SCREENING FOR NEW ANTIFOLATES OF MICROBIAL ORIGIN AND A NEW ANTIFOLATE AM-8402

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A screening method was established for new specific inhibitors of folate metabolism. Culture broths of soil isolates were selected based on relative microbial activity. A culture, to be retained, must be active against *Enterococcus faecium* grown in a medium which contains a limited amount of pteroic acid but lacks activity against the microorganism grown in a medium supplemented with thymidine. By this screening method, three new antibiotics, diazaquinomycins A and B and AM-8402 were selected from eight thousand soil isolates. The isolation and structures of diazaquinomycins have been reported. AM-8402 is a new antifolate active against Gram-positive bacteria and mycoplasmas. It consists of a nanaomycin D moiety as chromophore and a deoxysugar and is structurally related to medermycin.

The folate metabolic pathway should be regarded as an important target for chemotherapy[1,2]. Tetrahydrofolic acid (THF) and its derivatives are essential in cell metabolism. They are involved in the transfer of one-carbon-atom units and are vital for the biosynthesis of purine and pyrimidine nucleotides and therefore the biosynthesis of nucleic acids.

In fact, sulfa drugs, which had been main chemotherapeutics before penicillins were used, inhibit the biosynthesis of THF at the site of dihydropteroate (DHP) synthase by competition with *p*-aminobenzoic acid[3]. Methotrexate[4] (MT), trimethoprim[5] (TP) and pyrimethamine[6], which are well known as antifolate drugs, inhibit dihydrofolate (DHF) reductase and are clinically used as anticancer, antibacterial and antimalarial drugs, respectively.

TMP synthase has an essential role in DNA biosynthesis and is regarded as an attractive target enzyme in cancer chemotherapy[7]. 5-Fluorouracil (5-FU) which inhibits the enzyme after conversion to 5-fluorodeoxyuridine monophosphate (5-F-dUMP) in cells is employed in cancer treatment. 5,10-Methylene-THF is the substrate of the enzyme and the sole source of the transfer of one carbon fragment to dUMP. Fig. 1 shows folate metabolism[1,8,9] and the site of action of related drugs. As described above, synthetic chemotherapeutics which interfere with folate metabolism are now used clinically. However, such compounds of natural origin are hardly known[10,11]. So, we tried to develop a screening method for new antifolates from fermented broths of microorganisms in order to find useful antibacterial and anticancer drugs.

The method described in this paper is based on two points. The first is that most general microorganisms cannot incorporate folate-related compounds, but some special microorganisms such as *Streptococcus* sp. and *Lactobacillus* sp. require folate-related compounds and can incorporate them.

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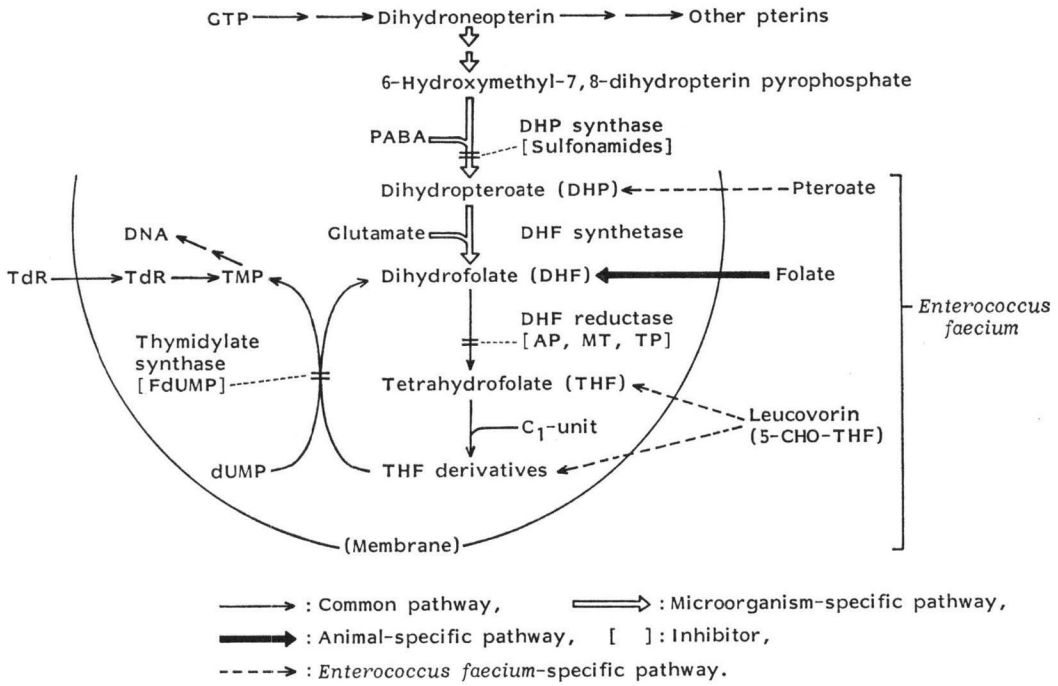
Fig. 1. Folate metabolism and its inhibitors.



