IN VITRO ACTIVITY OF TIAMULIN (81.723 HFU), A NEW PLEUROMULIN DERIVATIVE, AGAINST CLINICALLY SIGNIFICANT ANAEROBES

H. WERNER, G. LABER, E. SCHÜTZE, C. KRASEMANN and P. MAY

The Institute of Medical Microbiology and Immunology, University of Bonn, FR of Germany
and Sandoz Forschungsinstitut, Vienna, Austria

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The susceptibility of more than 40 strains of Gram-negative and Gram-positive anaerobes to tiamulin (Sandoz 81.723 hfu), a new pleuromulin (pleuromutilin) derivative, was determined by broth dilution and agar dilution tests. The influence of density of the inoculum upon MICs was studied by a specially designed pour plate-technique. *Bacteroides fragilis, B. vulgatus, B. splanchnicus, B. oralis, B. asaccharolyticus, B. melaninogenicus, Fusobacterium fusiforme* (*F. nucleatum*), *Sphaerophorus necrophorus, Clostridium perfringens, C. fallax, Propionibacterium acnes* and several species of Peptococcaceae showed broth dilution MICs of 0.03~1 μg/ml. Members of *B. thetaiotaomicron, B. distasonis* and *S. freundii* (*F. mortiferum*) were inhibited by 8~32 μg/ml and 2 strains of *S. varius* had a broth dilution MIC of 256 μg/ml. With most strains, the agar dilution MICs were 2~4~8 times the broth dilution MICs. In pour plate-tests, the MICs were not considerably influenced by varying initial concentrations of viable cells. With most anaerobes, the MBCs of tiamulin were more than 100-fold higher than the MICs. The results obtained indicated that, apart from *S. varius, B. thetaiotaomicron, B. distasonis* and *S. freundii* (*F. mortiferum*), members of 16 other anaerobic species including *B. fragilis* were without exception sensitive to tiamulin.

In recent years, it has been demonstrated by numerous study groups that Gram-negative and Gram-positive anaerobes are common causes of pyogenic and septic infections. Certain anaerobic bacteria, *e.g. Bacteroides fragilis* and related organisms, are of special interest because of their virulence and resistance to antimicrobial agents.[1,2]

A new pleuromulin derivative, 14-deoxy-14(2-diethylaminoethyl)mercaptoacidoxymutilin hydrogen fumarate, tiamulin (Sandoz 81.723 hfu), was shown by DREWS *et al.*[2] to be active against a variety of Gram-positive bacteria and against mycoplasmas. A number of *Shigella, Klebsiella* and *Escherichia coli* strains were also found to be susceptible. The new compound acted bacteriostatically.[2,5] Bactericidal effects were observed only at concentrations which were 100-fold higher than the MICs.[2]

The present paper deals with studies to evaluate the efficacy of tiamulin against clinically important anaerobes.

Materials and Methods

Antibiotic

Tiamulin was synthesized by procedures described elsewhere.[3] Lot No. 74903 was used throughout the study (purity 100%).

Organisms

Most of the anaerobic bacteria tested were isolated from clinical specimens during 1975~1977.

Anaerobic Gram-negative non-sporing rods (Bacteroidaceae) were differentiated by procedures described elsewhere.[11~14] Among the saccharolytic non-pigmented *Bacteroides* species identified were *B. fragilis, B. thetaiotaomicron, B. vulgatus, B. distasonis*[1,12], and the newly described species

B. splanchnicus.[16] The butyric acid-producing strains of *B. melaninogenicus* have recently been assigned to a new species, *B. asaccharolyticus*.[4] A strain of carbohydrate-fermenting, butyric acid-producing, pigmented *Bacteroides* could not be properly assigned to one of the remaining subspecies of *B. melaninogenicus*[6,7] and is referred to as *B. melaninogenicus*, saccharolytic, butyrate-positive. The species designation of *B. oralis* is used as described by WERNER *et al.*[18] The genus designation of *Sphaerophorus* was retained for the rod-like Gram-negative anaerobes which do not accumulate isobutyric and isovaleric acids but form butyrate as one of the major fermentation products.[11] According to our studies,[14] there is in existence only one species of fusiform butyrate-producing anaerobes, *Fusobacterium fusiforme* (*F. nucleatum*).

