BIOLOGICAL STUDIES ON AMICLENOMYCIN

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The action of amiclenomycin (AM) in inhibiting growth of microorganisms is specific against mycobacteria *in vitro*, but the antibiotic does not show a therapeutic effect against tubercle bacilli *in vivo*. The action of AM is reversed by biotin, desthiobiotin (DTB) and 7, 8-diaminopelargonic acid (DAPA), but not by 7-keto-8-aminopelargonic acid (KAPA), pimelic acid and glutaric acid. In the presence of AM, cultures of *Mycobacterium smegmatis* and *Bacillus sphaericus* accumulated KAPA, whereas the formation of DTB decreased. Therefore, AM is thought to inhibit KAPA-DAPA transamination in biotin biosynthesis. In *M. smegmatis* and *B. sphaericus* the conversions of KAPA to DAPA and of DTB to biotin were rate limiting in biotin synthesis. Accordingly, the synergistic antibiotic activity of AM, inhibiting the former, and actithiazic acid, inhibiting the latter reaction, would be simply explained.

As reported in a previous paper,[1] amiclenomycin produced by *Streptomyces lavendulae* subsp. *amiclenomycini* is a new amino acid antibiotic which inhibits the growth of mycobacteria including drug resistant tubercle bacilli. It can also be regarded as an antimetabolite antibiotic, because its activity is reversed by biotin and desthiobiotin.

The present paper deals with the biological activity of AM with reference to its mode of action.

Materials and Methods

Culture conditions. *Mycobacterium smegmatis* ATCC 607 was stationary-cultured at 37°C in a test tube containing 3 ml of the following medium (KIRCHNER's medium): glycerol 20 ml, asparagine 5 g, sodium citrate 2.5 g, KH_2PO_4 4 g, $Na_2HPO_4 \cdot 12H_2O$ 3 g, $MgSO_4 \cdot 7H_2O$ 0.6 g, 10 % Tween 80 5 ml, deionized water 1,000 ml, pH 7.2, unless otherwise mentioned.

In order to examine the mechanism of action with reference to biotin biosynthesis, *M. smegmatis* ATCC 607 and *Bacillus sphaericus* IFO 3525 were shake-cultured at 27°C in a medium containing pimelic acid to accumulate biotin vitamers according to the method of OGATA *et al.*[2]

Measurement of antimicrobial activity. Antimicrobial activity of AM was examined by the agar dilution method. Bacteria, molds, yeasts and actinomycetes were incubated in the following media: nutrient agar for bacteria, nutrient agar containing 1 % glycerol for mycobacteria, nutrient agar containing 1 % glucose for molds and yeasts, maltose (1 %)-yeast extract (0.4 %)-agar for actinomycetes.

In vivo test. A 2-week culture of *M. tuberculosis* H37Rv was harvested from SAUTON medium and 1 mg of cells (3.1×10^7 U.V.) per mouse were intravenously inoculated into male mice of *ddY* strain weighing 19~20 g. AM was subcutaneously administrated on the first day after infection. A daily check was made on surviving mice until 28 days after infection. The result was compared with that of kanamycin (KM).

Determination and identification of biotin vitamers. Biotin vitamers were determined by a microbiological assay with *Saccharomyces cerevisiae* ATCC 7754[3] (for "total biotin") and *Lactobacillus arabinosus* ATCC 8014[4] (for true biotin). Biotin vitamers, accumulated in culture broths were identified by bioautography with *Sacch. cerevisiae*[5] after ascending paper chromato-

graphy or thin-layer chromatography (silica gel plate), carried out by using the following solvent systems: *n*-butanol - 1NHCl (6:1) for paper chromatography, and benzene - methanol - acetone - acetic acid (14:4:1:1), *n*-butanol - acetic acid - H_2O (3:1:1) and 95% ethanol - H_2O (6:4) for thin-layer chromatography. Rf values were compared with authentic samples.

Results

Antimicrobial Activities of Amiclenomycin

The activity of AM in inhibiting the growth of microorganisms including 40 strains of bacteria, 7 strains of yeasts, 8 strains of molds and 6 strains of actinomycetes was examined by the agar dilution method. AM showed a specific activity (minimal inhibitory concentration (M. I. C.) 6.25 mcg/ml) in inhibiting the growth of mycobacteria and weak activity (M. I. C. 100 mcg/ml) against *Cryptococcus neoformans* F 10 and *Xanthomonas oryzae*.

However, AM did not significantly inhibit tubercle bacilli *in vivo*. With daily subcutaneous injection of 2 mg of AM per mouse for 21 days, the number of mice surviving were only 2 out of 10 of those infected with tubercle bacilli, while 5 out of 10 survived with kanamycin treatment (daily dose: 1 mg per mouse). Thus, prolongation of survival time by AM treatment was not significantly improved in contrast to kanamycin treatment.