Secondly, the microorganism which requires folate can grow in the folate-free medium supplemented with amino acids such as glycine, serine, histidine and methionine and purine and pyrimidine bases such as adenine, guanine and thymine, because folate and its derivatives are essential to the biosynthesis of those compounds. As a result, we selected the culture broths of soil isolates having inhibitory activity against a *Streptococcus* grown in a medium containing a limited amount of pteroic acid and enough amino acids and bases or nucleosides except thymine or thymidine (TdR), but lacking activity against the organism grown in the medium supplemented with a sufficient amount of TdR.

In this paper we present the new method with evidence for its applicability and the results of the practical screening using the method.

Table 1. Composition of Lactobacilli Inoculum Broth "Nissui".

Yeast extract	5.5 g
Peptone	12.5
Glucose	11.0
K_2HPO_4	0.25
KH_2PO_4	0.25
Na-Acetate	10.0
$MgSO_4 \cdot 7H_2O$	0.1
$MnSO_4 \cdot H_2O$	5.0 mg
$FeSO_4 \cdot 7H_2O$	5.0 mg
Distilled water	1,000 ml
pH 6.8	

Materials and Methods

Bacterial Strain and Medium

Enterococcus faecium IFO 3181 was used as test organism. Lactobacilli Inoculum Broth "Nissui" (Table 1) and Folic Acid Assay Medium "Nissui" (Table 2) were used for growth of the organism and for assay of antimicrobial activity, respectively.

Assay Method of Antimicrobial Activities

Antimicrobial activities were assayed by the paper disk method. Agar plates were prepared as follows. The cells of a stock culture of the

organism were transferred into 5 ml of the medium and incubated statically at 37°C for 16~18 hours. The culture was then centrifuged. The harvested cells were washed three times with sterile distilled water and suspended. The cell suspension was transferred at the rate of 4% to an assay medium (Folic Acid Assay Medium "Nissui") containing a folate-related compound (for example, 1.0 ng/ml pteroic acid) and Difco agar (1.0%), and aliquots (10 ml) of the culture were poured into Petri dishes. The plates were incubated at 37°C for 16~18 hours after paper disks were put on the plates.

Screening Method

A counter diffusion disk assay was used to screen for antifolates in fermented broths. A paper disk containing a fermented broth which showed antibacterial activity against *E. faecium* was applied to the assay agar plate and then a paper disk containing a folate-related compound was placed near the former disk.

Materials

Leucovorin (5-formyl-THF) and pteroic acid were gifts from Lederle Laboratories. TP, MT and 5-FU were purchased from Burroughs Wellcome Co., ICN Pharmaceuticals Inc. and Kyowa Hakko Kogyo Co., Ltd., respectively. Other antibiotics were stock materials of our laboratory.

