.39 mcg/ml as shown in Table 2.

On the other hand, aspiculamycin was not effective against L-forms of bacteria, showing a minimal inhibitory concentration

of more than 100 mcg/ml against all strains of the L-forms tested.

The influence of inoculum size on the activity of aspiculamycin was not observed in the most of the species of *Mycoplasma* except *M. hominis* type 1 (Table 3). Table 4 indicates that the antimycoplasma activity is not influenced by the pH of assay medium between 6.0 and 9.0.

The effect of serum on the activity of the antibiotic against *A. laidlawii*, which does not require serum for growth, was examined. The MIC value was 6.25 mcg/ml in the absence of serum and 50 mcg/ml in the presence of 20 % horse serum by the agar dilution method at pH 7.8 and 10^3 CFU/ml of the inoculum size.

Aspiculamycin was very stable in aqueous solution and complete recovery of the activity against 12 strains of *Mycoplasma* was detected even after 5 weeks storage at 37°C.

The cytotoxic effects of aspiculamycin were observed against both BHK-21 and L-929 cells in the same order and the end points of cytotoxic and lethal effects for both cell lines were 125 and 500 mcg/ml, respectively.

The sensitivities of 12 strains of macrolide resistant *M. gallisepticum* for aspiculamycin

Table 3. Influence of inoculum size on the MIC of aspiculamycin.

Mycoplasma species	MIC (mcg/ml)				
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
M. mycoides var. *mycoides*	50	25	25	25	25
M. agalactiae	3.12	3.12	3.12	3.12	3.12
M. mycoides var. *capri*	6.25	3.12	3.12	3.12	3.12
M. arthritidis	6.25	3.12	3.12	3.12	1.56
M. bovigenitalium	6.25	6.25	3.12	1.56	1.56
M. pulmonis	3.12	1.56	1.56	1.56	1.56
M. gallisepticum	1.56	1.56	1.56	1.56	0.78
M. canis	3.12	1.56	1.56	1.56	1.56
M. felis	3.12	3.12	3.12	3.12	1.56
M. hominis type 1	3.12	1.56	1.56	1.56	0.39
A. laidlawii	50	50	50	50	25

Table 4. Influence of medium pH on the MIC of aspiculamycin.

Mycoplasma species	MIC (mcg/ml)		
	pH 6.0	pH 7.0	pH 9.0
M. mycoides var. *mycoides*	25	25	25
M. agalactiae	6.25	3.12	3.12
M. mycoides var. *capri*	3.12	3.12	3.12
M. arthritidis	3.12	3.12	3.12
M. bovigenitalium	3.12	1.56	1.56
M. pulmonis	3.12	1.56	1.56
M. gallisepticum	3.12	1.56	1.56
M. canis	3.12	1.56	6.25
M. felis	12.5	3.12	6.25
M. hominis type 1	1.56	1.56	1.56
A. laidlawii	25	50	25

were all the same as a sensitive strain (MIC; 1.56 mcg/ml).

The minimal mycoplasmacidal concentration of aspiculamycin against *M. gallisepticum* was 25 mcg/ml as shown in Fig. 1.

In Vivo Activity of Aspiculamycin

The mice infected intranasally with *M. pulmonis* were treated with several doses of the antibiotic for 7 days. *Mycoplasma* could not be recovered from lung and trachea of the treated mice in the groups at doses of 0.04 % and 0.06 % of the antibiotic in the diet (Table 5). Aspiculamycin was also effective against mice arthritis induced with *M. pulmonis* by intravenous inoculation. Inversion on *Mycoplasma* into the joints of mice was completely protected at doses of 0.05 and 0.06 % of aspiculamycin in the diet as shown in Table 6.

Fig. 1. Mycoplasmacidal effect of aspiculamycin against *Mycoplasma gallisepticum*.

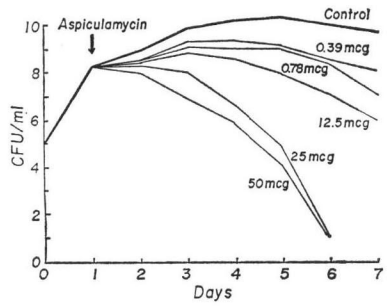

Table 5. Effect of aspiculamycin in the diet on nasal infection with *M. pulmonis* in mice.

Dose (% in feed)	Tested mice	Recovery of *Mycoplasma*	
		Lung	Trachea
Infected-nontreatment	10	10	10
Noninfected-nontreatment	10	0	0
0.06 %	10	0	0
0.04 %	10	0	0
0.02 %	10	7	7
0.01 %	10	10	10

Table 6. Effect of aspiculamycin against mice with arthritis induced by *M. pulmonis*.

Dose (% in feed)	No. of mice tested	Pathogenicity		Recovery of *Mycoplasma*				
		Arthritis	Pneumonia	Joint	Lung	Bronchus	Blood	Spleen
Infected-nontreatment	10	10	0	10/10	0/10	0/10	0/5	0/5
Noninfected-nontreatment	10	0	0	0/10	0/10	0/10	0/5	0/5
0.06%	10	0	0	0/10	0/5	0/5	0/5	0/5
0.05%	10	0	0	0/10	0/5	0/5	0/5	0/5
0.04%	10	1	0	1/10	0/5	0/5	0/5	0/5
0.03%	10	3	0	3/10	0/5	0/5	0/5	0/5
0.02%	10	4	0	4/10	0/5	0/5	0/5	0/5
0.01%	10	4	0	4/10	0/5	0/5	0/5	0/5
0.005%	10	5	0	5/10	0/5	0/5	0/5	0/5

The data described above indicates the effectiveness of aspiculamycin against a number of *Mycoplasma* species *in vitro* and also against *M. pulmonis in vivo* by oral administration. The efficacy of this antibiotic was also demonstrated by clearance of the pathogen from the lungs, trachea and joints, which were the target organs of *M. pulmonis* infection.

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