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In Vitro Activity of A-16686, a Potential Antiplaque Agent

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A-16686, a new glycoproteide antibiotic from *Actinoplanes* sp., was evaluated as a potential antiplaque agent in comparison with chlorhexidine, benzalkonium chloride, and cetylpyridinium chloride. A-16686 had good activity against gram-positive organisms associated with dental plaque (various streptococci, *Streptococcus mutans* in particular, lactobacilli, *Actinomyces viscosus*, and *Actinomyces naeslundii*); most of the strains tested were clinical isolates. It was bactericidal for streptococci (MBC/MIC ratio of ≤8 for 92% of the strains) and for growing cells of *S. mutans* briefly exposed to antibiotic (99.9% killing within 5 min of contact with 200 μg of A-16686 per ml). It also inhibited the in vitro plaque formation by *S. mutans* and had good activity against preformed plaques. For most cases, its activity was comparable to those of chlorhexidine, benzalkonium chloride, and cetylpyridinium chloride. A-16686 appears to be a promising antiplaque agent because of the following attributes: narrow spectrum of activity, rapid bactericidal action, lack of selection of resistant mutants, absence of cross-resistance with clinically used antibiotics, nonabsorption by oral route, good tolerability by the oral mucosa (rats and dogs), and physical characteristics (white powder, soluble in water).

Coronal plaque, which adheres to the surface of teeth, is a dense bacterial mass containing up to 10^{11} organisms per g, wet weight (11). Certain oral bacteria involved in plaque formation play a key role in the initiation and progression of dental caries; they initiate the development of enamel lesions and can also cause destruction of periodontal tissue. Organisms most frequently found in the supragingival plaque are gram-positive aerobic bacteria (streptococci, in particular *Streptococcus mutans*; lactobacilli; and *Actinomyces viscosus* and *Actinomyces naeslundii*) (11, 12, 21, 22).

Physical removal of the plaque leads to the remission of symptoms of gingivitis, periodontitis, and dental caries (21). Therefore, topical, rather than systemic, treatment with antibacterial agents would seem to be indicated for control of dental caries and periodontal diseases. An antiplaque agent should permeate the plaque and be rapidly bactericidal against pathogenic organisms after brief exposure (24).

We have evaluated A-16686, a new glycoproteide antibiotic from *Actinoplanes* sp. (4), as a potential antiplaque agent because of its spectrum of activity and high bactericidal action against various species of gram-positive bacteria, including *Streptococcus mutans* (20). As the plaque-forming organisms are more susceptible to the antimicrobial agents than is the intact plaque, the efficacy of this new agent was also tested on pregrown bacterial plaques; plaque inhibition was also studied. Cationic compounds were used as comparison drugs.

MATERIALS AND METHODS

Antibacterial agents. A-16686 (Lepetit), chlorhexidine diacetate (CHX), benzalkonium chloride (BKC), and cetylpyridinium chloride (CPC) were used in these experiments.

Microorganisms. The microorganisms used in these studies were: *Streptococcus mutans* strains ATCC 27351, ATCC 27352, NCTC 10919, ATCC 27607, ATCC 25175, and 15 clinical isolates, belonging to different serological groups; *Streptococcus salivarius*, 4 clinical isolates; *Streptococcus mitis*, 2 strains; *Streptococcus sanguis*, 2 strains; *A. viscosus* ATCC 19246; *A. naeslundii* ATCC 12104 and ATCC 19039); and various species of lactobacilli, 6 strains.

Media. Todd-Hewitt broth (Difco Laboratories) was used for susceptibility testing and killing curves with streptococci; colonies were counted on Todd-Hewitt agar. Brain heart infusion agar (Difco) and Lactobacilli MRS broth (Difco) were used for *Actinomyces* sp. and lactobacilli, respectively.

Jordan medium, supplemented with 5% (wt/vol) sucrose, which was sterilized separately (14), was used for both plaque inhibition and pregrown plaque assays; for the latter test, 0.005% (wt/vol) sodium carbonate and 0.001% (wt/vol) bromcresol purple (pH indicator) were added (24). For these tests, *Streptococcus mutans* strains were maintained in brain heart infusion broth to which 2% glucose was added.

Susceptibility testing: MICs. MICs were determined by serial twofold dilutions either in broth or in agar. Overnight cultures of streptococci and lactobacilli, diluted to obtain 10^6 to 10^7 CFU/ml, were used as inocula: high inoculum sizes were tested to reproduce in vivo conditions. Suspensions of *Actinomyces* sp. from slants of 72 h, standardized to an optical density of 0.3 with a Spectronic 70 spectrophotometer at 590 nm, were inoculated on agar plates with a multipoint inoculator (Dynatech). The MIC was defined as the lowest concentration of a compound which prevented visible growth after incubation at 37°C for 24 h (or for 72 h in an atmosphere of N_2-CO_2-H_2 (80:10:10) for *Actinomyces* sp.