Results and Discussion

Effect of Folate-related Compounds on the Growth of *E. faecium*

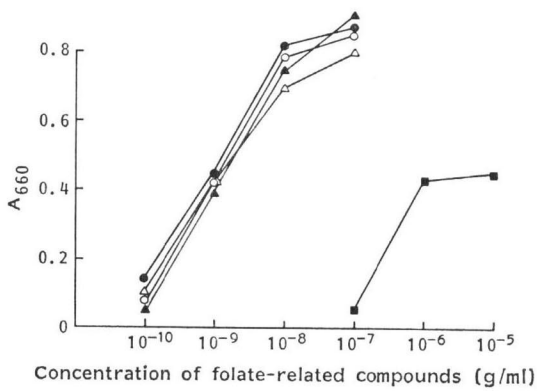
Because folate-related compounds are essential to supply C_1 atom units but *E. faecium* lacks DHP synthase, it requires them for the growth. To establish a screening method for new antifolates, at first we examined the growth response of the organism to folate-related compounds. As shown in Fig. 2, the growth responded well to very small amounts of pteroic acid, folic acid, DHF and leucovorin, and to a relatively large amount of TdR. The medium used, Folic Acid Assay Medium "Nissui" (Table 2), contained such amino acids and purine/pyrimidine bases except thymine that require C_1 atom units for

Table 2. Composition of Folic Acid Assay Medium "Nissui".

Vitamine-free Casamino Acids	5 g
L-Cysteine	380 mg
L-Tryptophan	100 mg
L-Asparagine	300 mg
Adenine·H_2SO_4	5 mg
Guanine·HCl	5 mg
Uracil	5 mg
Xanthine	10 mg
Glutathione	2.6 mg
Thiamine·HCl	200 μg
Riboflavin	500 μg
Pyridoxine·HCl	2 mg
Nicotinic acid	400 μg
Ca-Pantothenate	400 μg
Biotin	10 μg
p-Aminobenzoate	500 μg
K_2HPO_4	3.2 g
$MgSO_4 \cdot 7H_2O$	200 mg
NaCl	10 mg
$FeSO_4 \cdot 7H_2O$	10 mg
$MnSO_4 \cdot H_2O$	110 mg
Na-Citrate	26 g
Glucose	20 g
Tween 80	50 mg
Difco agar	10 g
Distilled water	1 liter
pH 6.8±0.1	

Fig. 2. Growth response of *Enterococcus faecium* to folate-related compounds.

●, Pteroic acid; ○, folic acid; △, DHF; ▲, leucovorin; ■, TdR.



their *de novo* biosynthesis. Consequently, folate-related compounds are considered to be necessary only for the biosynthesis of TMP when the organism grows in the above medium. In fact, TdR could support the growth without folate-related compounds. From the results in Fig. 2, the amount of pteroic acid contained in an assay medium used in this screening program was chosen to be 1.0 ng/ml.

The Inhibitory Effect of Antifolate Drugs and Some Antibiotics against *E. faecium*

Antibacterial activities of antifolate drugs and some antibiotics against *E. faecium* were compared to examine whether the comparative activity is useful in the screening for specific inhibitors of folate metabolism. As shown in Table 3, sulfa drugs which inhibit DHP synthase were not active against the bacterium. TM, aminopterine (AP) and MT which inhibit DHF reductase were active against the bacterium grown in the medium which contained pteroate, folate or DHF, but did not show antibacterial activity in the medium supplemented with leucovorin or TdR. 5-FU, which is known to be converted to 5-F-dUMP in cells and to inhibit TMP synthase, was active against the organism in the medium containing pteroate, folate, DHF or leucovorin, but showed no activity in the medium containing TdR.

