STUDIES ON CIRRAMYCIN A_1. II
BIOLOGICAL ACTIVITY OF CIRRAMYCIN A_1

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(Received for publication December 10, 1968)

Cirramycin A_1 is primarily active against gram-positive bacteria and exhibits the antimicrobial spectrum typical to that of the macrolide antibiotics. It exerts remarkable activity against a variety of *Mycoplasma* species. The *in vivo* activity of cirramycin A_1 was evaluated against three gram-positive pathogens, and the absorption and excretion of the antibiotic were studied on rats and dogs.

In our previous biological studies on cirramycin[1], the *in vivo* evaluations were made with a mixture of cirramycins A and B. Recent progress in the work on fermentation and purification[2] has led to the isolation of a new single component of cirramycin A_1 which has been evaluated in detail. This paper reports the biological activity of cirramycin A_1 both *in vitro* and *in vivo*.

In Vitro Antibacterial Activity

The serial two-fold agar dilution method was employed to determine the minimum inhibitory concentration (MIC) of cirramycin A_1 against a variety of bacteria and fungi. Nutrient agar was used for most of the test organisms, blood agar for streptococci and diplococci, glucose-peptone-yeast extract agar for lactic bacilli, glucose(2 %)-SABOURAUD agar for fungi and KIRCHNER's liquid medium for tubercle bacilli. The MICs of cirramycin A_1 are shown in Table 1 along with those of erythromycin which was comparatively tested as a reference antibiotic. As can be seen in the table, cirramycin A_1 is as active as erythromycin against staphylococci, slightly less active against streptococci and diplococci, and slightly more active against gram-negative bacteria.

In order to examine the effect of serum on the antibacterial activity of cirramycin A_1, the serial broth dilution method was used to determine the MIC of the antibiotic in presence of 0, 5, 10, 25 and 50 % of pooled human serum using *Staph. aureus* Smith as a test organism. As shown in Table 2, the activity of cirramycin A_1 is only slightly affected by serum at the concentrations tested.

Anti-mycoplasma Activity

The anti-mycoplasma activity of cirramycin A_1 was evaluated with 6 mycoplasma species, *Mycoplasma pneumoniae* Mac, *M. fermentans*, *M. pulmonis*, *M. hominis*, *M. salivarium* and *M. orale*. The liquid medium used for the growth of all the above

Table 1. Antimicrobial spectrum of cirramycin A_1

	Test organisms	Minimum inhibitory concentration (mcg/ml)	
		Cirramycin A_1	Erythromycin
Gram-positive bacteria	*Staphylococcus aureus* FDA P209	0.39	0.39
	// FDA P209 (ST, SM-R)*	0.78	0.78
	// FDA P209 (Novobiocin-R)	12.5	25
	// #193 (PC, TC-R)*	0.78	0.78
	// #193 (PC, TC, EM, CRM-R)*	100	50
	// #193 (EM-R)*	100	>100
	// Smith strain	0.78	0.78
	// #52-34 (PC, TC, EM, CRM-R)*	100	100
	Staphylococcus albus	0.39	0.78
	Sarcina lutea PCI 1001	0.1	0.1
	Micrococcus flavus	0.2	0.05
	Corynebacterium xerosis 53-K-1	0.1	0.05
	Bacillus mycoides strain "O"	0.39	0.39
	Bacillus sphericus strain #122	0.39	3.12
	Bacillus cereus ATCC 10702	0.39	0.2
	Bacillus subtilis PCI-219	0.1	0.1
	Bacillus anthracis 115	0.2	0.78
	Bacillus megaterium	0.39	0.1
	Lactobacillus acidophilus B-406-1	0.05	0.025
	Lactobacillus casei IAM 1118 7469	0.05	0.025
	Lactobacillus arabinosus strain 17-5	0.05	0.025
	Leuconostoc mesenteroides P-60 ATCC	0.05	0.05
	Streptococcus faecalis B-402-3	0.05	0.025
	Streptococcus hemolyticus Dick	1.56	0.2
	Streptococcus pyogenes Dig 7 Type 3	1.56	0.39
	Diplococcus pneumoniae Type II	0.39	0.2
	Diplococcus pneumoniae DP-3-5A	0.1	0.013
Acid-fast bacteria	*Mycobacterium* 607	>100	100
	Mycobacterium phlei	100	50
Gram-negative bacteria	*Escherichia coli* NIHJ	12.5	50
	// PO 1495	50	100
	// PO 1495 (CP, TC-R)*	100	100
	Pseudomonas aeruginosa	25	50
	Klebsiella pneumoniae julianelle type A	25	100
	Shigella dysenteriae A	12.5	50
	Shigella flexneri	12.5	50
	Salmonella typhi	25	50
	Neisseria sp. (CP-R)*	25	12.5
Fungi	*Candida albicans*	>100	>100
	Saccharomyces cerevisiae ATCC 9763	>100	>100
	Aspergillus niger var. Tieghen	>100	>100
	Trichophyton mentagrophytes	>100	>100