Effect of Actithiazic Acid, Antitubercular Compounds and Amino Acids on the Antimicrobial Activity of Amiclenomycin

As described in a previous paper,[1] the effect of AM is reversed by both biotin and DTB. The action of actithiazic acid, another antibiotin antibiotic, is known to be reversed by biotin, but not by DTB. Therefore, we experimented to see if these compounds would be synergistic in inhibiting *M. smegmatis*. Activity was measured by a serial dilution method using nutrient broth containing 1% glycerol. The M. I. C. values of AM and actithiazic acid against this strain were 12.5 mcg/ml and 0.39 mcg/ml, respectively.

As shown in Table 1, the M. I. C. value was significantly reduced when they were both added at the same time. The growth was still completely suppressed even when they were added at 1/8 of their M. I. C. Thus, it was confirmed that AM and actithiazic acid are synergistic. However, the action of AM alone or in combination with actithiazic acid was reversed by biotin at a concentration of 0.01 mcg/ml, but not at 0.001 mcg/ml.

Table 1. Synergism between amiclenomycin and actithiazic acid

Antibiotic	mcg/ml*	Dilution rate**				
		×1	×2	×4	×8	×16
Amiclenomycin	12.5	−	+	+	+	+
Actithiazic acid	0.39	−	+	+	+	+
Amiclenomycin + actithiazic acid	12.5	−	−	−	−	+
	0.39					

+: Growth, −: No growth
* The concentration of the compounds in a medium which was diluted in double fold series.
** Incubated at 37°C for 40 hours in nutrient broth containing 1.0% glycerol.

On the other hand, AM showed no synergistic action with other antitubercular agents such as kanamycin, cycloserine and *p*-aminosalicylic acid. Arginine, glutamic acid, lysine, methionine, ornithine and phenylalanine at 200 mcg/ml also had no effect.

Bacteriostatic Action of Amiclenomycin

In order to examine whether AM is bactericidal or bacteriostatic against *M. smegmatis*, this organism was incubated at 37°C for 5 or 48 hours in KIRCHNER's medium supplemented

with 1,000 mcg/ml of AM. Cells were harvested, washed twice with 0.85 % saline and suspended in 0.85 % saline. Then, one drop of the suspension was transferred into fresh KIRCHNER's medium without addition of AM.

As shown in Table 2, cells pretreated with AM and washed with saline grew as well as the control cells which had been cultured in medium without AM, indicating that AM is bacteriostatic, not bactericidal.

Table 2. Bacteriostatic effect of amiclenomycin

Amiclenomycin concentration (mcg/ml)	Time of pre-treatment (hr)	Growth*			
		before**		after**	
		5 hr	48 hr	0 hr	48 hr
0 (Control)	5	0	—	0	40
	48	—	62	0	50
1,000	5	0	—	0	40
	48	—	9	0	75

Medium: KIRCHNER's liquid medium without horse serum.
* Reading of Klett-Summerson colorimeter at 560 nm.
** Before or after washing with physiological saline.

Effect of Biotin Vitamers on the Activity of Amiclenomycin

We previously reported[1] that the activity of AM (10~1,000 mcg/ml) was reversed by biotin (0.01 mcg/ml) and DTB (0.1 mcg/ml). Since actithiazic acid inhibits the conversion of DTB to biotin,[6] the effects of biotin vitamers on the action of AM were expected to be different from those on actithiazic acid. Therefore, the effects of biotin precursors such as 7, 8-diaminopelargonic acid (DAPA), 7-keto-8-amino-pelargonic acid (KAPA) and desthiobiotin (DTB) on the action of AM and actithiazic acid were examined by measuring the growth of *M. smegmatis* in KIRCHNER's medium with or without AM. In addition, the effects of pimelic acid, glutaric acid,[7] L-alanine[8] and S-adenosyl-L-methionine (SAM),[9,10] which are also involved in biotin biosynthesis, were examined in the same way.

As shown in Table 3, the action of AM (10~100 mcg/ml) was reversed by biotin (0.01 mcg/ml), DTB (0.1 mcg/ml) and DAPA (1 mcg/ml), but not by KAPA, pimelic acid, glutaric acid, L-alanine and SAM at any concentrations tested. It was also confirmed that the action of actithiazic acid could be reversed only by biotin. Those of biotin vitamers involved in later steps of biotin biosynthesis were more effective in reversing AM.

Fig. 1 shows the time course of growth of *M. smegmatis* in media containing AM at 50 mcg/ml and biotin vitamers at various concentrations. Growth of *M. smegmatis* was restored with increase of the concentrations of biotin, DTB and DAPA in AM-containing media. However, KAPA and pimelic acid did not restore growth.