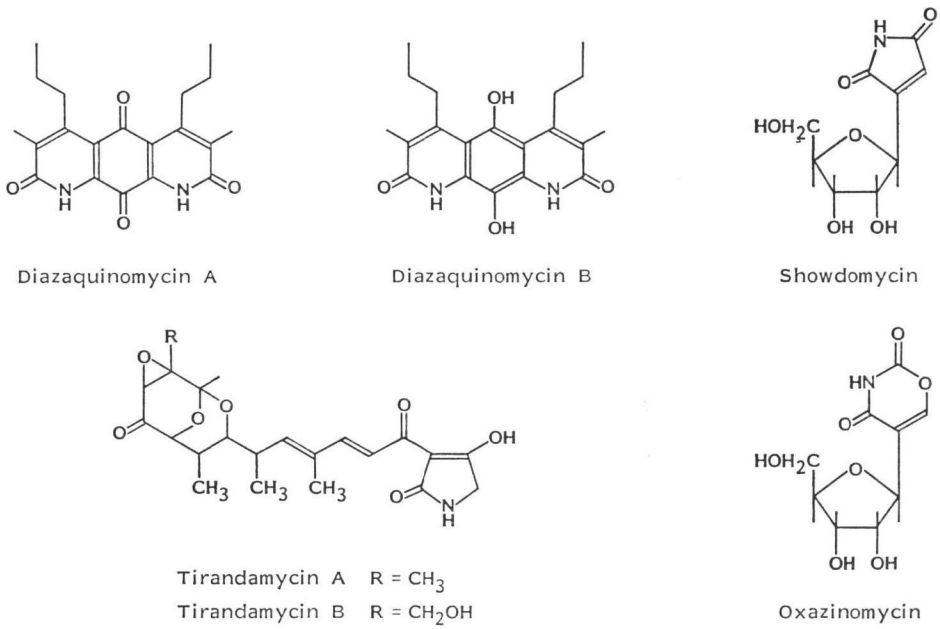
Inhibitors which interfere with the sites other than folate metabolism such as protein synthesis, cell wall synthesis and nucleic acid synthesis were active against the bacterium in any media supplemented with folate-related substances and TdR, although some antibiotics exhibited no activity in any media (Table 3).

From these results, it was ascertained that antifolates can be obtained by selecting out substances that are active against *E. faecium* in the medium containing a limited amount of pteroate but are inactive in the medium supplemented with a sufficient amount of TdR.

Table 3. Antibacterial activities of antifolates and some antibiotics against *Enterococcus faecium* in media supplemented with folate-related compounds as growth factor.

Site of inhibition	Compounds (100 μg/ml)	Growth factor				
		Pteroate (1 ng/ml)	Folate (1 ng/ml)	DHF (1 ng/ml)	Leucovorin (1 ng/ml)	TdR (10 μg/ml)
DHP synthase	Sulfa drugs	—	—	—	—	—
DHF reductase	TP	33.4	31.0	30.0	—	—
	AP	30.0	29.4	27.0	—	—
	MT	35.8	34.0	32.5	—	—
TMP synthase	5-FU	29.2	29.0	29.0	28.5	—
Protein synthesis	Streptomycin	—	—	—	—	—
	Chlortetracycline	25.0	23.0	23.4	24.0	22.9
	Leucomycin	18.3	17.0	17.0	18.2	18.0
	Blasticidin S	16.2	15.8	15.5	16.0	16.0
Cell wall synthesis	Benzylpenicillin	—	—	—	—	—
	Amoxicillin	16.8	15.0	15.4	16.0	15.3
	D-Cycloserine	—	—	—	—	—
Nucleic acid synthesis	Bleomycin	—	—	—	—	—
	Chromomycin A_3	21.6	19.0	19.5	18.0	18.7
	Rifampicin	17.2	15.5	16.0	14.0	13.4
Fatty acid synthesis	Cerulenin	—	—	—	—	—
Respiration	Antimycin	—	—	—	—	—

Fig. 3. Structures of diazaquinomycins A and B, showdomycin, oxazinomycin and tirandamycins A and B.