MBCs. For streptococci, MBCs were determined by the standard method (1). For each strain the number of CFU per milliliter was determined in the initial inoculum (10^6 to 10^7 CFU/ml). Subcultures were made by spreading 10 μl in triplicate on agar plates from each tube showing no visible growth. Colonies were scored after incubation of the plates for 48 h at 37°C in an anaerobic atmosphere. The MBC was defined as the lowest concentration of a drug which killed at least 99.9% of the cells present in the initial inoculum.

Killing curves. Various concentrations of A-16686 and CHX (10, 50, 100, and 200 μg/ml, corresponding to 20, 100, 200, and 400 times the MIC) were added to stationary or growing cells of *Streptococcus mutans* type c L659, a clinical isolate; inoculum sizes were 6.6×10^6 and 2.8×10^6 CFU/ml, respectively. To determine surviving bacteria, the

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TABLE 1. In vitro activity of A-16686, CHX, BKC, and CPC against bacterial strains of the oral flora, most of them clinical isolates

Organism (no. of strains)	Geometric mean MIC (range) (μg/ml)			
	A-16686	CHX	BKC	CPC
Streptococcus mutans (20)[a]	1.11 (0.5–4)	0.97 (0.5–4)	2 (1–4)	1.37 (1–8)
Streptococcus salivarius (4)	0.42 (0.25–0.5)	0.5 (all 0.5)	2 (all 2)	1 (all 1)
Streptococcus sanguis (2)	1 (all 1)	2 (all 2)	4 (all 4)	2 (all 2)
Streptococcus mitis (2)	0.5 (0.125–0.5)	0.35 (0.125–1)	1.41 (1–2)	0.71 (0.5–1)
Actinomyces viscosus ATCC 19246 (1)	0.125	2	4	4
Actinomyces naeslundii (2)	0.5 (all 0.5)	2 (all 2)	4 (all 4)	5.66 (4–8)
Lactobacilli (6)	0.025 (0.004–0.25)	3.17 (2–8)	5.04 (2–8)	3.17 (1–8)

[a] Including types a, c, and d.

cultures, incubated at 37°C, were sampled and plated before the substances were added and after 5, 10, 15, and 30 min of contact. The plates were incubated as described above.

Plaque inhibition test (19). Sterile 20-gauge stainless steel wires were incubated at 37°C in 10 ml of medium containing serial twofold dilutions of an antibacterial substance and 0.1 ml of an overnight culture of a *Streptococcus mutans* strain. A tube without any antibacterial compound served as a control. Each day (for 4 to 5 days) the wires were transferred to fresh medium containing corresponding concentrations of the compound, which was freshly inoculated with the same strain. The MIC was defined as the lowest concentration which inhibited plaque formation.

Pregrown plaque inhibition test (modification of Tanzer test) (24). Plaques of *Streptococcus mutans* were grown for 24 h in vitro on stainless steel wires in Jordan medium. The wires, with the adherent plaques, were rinsed in distilled water and immersed in various concentrations of the compounds to be tested. After 3 min of contact, the plaques were rinsed in distilled water for 15 min and then transferred to fresh medium. After 24 h of incubation at 37°C, a color change from purple (pH > 6.6) to yellow (pH < 5.2) indicates acid production by the plaques. The MIC was defined as the concentration which inhibited acid formation (and therefore color change).

RESULTS

Determination of MICs and MBCs. Table 1 shows the antibacterial activity of A-16686 in comparison with those of CHX, BKC, and CPC. All of the compounds tested had comparable activities against streptococci, but A-16686 was the most active on *Actinomyces* sp. and lactobacilli strains.

A-16686 was very bactericidal, with MBC/MIC ratios ranging from 1:1 to 8:1 for 90% of *Streptococcus mutans* strains and for 100% of other streptococci. Against *Streptococcus mutans*, A-16686 was generally more bactericidal than CHX but less active than BKC and CPC; on the other streptococci, the bactericidal activities of the various compounds were comparable (Table 2). Standard strains and clinical isolates had similar susceptibilities to antibacterial agents tested.

TABLE 2. Bactericidal activity of A-16686, CHX, BKC, and CPC

Compound	MBC range (μg/ml)	No. of strains for which the MBC/MIC ratio is the following[a]:					
		Streptococcus mutans			Other streptococci[b]		
		1–2	4–8	≥16	1–2	4–8	≥16
A-16686	0.5–32	10	8	2	5	1	
CHX	0.5–16	4	9	7	5		1
BKC	2–8	15	5		5	1	
CPC	1–8	19	1		5	1	

[a] Total number of strains used: *S. mutans*, 20; and other streptococci, 6.
[b] Including strains of *S. salivarius* and two strains of *S. mitis*.