Accumulation of 7-Keto-8-aminopelargonic Acid in AM-containing Media

The effects of AM on the biotin biosynthesis in *M. smegmatis* and *B. sphaericus* were studied in the following manner.

M. smegmatis was cultured at 27°C for 4 days in 3 ml of a medium containing pimelic acid (20~500 mcg/ml) and AM (2~6 mcg/ml) on a reciprocal shaker. Tween 80 (0.05 % in concentration) was added to disperse the growing cells.

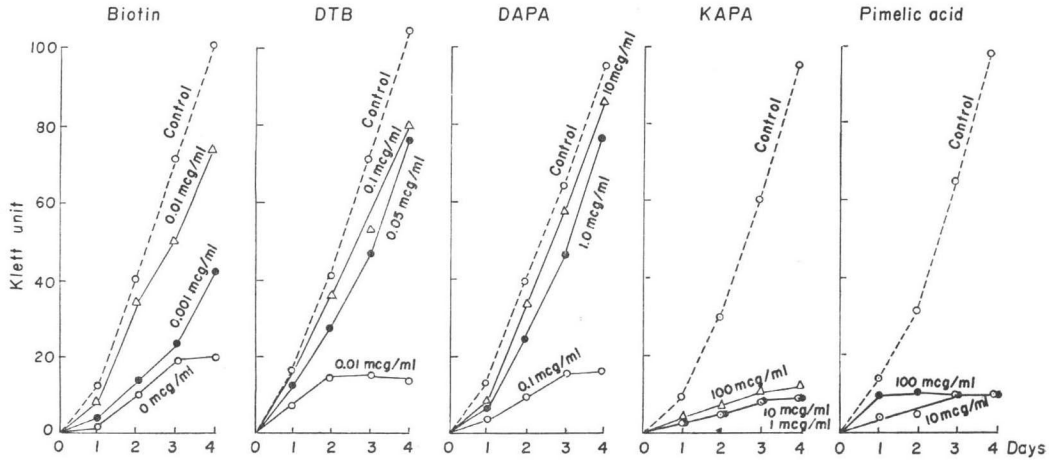
When the amounts of biotin vitamers (" total biotin ") and true biotin accumulated in cultures without AM were measured, it was found that biotin vitamers were accumulated in concentrations of 168~256 ng/ml. The presence of AM resulted in significant inhibition of growth, coupled with a slight increase of biotin vitamers accumulation (208~320 ng/ml). True biotin

Table 3. Effects of biotin and its vitamers on the activity of amiclenomycin and actithiazic acid

Compound	Conc. (mcg/ml)	Amiclenomycin (mcg/ml)				Actithiazic acid (mcg/ml)		
		0	10	50	100	5	50	500
Control	0	+	−	−	−	−	−	−
Biotin	0.001	+	−	−	−	−	−	−
	0.01	+	+	+	+	+	+	+
	0.1	+	+	+	+	+	+	+
DTB	0.01	+	−	−	−	−	−	−
	0.1	+	+	+	+	−	−	−
	1.0	+	+	+	+	−	−	−
DAPA	0.1	+	−	−	−	−	−	−
	1.0	+	+	−	−	−	−	−
	10.0	+	+	+	+	−	−	−
KAPA	1.0	+	−	−	−	−	−	−
	10.0	+	−	−	−	−	−	−
	100.0	+	−	−	−	−	−	−
Pimelic acid	10.0	+	−	−	−	−	−	−
	100.0	+	−	−	−	−	−	−
Glutaric acid	10.0	+	−	−	−	−	−	−
	100.0	+	−	−	−	−	−	−
L-Alanine	100.0	+	−	−	−	−	−	−
	500.0	+	−	−	−	−	−	−
SAM	100.0	+	−	−	−	−	−	−
	1,000.0	+	−	−	−	−	−	−

+: ≧30 KLETT units after 2 days incubation.
−: no growth or ≪30 KLETT units after 2 days incubation.

Fig. 1. Effect of biotin vitamers on the growth of *Mycobacterium smegmatis* ATCC 607 in the presence of amiclenomycin (50 mcg/ml).



was only slightly accumulated regardless of AM-addition.

The accumulated biotin vitamers were shown bioautographically to be KAPA and DTB (Fig. 2). This was confirmed by bioautography combined with paper chromatography or thin layer chromatography using various solvent systems as described in Materials and Methods.