* ST=Streptothricin, SM=Streptomycin, PC=Penicillin G, TC=Tetracycline, EM=Erythromycin, CRM=Carbomycin, CP=Chloramphenicol, -R=resistant.

organisms has the follwing composition based on the method of CHANOCK *et al.*[3]

2.1 % PPLO broth (Difco)	70 ml	Thallium acetate	50 mg
Fresh horse serum	20 ml	Benzyl penicillin	50,000 units
25 % yeast extract	10 ml	pH 7.6~7.8,	Total : 100 ml

As the growth indicator of the mycoplasmas, 1 % of glucose and 0.002 % of phenol red were added to the medium for *M. pneumoniae*, *M. fermentans* and *M. pulmonis*,

Table 2. Serum effect on cirramycin A_1

Serum %	MIC *vs.* *S. aureus* Smith
0	0.39 mcg/ml
5	0.78
10	0.78
25	0.78
50	0.78

and 1 % of arginine and 0.002 % of phenol red for the other three oropharyngeal mycoplasmas. Incubation of the cultures at 37°C for 3 days generally gives a maximum titer of $10^7 \sim 10^8$ colony-forming-unit (CFU) per ml, which is used as a seed culture for inoculation.

Table 3. Antimycoplasma activity of cirramycin A_1 by tube dilution method

Test organism	Minimum inhibitory concentration (mcg/ml)		
	Cirramycin A_1	Erythromycin	Tetracycline
Mycoplasma pneumoniae Mac	0.001	0.1	10
Mycoplasma fermentans	0.025	50	0.4
Mycoplasma pulmonis	6.3	50	1.6
Mycoplasma hominis	100	>100	50
Mycoplasma salivarium	>100	>100	50
Mycoplasma orale	25	50	25

Table 4. Antimycoplasma activity of cirramycin A_1 by agar-plate diffusion assay

Test organism	Minimum inhibitory concentration (mcg/ml)		
	Cirramycin A_1	Erythromycin	Tetracycline
Mycoplasma pneumoniae Mac	0.016	0.25	25
Mycoplasma fermentans	3.1	>100	12.5
Mycoplasma pulmonis	12.5	>100	6.3
Mycoplasma hominis	0.8	100	25
Mycoplasma salivarium	1.6	>100	25
Mycoplasma orale	0.2	>100	25

The anti-mycoplasma activity of cirramycin A_1 was tested by two methods: the serial tube dilution assay and the agar plate diffusion assay. In the tube assay, serial dilutions of cirramycin A_1 with the above liquid medium were inoculated with 1/10 volume of the seed culture of the test organism and incubated at 37°C for 3 days to determine the minimum inhibitory concentration (MIC). When the growth of the organism is inhibited the color of the medium is reddish orange, and when grown it turns yellow for *M. pneumoniae*, *M. fermentans* and *M. pulmonis*. In the other three oropharyngeal mycoplasma the color change is reddish orange to dark pink with the progress of growth. The results are shown in Table 3 along with the MICs of erythromycin and tetracycline which were tested as reference antibiotics.