Results of This Screening Program

Broth filtrates of about eight thousand strains of actinomycete soil isolates were submitted to this screening program. Three new antibiotics, diazaquinomycins A and B and AM-8402 were discovered, and four known antibiotics, showdomycin, oxazinomycin (minimycin) and tirandamycins A and B (Fig. 3) were identified in this program.

Showdomycin[12,13] and oxazinomycin[14~17] are C-nucleosides active against Gram-positive and Gram-negative bacteria and Ehrlich ascites tumor. Their activity is known to be reversed with various nucleosides[18]. The inhibition against *E. faecium* was reversed by adenosine, uridine and cytidine as well as TdR, but not by leucovorin. Thus, these antibiotics were not considered to be folate metabolism inhibitors. After that, we did not select the cultured broths exhibiting an inhibitory activity reversed by various nucleosides in addition to TdR.

Tirandamycins A and B[19~22] are potent inhibitors of RNA polymerase, and are active against Gram-positive bacteria. Their activity against *E. faecium* was slightly reversed by leucovorin and TdR but not by other nucleosides.

Diazaquinomycins A and B, the new antibiotics discovered during this screening, are new folate metabolism inhibitors active against Gram-positive bacteria. Their activity was reversed by folate, DHF, leucovorin and TdR, but was not reversed by other nucleosides. The taxonomy, isolation, properties and structures have been reported by ŌMURA *et al.*[23,24]. The mode of action will be reported elsewhere[25].

AM-8402 is another new antibiotic discovered in this screening program. The activity of AM-8402 against *E. faecium* was completely reversed by leucovorin and TdR and partially reversed by DHF. The production, isolation and properties of the antibiotic are described below.

Production and Isolation of AM-8402

A mature spore chain of the producing organism, strain AM-8402, consists of more than 20 spores and forms spiral. LL-Diaminopimelic acid was detected in whole cell analysis. The color of aerial mycelia was shell pink on various agar media. Thus, the strain is considered to belong to the genus *Streptomyces*.

A loopful of the aerial mycelia of a slant culture of the strain was transferred into a seed medium (pH 7.0, 100 ml) containing glycerol 2.0%, soybean meal 2.0% and NaCl 0.3% in a 500-ml Sakaguchi flask, and incubated with reciprocal shaking for 2 days at 27°C to give a seed culture for production of AM-8402. The seed culture was transferred at the rate of 1.0% into a production medium (pH 7.0) containing glucose 2.0%, peptone 0.5%, dried yeast 0.3%, meat extract 0.5%, NaCl 0.5% and $CaCO_3$ 0.3%.

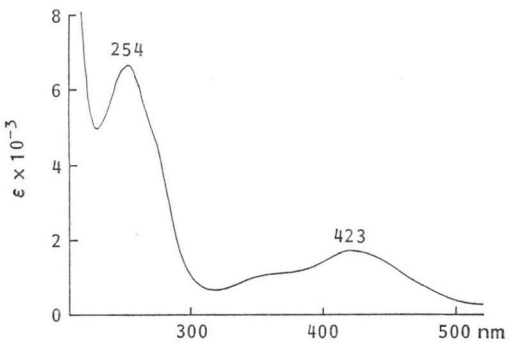
The cultured broth (30 liters) obtained by incubation in a 50-liter tank at 27°C for 24 hours with 10 liters of air per minute and agitation of 250 rpm was centrifuged and the supernatant fluid was applied to a column of Diaion HP-20. After washing with H_2O, the adsorbed material was eluted with 50% Me_2CO - 1% AcOH. The active eluate was concentrated *in vacuo* and was adjusted to pH 6.0 with $NaHCO_3$ powder. It was applied to an Amberlite IRC-50 (H^+) column. After washing with H_2O, the adsorbed material was eluted with 1 N HCl. Active eluate was adjusted to pH 6.0 with $NaHCO_3$ and applied to a Diaion HP-20 column. Active material was eluted with 50% Me_2CO - 1% AcOH, concentrated *in vacuo* and freeze-dried. The crude powder was applied to a silica gel column and developed with Me_2CO - MeOH - H_2O (4: 2: 1, 1% AcOH). Active eluate was applied to a Lobar column and developed with 2-butanone - Me_2CO - H_2O (8: 3: 2, 1% AcOH) at a pressure of 10~20 kg/cm^2. Active eluate was concentrated *in vacuo* and applied to a Sephadex G-10 column. It was developed with water. Active eluate was freeze-dried to give a yellowish powder of AM-8402 (5.4 mg).