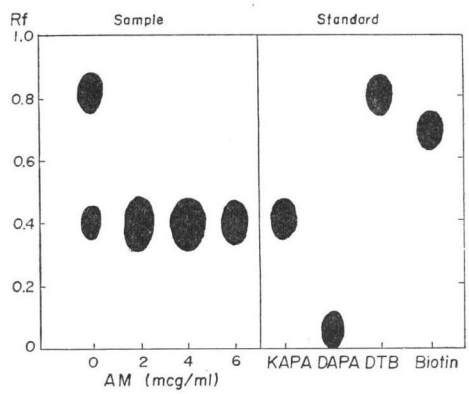
KAPA accumulated in appreciable amounts in all culture broths. In media containing 20~500 mcg/ml of pimelic acid, its accumulation was significantly higher in the presence of AM than in its absence. On the other hand, DTB was detected only in the medium without addition of AM, but not in that containing this antibiotic.

These results suggest that the conversions of KAPA to DAPA and of DTB to biotin reactions might be the rate limiting steps in biotin biosynthesis in *M. smegmatis*. AM would inhibit transamination of KAPA to DAPA.

Fig. 2. Effect of amiclenomycin on production of biotin vitamers.

Sample: 3 days culture filtrate (4 μl) of *Mycobacterium smegmatis* ATCC 607.

PPC: Toyo-Roshi No. 53; *n*-BuOH-1N HCl (6:1) Bioautography: *Saccharomyces cerevisiae* ATCC 7754.



To confirm the above observations, *B. sphaericus* was employed, since it is known to produce large amounts of DTB. This organism was cultured in a medium containing pimelic acid (500~2,000 mcg/ml) and AM (500 and 1,000 mcg/ml) in a manner similar to *M. smegmatis* except the omission of Tween 80.

Grown of *B. sphaericus* was considerably suppressed by 500~1,000 mcg/ml of AM. Accumulation of biotin vitamers increased distinctly with addition of pimelic acid and reached to the concentrations of 3.6~49.6 mcg/ml and 22.5~60.9 mcg/ml respectively after 2 and 4 day of cultivation. These amounts are approximately 100~200 times larger than those obtained with *M. smegmatis*.

Furthermore, when the biotin vitamers were examined bioautographically, only KAPA and DTB were detected as in the case of *M. smegmatis*. In all cases KAPA accumulated in large amounts, whereas DTB accumulation decreased with increasing concentration of added AM and could not be detected at all in the presence of 1,000 mcg/ml of AM.

Discussion

Amiclenomycin is a new amino acid antibiotic which exhibits specific growth inhibition against mycobacteria. Its action is reversed by biotin, DTB and DAPA. This means that the step in biotin biosynthesis inhibited by AM is different from that inhibited by actithiazic acid, the action of which is reversed by biotin but not by DTB or DAPA.

Three different pathways[12] have been postulated for biotin biosynthesis in microorganisms after extensive studies using *Escherichia coli*,[9,13,14,15] *Brevibacterium*,[10,15] *Bacillus sphaericus*,[11,17,18] *Achromobacter*[19] and *Agrobacterium*.[7] In our experiments, *M. smegmatis* accumulated KAPA, DTB and biotin in a medium containing pimelic acid, and in addition, DAPA, DTB and biotin supported growth of *M. smegmatis* even in the presence of 100 mcg AM per ml medium. Therefore, mycobacteria seem to have a biosynthetic pathway common with that of *E. coli*: pimelic

acid→pimelyl CoA→KAPA→DAPA→DTB→biotin.

Addition of AM to *M. smegmatis* resulted in inhibition of DTB synthesis and an increase of KAPA accumulation. The same observation was made in the case of *B. sphaericus* which is known to be a DTB producer. This indicates that AM inhibits the reaction step KAPA to DAPA in the pathway of biotin biosynthesis and thereby causes the growth inhibition.

BAGGALEY *et al.*[20] reported on stravidin, which contains a structural moiety similar to AM, and briefly pointed to a structural relationship between KAPA and stravidin. In view of the results described in this paper, it is possible that stravidin may have the same mode of action as AM. However, it should be noted here that there is also a structural resemblance between AM and DAPA as well as between AM and KAPA. A more detailed study of action of AM on KAPA-DAPA aminotransferase will be published in our next paper.

In *in vivo* tests, AM did exhibit practically no therapeutic effect against infection with tubercle bacilli in mice. This seems to be due to a reversion of the antibiotic effect by biotin *in vivo*.

M. smegmatis was confirmed to produce biotin vitamers, especially KAPA and DTB, in a concentration of 168~256 ng/ml. This corresponds approximately to only 1/100~1/200 of that produced by *B. sphaericus*. It suggests that the reactions converting KAPA to DAPA and DTB to biotin would be rate limiting steps in biotin biosynthesis by mycobacteria. If both steps, KAPA to DAPA and DTB to biotin, are rate limiting, it can be understood that specific inhibitors of these steps, such as AM and actithiazic acid, are synergistic in growth inhibition of mycobacteria.

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