The method used for the agar plate diffusion assay was based on the paper described by ARAI *et al.*[4] Composition of the solid medium and the growth indicator are the same as in the liquid medium except for that 3.4 % PPLO agar (Difco) is used instead of 2.1 % PPLO broth in the latter. The agar plates are prepared by mixing 1.0 ml of the liquid culture of mycoplasmas at maximum growth with 9.0 ml of the solid medium. The paper discs of 8 mm in diameter impregnated with serial concentrations of cirramycin A_1 are placed on the plates. After incubation of the plates at 37°C for 2~3 days the growth inhibition zone (reddish orange) around the disc is read microscopically. The concentration which gives the inhibition zone of 10 mm was tentatively defined as the minimum inhibitory concentration by the plate assay. Again, erythromycin and tetracycline were comparatively tested. As shown in Table 4, cirramycin A_1 demonstrated broad and marked antimycoplasma activity.

In Vivo Activity of Cirramycin A_1 against Experimental Infections

Cirramycin A_1 was evaluated *in vivo* against three bacterial infections, *Staphylococcus aureus* Smith, *Streptococcus hemolyticus* Dick and *Diplococcus pneumoniae* Type II. The virulences of the pathogens (LD_{50}) were determined prior to the test. In our experiments, an intraperitoneal inoculation of about 50 viable cells per mouse of *Staphylococcus aureus* Smith in a 5% suspension of hog gastric mucin generally killed 50% of the mice within 24 hours. Likewise, the LD_{50}s of the streptococcus and diplococcus strains were 75,000 and 75 viable cells per mouse, respectively. For the chemotherapeutic experiments, a dose equivalent to 100 LD_{50} of the microorganism was given to each mouse intraperitoneally. Immediately after the bacterial challenge, cirramycin A_1 was administered to mice either subcutaneously or orally. Groups of 40 mice were used for each dosage level and the animals were observed for 4 days to determine the median curative dose (CD_{50}). As shown in Tables 5 and 6, cirramycin A_1 exerts remarkable therapeutic effect against the three gram-positive pathogens especially when given parenterally.

Table 5. *In vivo* activity of cirramycin A_1 (subcutaneous CD_{50} determination)

Dose (subcutaneous)	Challenge organism		
	S. aureus Smith	*S. hemolyticus* Dick	*D. pneumoniae* Type II
40 mg/kg	37/40	40/40	28/40
10	28/40	36/40	22/40
2.5	19/40	26/40	14/40
0.6	7/40	11/40	4/40
CD_{50}	3.2 mg/kg	1.5 mg/kg	7.6 mg/kg

Table 6. *In vivo* activity of cirramycin A_1 (oral CD_{50} determination)

Dose (oral)	Challenge organism		
	S. aureus Smith	*S. hemolyticus* Dick	*D. pneumoniae* Type II
400 mg/kg	36/40	32/40	30/40
200	26/40	27/40	14/40
100	19/40	19/40	9/40
50	13/40	11/40	3/40
CD_{50}	110 mg/kg	110 mg/kg	230 mg/kg

Absorption and Excretion Studies

Determination of Serum Levels of Cirramycin A_1 in Dogs

Groups of 3 dogs were dosed with 10 mg/kg of cirramycin A_1 intramuscularly or with 50 mg/kg of the antibiotic orally, and the blood samples were collected at certain time intervals. The blood was mixed with the same volume of 0.8% citrate saline, and the serum levels were determined by the usual cylinder-agar plate method using *Bacillus subtilis* as an assay

Table 7. Serum levels of cirramycin A_1 in dogs

Dose and route	Dog No.	Serum level in mcg/ml				
		0.5 hr	1 hr	3 hr	5 hr	peak
10 mg/kg i. m.	124	1.28	0.79	0.94	0.78	1.28
	127	<0.1	0.44	<0.1	<0.1	0.44
	141	<0.1	<0.1	0.72	<0.1	0.72
	mean					0.81 mcg/ml
50 mg/kg p. o.	128	—	0.38	<0.1	<0.1	0.38
	134	—	0.33	0.18	<0.1	0.33
	141	—	<0.1	0.42	0.38	0.42
	mean					0.38 mcg/ml

organism. As shown in Table 7, an average peak level of 0.81 mcg/ml was attained by an intramuscular administration of 10 mg/kg of cirramycin A_1, and 0.38 mcg/ml by an oral dose of 50 mg/kg.