Physico-chemical Properties of AM-8402

AM-8402 obtained as yellowish powder was basic and soluble in H_2O and lower alcohols. It was extremely unstable in a neutral or alkaline solution and comparably more stable in an acidic solution. It shows positive color reaction to anthrone-H_2SO_4, $FeCl_3$ and Dragendorff reagents.

The UV spectrum of AM-8402 (Fig. 4) exhibited two characteristic absorption maxima at 254 (ε 6,500) and 423 (ε 1,700) nm, which corresponded to those of nanaomycin D. This suggests that AM-8402 has the same chromophore as nanaomycin D[26].

Fig. 4. UV spectrum of antibiotic AM-8402 (in MeOH).



The IR spectrum of AM-8402 (Fig. 5) exhibited the absorptions of a carbonyl group of a five-membered ring lactone at 1785 cm^{-1}, a quinone carbonyl group chelated with a hydroxyl group at 1625 cm^{-1}, an alcoholic hydroxyl group at 1150 cm^{-1}, a phenolic hydroxyl group at 1245 cm^{-1}, and another quinone carbonyl group at 1655 cm^{-1}. The ^{1}H NMR spectrum data of AM-8402 (Table 4) showed the existence

Fig. 5. IR spectrum of antibiotic AM-8402 (KBr).

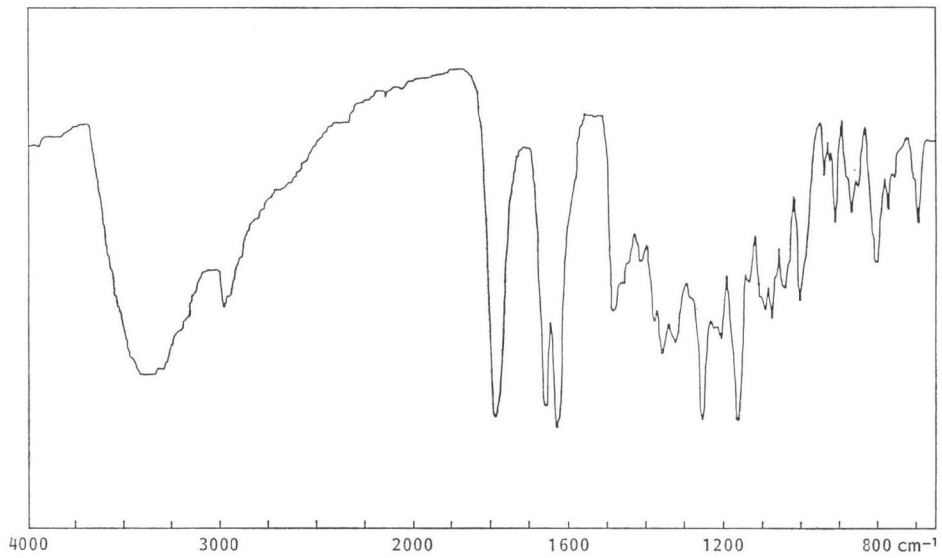
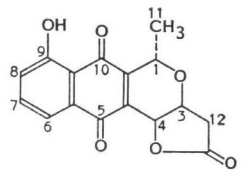


Table 4. ^{1}H NMR data of antibiotic AM-8402 and nanaomycin D in CD_3OD.