Species differentiation of anaerobic Gram-positive cocci (Peptococcaceae) was based on formation of fatty acids and pH values in peptone-yeast extract-glucose medium.[15]

Strains of *Propionibacterium acnes* were characterized by formation of propionic and isovaleric acids, indole-production and fermentation of carbohydrates.[7]

Clostridium strains (*C. perfringens*, *C. fallax*) were identified by their acid and alcohol products as well as other biochemical and morphological features.[7,17] A member of *Bifidobacterium* showed acetic acid production and other biochemical features of the genus.[7]

Susceptibility testing

The MICs were determined by broth and agar dilution tests. Medium 156 according to SIMON[10] was inoculated with approximately 10^7 organisms per ml cultivated in ROSENOW broth. The tubes were incubated 15~48 hours at 37°C, according to the species tested. The MIC was defined as the lowest concentration of tiamulin permitting no growth. The agar dilution test was performed with glucose-cysteine-yeast extract agar, to which 5% laked sheep blood was added.[8] The plates were inoculated with 10^5 bacteria and incubated in GasPak jars for 48 hours. Here again the MIC was determined as the lowest concentration of tiamulin permitting no growth.

The MBC was designated as either that concentration of tiamulin that completely inhibited growth, or that concentration yielding fewer than 10 colonies after subculture to a blood-agar plate.[9]

The influence of density of the inoculum upon MIC was studied by a special pour plate-technique. The several concentrations of test organisms (=serial tenfold dilutions of ROSENOW broth cultures) were added to melted peptone - yeast extract - glucose agar containing the several concentrations of tiamulin and plates were poured. With each test organism, about 10 concentrations of tiamulin were used ranging between 0.25~4 times the MIC as determined by broth dilution tests. After 4~6 days incubation at 37°C in GasPak jars the numbers of colonies formed were determined and zero end points evaluated as MICs.

Results

The MIC and MBC values of 41 strains are shown in Table 1. The majority of the strains belonging to *Bacteroides fragilis, B. vulgatus, B. splanchnicus, B. oralis, B. asaccharolyticus, B. melaninogenicus, Fusobacterium fusiforme* (*F. nucleatum*), *Sphaerophorus necrophorus, Clostridium perfringens, C. fallax, Propionibacterium acnes, Bifidobacterium* sp. and Peptococcaceae spp. had broth dilution MICs of 0.03~1 μg/ml, whereas members of *B. thetaiotaomicron, B. distasonis* and *S. freundii* (*F. mortiferum*) were inhibited by 8~32 μg/ml. The only species to show broth dilution MICs of 256 μg/ml was *S. varius* (Table 1).

Agar dilution MICs tended to be 2~4~8 times higher than those determined by broth dilution tests (Table 1). However, with some strains, *e.g.* members of *B. oralis, B. asaccharolyticus, Clostridium* spp. and *Bifidobacterium* sp., the results obtained by means of the two methods were identical (Table 1).

MBC values were in many instances, especially with those strains exhibiting low MICs, more than 256 times higher than the MICs as determined either in broth or in agar (Table 1).

The MICs of 33 strains were determined in agar plates containing $10^1 \sim 10^9$ colony-forming units

Table 1. *In vitro* activity of 81.723 hfu against Gram-negative and Gram-positive anaerobic bacteria

Species	No. of strains	MIC (μg/ml)		MBC (μg/ml)
		Broth	Agar	
Bacteroides fragilis	1	0.5	1	256
	1	0.5	1	>256
	1	0.5	2	>256
	1	1	1	>256
	1	1	4	>256
	1	1	8	>256
B. thetaiotaomicron	1	16	256	>256
	2	32	>256	>256
B. vulgatus	1	0.25	1	>256
B. distasonis	1	16	128	>256
B. splanchnicus	1	0.06	0.25	256
	1	0.125	0.25	256
B. oralis	1	0.125	0.025	4
	1	0.125	0.125	4
B. asaccharolyticus	1	0.03	0.03	64
	1	0.03	0.06	16
	1	0.06	0.06	32
B. melaninogenicus (saccharolytic, butyrate-positive)	1	0.06	0.125	128
Fusobacterium fusiforme (=*F. nucleatum*)	1	0.125	0.25	0.5
	1	0.5	0.5	32
Sphaerophorus necrophorus	1	0.125	0.25	64
	1	0.125	0.125	256
S. varius	2	256	>256	>256
S. freundii (=*F. mortiferum*)	1	8	8	256
	1	16	32	128
Clostridium perfringens	2	0.25	0.25	>256
C. fallax	1	0.25	0.25	256
Propionibacterium acnes	1	0.06	0.125	>256
	1	0.06	0.25	128
Bifidobacterium sp.	1	0.06	0.06	64
Peptostreptococcus anaerobius	1	0.25	0.5	2
	1	0.25	1	4
Peptococcus asaccharolyticus	1	0.25	0.25	2
	1	0.5	0.25	16
P. prevotii	1	0.06	0.125	128
	1	0.25	2	256
P. variabilis	1	0.06	0.25	>256
	1	0.125	0.125	>256

per ml (Table 2). With many of the cultures, only 3, 4 or 5 concentrations of test organisms could be evaluated. The increase in numbers of cells inoculated from 10^1 or 10^2 to 10^5, 10^7 or 10^9 per ml resulted in relatively inconspicuous rises of MIC values mostly not exceeding a doubling or trebling. With a strain of *S. necrophorus* and *S. varius* each, MICs increased 16-fold (Table 2).