Determination of Urine Recovery of Cirramycin A_1 in Rats

The absorption and excretion following the oral or parenteral administration of cirramycin A_1 were also studied on rats. Groups of male rats were fasted overnight and then placed in metabolism cages (3 rats per cage). The antibiotic was administered either orally or subcutaneously at a dose of 25 mg/kg. The animals were also given 10 ml of water by stomach tube at the time of antibiotic dosing and another 5 ml of water after 5 hours. The urine samples were collected at 7 and 24 hours after dosing. Antibiotic levels in urine samples were determined by the agar plate assay as above, and the percent recoveries were calculated from the antibiotic concentration, the urine volume and the total dose. The average percent recovery following oral administration of cirramycin A_1 obtained with 14 groups of rats was 1.0 %, and that after subcutaneous dosing was 5.1 %, suggesting about five times greater absorption and/or excretion by the parenteral route than by oral route.

Acute Toxicity and Local Tissue Toxicity

The acute toxicity of cirramycin A_1 was determined with mice by intravenous, subcutaneous and oral routes, the LD_{50} being found to be 250 mg/kg, 280 mg/kg and >2,000 mg/kg, respectively.

The local tissue toxicity of cirramycin A_1 was studied in order to assess the irritation or pain when the antibiotic is given parenterally. Two experimental methods were employed: the rabbit eye method and the rat paw method.

Cirramycin A_1 was dissolved in isotonic saline at various concentrations. Rabbits were placed in stocks and one drop of the antibiotic solution was instilled into the conjunctival sac of one eye. The other eye served as a control and was treated with the saline vehicle. After insuring thorough contact of the test solution with the eye, periodic observations of the conjunctiva, the mucous membranes of the lid and nictitating membrane were made. Solutions of cirramycin A_1 (0.5 %, 1.0 % and 2.0 %) did not show any irritation of the mucosal membranes during the observation period of 24 hours.

In the rat paw experiment, 0.1 ml of 0.5 %, 1.0 % and 2.0 % saline solutions of cirramycin A_1 was injected into the plantar surface of the left hind paw of groups of 5 rats and 0.1 ml of saline vehicle into the right hind paw. The paw size was measured 3 hours after the injection by fluid displacement, and the difference between the foot volume of the treated and the control paws of the same rat was

Table 8. Local tissue toxicity of cirramycin A_1 (Rat paw method)

	Concentration (%)	Average volume of foot swelling (ml)
Carrageenin	2.0	1.05
Cirramycin A_1	0.5	0.38
〃	1.0	0.94
〃	2.0	1.18
Benzylpenicillin	2.0	0.09
Erythromycin	2.0	0.85
Streptomycin	2.0	0.16
Tetracycline	2.0	0.71

determined. Carrageenin (2.0 % suspension) was used as a painful reference material showing an average swelling volume of 1.05 ml. Non-irritating compounds generally produce less than 0.2 ml of the foot swelling. As can be seen in Table 8, 1.0 % and 2.0 % solutions of cirramycin A_1 seem to be considerably irritating.

Conclusions

1. Cirramycin A_1 exhibits a typical antibacterial spectrum to the macrolide group antibiotics. It is as active as erythromycin against staphylococci, slighly less active against streptococci and diplococci, and slightly more active against gram-negative bacteria.

2. Cirramycin A_1 shows high activity against a variety of mycoplasma species.

3. Cirramycin A_1 exerts remarkable *in vivo* activity against experimental infections caused by three gram-positive pathogens especially when it is given parenterally.

4. An intramuscular dose of 10 mg/kg in dogs gives an average peak serum level of 0.81 mcg/ml, and an oral dose of 50 mg/kg produces average peak of 0.38 mcg/ml.

5. Cirramycin A_1 is not irritating to mucosal membranes of rabbit eyes, but produces signs of pain when injected into rat paws.

Acknowledgements

The authors are greatly indebted to Prof. NAKAO ISHIDA and Dr. KATSUO KUMAGAI, Tohoku University, for their kind supply of mycoplasma strains and for their valuable advice in the mycoplasma experiment.

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