AM-8402		Nanaomycin D*	
3H	δ 1.66 (d, J=7.7)	11	δ 1.58
2H(a)	δ 2.60 (d, J=19.6)	12	δ 2.66
(b)	δ 3.23 (dd, J=5.9, 19.6)	12	δ 3.02
1H	δ 4.87	3	δ 4.34
1H	δ 5.10 (q, J=7.7)	1	δ 5.12
1H	δ 5.51 (d, J=3.3)	4	δ 5.19
1H	δ 7.52 (d, J=10.0)	6, 7, 8	δ 7.37~7.71
1H	δ 8.08 (d, J=10.0)		
	Nanaomycin D		
3H	δ 1.36 (d, J=7.3)		
2H(a)	δ 2.30 (m)		
(b)	δ 2.35 (m)		
2H(a)	δ 2.37 (ddt)		
(b)	δ 2.83 (ddt)		
	δ 3.76 (ddd, J=3.3, 8.9, 12.0)		
1H	δ 4.43 (dq, J=3.3, 7.3)		
1H	δ 5.35 (dd, J=5.3, 14.3)		
	Deoxysugar		
3H	δ 2.53 (brs)		
	N-CH_3		

*



Nanaomycin D

Table 5. Antimicrobial spectrum of antibiotic AM-8402.

Organism	MIC (μg/ml)*	Organism	MIC (μg/ml)*
Staphylococcus aureus FDA 209P	1.56	*Klebsiella pneumoniae* PCI 602	100
Bacillus subtilis ATCC 6633	1.56	*Serratia marcescens* IAM 1184	>100
Micrococcus luteus ATCC 9341	0.78	*Proteus vulgaris* IFO 3851	100
Escherichia coli NIHJ JC-2	>100	*Pseudomonas aeruginosa* NCTC 10490	>100

* Agar dilution method (medium, 0.5% peptone, 0.5% meat extract; 37°C, 20 hours).

Table 6. Reversion by TdR, leucovorin and DHF of the inhibitory activity of antibiotic AM-8402 and TP against *Enterococcus faecium*.

Compounds	Concentration (μg/ml)	Activity* (mm)	Reversion** by		
			TdR	leucovorin	DHF
AM-8402	1,000	22.3	++	++	+
TP	300	30.0	++	++	+
	1,500	33.1	++	++	+

* Inhibitory zone in paper disk method.

** Counter diffusion disk method: ++, Strongly reversed; +, weakly reversed; −, not reversed.

of a nanaomycin D moiety or its enantiomer, $>NCH_3$ and $-CHCH_2CH_2CH_2CHCH_3$. The FD mass spectrum showed the molecular ion peak $(M+1)^+$ at m/z 444.

These spectral informations supported each other and indicated that AM-8402 is a new antibiotic which contains a nanaomycin D moiety and a deoxysugar and that it resembles medermycin[27,28] structurally.

Biological Properties of AM-8402

Table 5 shows the antimicrobial spectrum of AM-8402. It inhibited Gram-positive bacteria but hardly inhibited Gram-negative bacteria, yeasts and fungi. It also inhibited mycoplasmas such as *Acholeplasma laidlawii* and *Mycoplasma gallisepticum* by paper disk method (data not shown).

The antitumor activity of AM-8402 was examined against Ehrlich ascites tumor and L1210 in mice. However, no activity was observed. The inhibitory activity against *E. faecium* was reversed by leucovorin and TdR and partially reversed by DHF (Table 6). This pattern corresponds to that of TP which inhibited DHF reductase.

The above data suggest that AM-8402 interferes with DHF reductase, but it exhibited no antitumor activity in mice and no inhibitory activity against DHF reductase from rat liver (data not shown). This seems to indicate that the antibiotic interferes with DHF reductase only from bacteria as TP does.

Acknowledgments

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