Discussion

In determining the possible efficacy of newer agents against anaerobes, numbers and species of the organisms tested should be representative of their respective clinical importance as reflected in frequency of isolation and severity of cases.

There are a number of problems associated with susceptibility testing of anaerobes which are not encountered in the testing of aerobes. Apart from the influence of media used and initial pH, anaero-

Table 2. Influence of density of the inoculum upon MIC values

Species	No. of strains	Log_{10} of conc. of test organisms/ml	MIC (μg/ml)
Bacteroides fragilis	1	2~4	1.6~4
	1	2~5	0.5~1
	1	1~5	1~2
	1	2~5	2~7.4
B. thetaiotaomicron	1	1~3	51~114
B. vulgatus	1	1~5	0.5~2
B. splanchnicus	2	1~7	0.125~0.25
B. oralis	1	2~9	0.06~0.25
	1	2~9	0.125~0.25
B. asaccharolyticus	1	2~9	0.03~0.125
	1	2~9	0.125~0.25
B. melaninogenicus ss. *melaninogenicus*	1	2~6	0.06~0.25
B. melaninogenicus (Saccharolytic, butyrate-pos.)	1	2~9	0.125~0.25
Fusobacterium fusiforme (=*F. nucleatum*)	1	1~5	0.25~0.5
	1	1~6	0.03~0.06
Sphaerophorus necrophorus	1	1~5	0.03~0.5
	1	1~5	0.16~0.43
	1	1~6	0.125~0.5
S. varius	1	1~4	256~1024
	1	1~5	64~1024
S. freundii (=*F. mortiferum*)	1	1~3	0.5~2
	1	1~6	8~16
	1	1~7	8~32
Clostridium perfringens	1	2~5	0.125~0.5
	1	1~7	0.35~1
C. fallax	1	1~6	0.2~0.35
Propionibacterium acnes	1	1~5	0.25~0.5
	1	1~6	0.125~0.25
Peptostreptococcus anaerobius	1	1~4	0.5~1
Peptococcus asaccharolyticus	1	1~4	0.125~0.5
P. prevotii	1	1~5	0.06~0.25
P. variabilis	1	1~5	0.06~0.25

biosis itself is known to affect the activity of some antibiotics and, consequently, the results of the tests. Possibly the majority of the problems occurring with susceptibility testing of anaerobes are due to the necessity for using relatively long incubation periods and high initial concentrations of viable cells to obtain satisfactory growth of the anaerobic organisms. For example, in broth dilution tests using thioglycollate medium, the number of viable cells inoculated cannot be reduced below 10^7/ml without considerably lengthening the incubation time or even running the risk of obtaining no growth at all. A special test using peptone-yeast extract-agar plates with $10^1 \sim 10^9$ viable cells per ml had therefore to be designed for studying the influence of density of the inoculum upon MICs (Table 2). As marked differences between the MICs determined by broth dilution tests and those obtained by agar dilution techniques have been described for certain antimicrobials, a comparative study by means of three quantitative methods of susceptibility testing was performed with tiamulin.

Unlike the activity of certain cephalosporins against β-lactamase-producing Bacteroidaceae,[8] with tiamulin, as a rule, the lower MICs were determined by broth dilution tests, the agar dilution MICs tending to be 2~4~8 times higher than the former (Table 1). Presumably, the interaction between pleuromulin molecules and anaerobic bacterial cells is facilitated in broth. It is shown by the experiments reported in Table 2 that in most instances the outcome of the growth inhibition tests with tiamulin was not markedly influenced by varying densities of the inoculum. With most anaerobes

tested, the MBCs of tiamulin were more than 100-fold higher than the MICs (Table 1). This clearly shows the pleuromulin derivative to be a bacteriostatic agent.[5] However, strains of *Fusobacterium fusiforme* (*F. nucleatum*), *Peptostreptococcus anaerobius* and *Peptococcus asaccharolyticus* were killed at concentrations of tiamulin that were only 4~8~16 times the broth dilution MICs (Table 1).

Although pharmacological and clinical data concerning the criteria for distinguishing between sensitivity and resistance to tiamulin are as yet lacking, the results obtained in the present study show only one species, *S. varius*, to be quite insensitive. The MICs observed with strains of *B. thetaiotaomicron*, *B. distasonis* and *S. freundii* (*F. mortiferum*) indicated a similarly reduced sensitivity, but members of 16 other anaerobic species, including *B. fragilis*, were without exception sensitive to tiamulin. This *in vitro* evidence of a broad activity may well deserve further attention during therapeutic trials of infected hosts.

References

1) CATO, E. P. & J. L. JOHNSON: Reinstatement of species rank of *Bacteroides fragilis, B. ovatus, B. distasonis, B. thetaiotaomicron* and *B. vulgatus*: Designation of neotype strains for *Bacteroides fragilis* (VEILLON and ZUBER) CASTELLANI and CHALMERS and *Bacteroides thetaiotaomicron* (DISTASO) CASTELLANI and CHALMERS. Int. J. Syst. Bact. 26: 230~237, 1976
2) DREWS, J.; A. GEORGOPOULOS, G. LABER, E. SCHÜTZE & J. UNGER: Antimicrobial activities of 81.723 hfu, a new pleuromutilin derivative. Antimicr. Agents & Chemoth. 7: 507~516, 1975
3) EGGER, H. & H. REINSHAGEN: New pleuromutilin derivatives with enhanced antimicrobial activity. J. Antibiotics 29: 915~927, 1976
4) FINEGOLD, S. M. & E. M. BARNES: Report of the ICSB Taxonomic Subcommittee on Gram-negative anaerobic rods: Proposal that the saccharolytic and asaccharolytic strains at present classified in the species *Bacteroides melaninogenicus* (OLIVER and WHERRY) be reclassified in two species as *Bacteroides melaninogenicus* and *Bacteroides asaccharolyticus*. Int. J. Syst. Bact. 27: 388~391, 1977
5) HÖGENAUER, G.: The mode of action of pleuromutilin derivatives. Eur. J. Biochem. 52: 93~98, 1975
6) HOLDEMAN, L. V. & W. E. C. MOORE: Bacteroidaceae. *In*: BERGEY's Manual of Determinative Bacteriology, *ed.* R. E. BUCHANAN & N. E. GIBBONS, pp. 384~418, Williams & Wilkins Company, Baltimore, 1974
7) HOLDEMAN, L. V. & W. E. C. MOORE (*Ed.*): Anaerobe laboratory manual. Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg, Virginia, 1975
8) KRASEMANN, C. & H. WERNER: Susceptibility of Bacteroidaceae, Peptococcaceae and *Clostridium* spp. to cefazolin and cefamandole. *In*: Current Chemotherapy. Proc. 10th Int. Congr. Chemoth., *ed.* W. SIEGENTHALER & R. LÜTHY, pp. 293~295, Amer. Soc. Microbiol., Washington, D.C., 1978
9) NASTRO, L. J. & S. M. FINEGOLD: Bactericidal activity of five antimicrobial agents against *Bacteroides fragilis*. J. Infect. Dis. 126: 104~107, 1972
10) SIMON, P. C.: Cultivation and maintenance of *Sphearophorus necrophorus*. I. An anaerobic medium for aerobic incubation. Canad. J. Comp. Med. 38: 94~96, 1974
11) WERNER, H.: A comparative study of 55 *Sphaerophorus* strains. Differentiation of 3 species: *Sphaerophorus necrophorus*, *Sph. varius*, and *Sph. freundii*. Med. Microbiol. Immunol. 157: 299~314, 1972
12) WERNER, H.: Differentiation and medical importance of saccharolytic intestinal *Bacteroides*. Arzneim. -Forsch. 24: 340~343, 1974
13) WERNER, H.; H. KUNSTEK-SANTOS, C. LOHNER, W. SCHÖPS, H. J. SCHMITZ & H. H. KARBE: Propionatnegative saccharolytische *Bacteroides*-Stämme (*B. oralis*)—Vorkommen, Identifizierung, Chemotherapeutika-Empfindlichkeit und serologisches Verhalten. Zbl. Bakt. Hyg., I. Abt. Orig. A 237: 536~547, 1977
14) WERNER, H.; F. NEUHAUS & H. HUSSELS: A biochemical study of fusiform anaerobes. Med. Microbiol. Immunol. 157: 10~16, 1971
15) WERNER, H. & G. RINTELEN: Anaerobe grampositive Kokken (*Peptococcus variabilis, P. asaccharolyticus, P. prevotii* und *P. saccharolyticus*) aus pathologischem Material. Zbl. Bakt. Hyg., I. Abt. Orig. A 223: 496~503, 1973
16) WERNER, H.; G. RINTELEN & H. KUNSTEK-SANTOS: Eine neue buttersäurebildende *Bacteroides-Art*: *B. splanchnicus* n. sp. Zbl. Bakt. Hyg., I. Abt. Orig. A 231: 133~144, 1975
17) WERNER, H.; G. RINTELEN & C. LOHNER: Identifizierung und medizinische Bedeutung von 8 aus pathologischem Material isolierten *Clostridium*-Arten. Zbl. Bakt. Hyg., I. Abt. Orig. A 224: 220~226, 1973

Address reprint requests to: Dr. E. SCHÜTZE, Sandoz Forschungsinstitut, Brunner Strasse 59, A-1235 Vienna, Austria