Killing curves. A-16686 was more bactericidal for cells in the logarithmic phase of growth than for cells from overnight

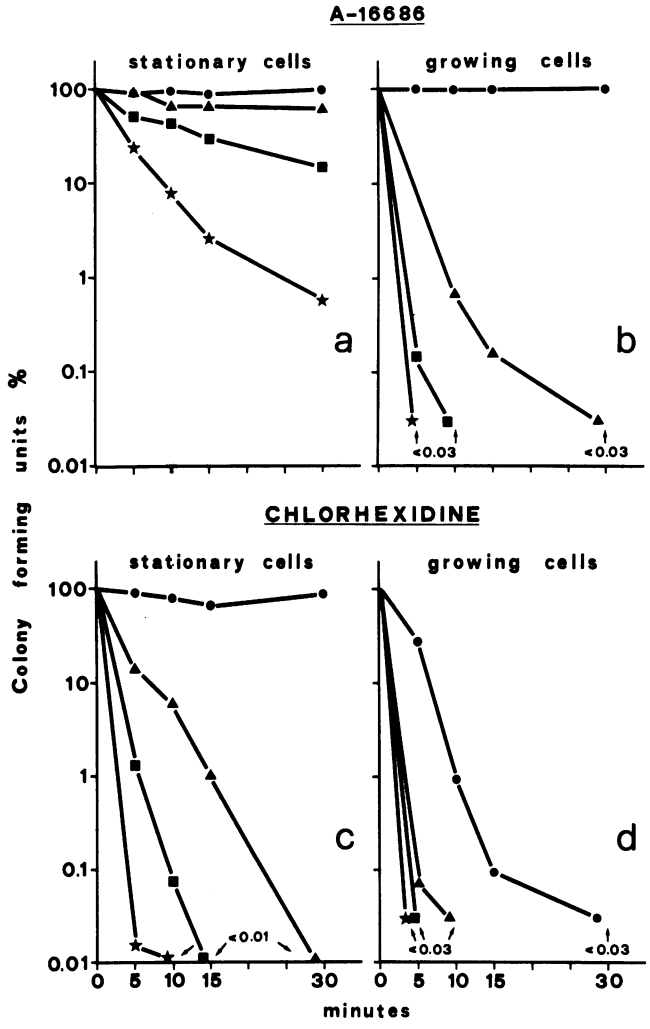

FIG. 1. Bactericidal activity of A-16686 and chlorhexidine against *S. mutans* L659. Symbols: ★, 200 μg/ml; ■, 100 μg/ml; ▲, 50 μg/ml; and ●, 10 μg/ml.

broth cultures. Growing cells were very rapidly killed (99.9% within 5 min of contact with 200 μg/ml); in contrast, 0.6% of cells from overnight cultures survived after 30 min of contact with A-16686 at the same concentration (Fig. 1a and b).

At 100 and 200 μg/ml, CHX had activity comparable with that of A-16686 against proliferating cells, but it was more active than A-16686 at lower concentrations (99.9% killing was achieved within 5 min of contact with 50 μg of CHX per ml).

On stationary cells, CHX was more active than A-16686 at concentrations of 50, 100, and 200 μg/ml; at this last concentration, 99.9% killing was achieved within 5 min of contact (Fig. 1c and d).

Plaque inhibition test. A-16686 had activity comparable with that of CHX on two of three *Streptococcus mutans* strains; on the third strain, it was less active. A-16686 was generally less active than BKC and CPC (Table 3).

Pregrown plaque inhibition test. The ability of A-16686 to inhibit pregrown plaques was compared with those of CHX, BKC, and CPC for five standard strains of *Streptococcus mutans*. In addition, plaques formed by one standard strain and four clinical isolates were exposed to A-16686 or CHX. A-16686 had activity comparable with that of CHX on all strains tested. With the exception of two strains, which were somewhat more susceptible to BKC and CPC, susceptibility to all four drugs was similar (Table 4).

DISCUSSION

A-16686, a new glycoproteide antibiotic, is active in vitro against microorganisms normally found in the mouth and against bacterial plaque.

From data available in the literature (8, 23), A-16686 would appear to be as active as penicillin, methicillin, cephalosporins, and erythromycin against *Actinomyces* sp. and lactobacilli, as active as vancomycin against *Streptococcus mutans*, and generally more active than aminoglycosides, tetracycline, polymyxin B, bacitracin, and various other antibiotics.

From the limited data available, various *Streptococcus mutans* serotypes (a, c, and d) seem to be equally sensitive to A-16686. This is in contrast to the serotype-dependent differences in susceptibility observed for other antibiotics (methicillin, polymyxin B, and bacitracin) (16) which, like A-16686, inhibit bacterial cell wall formation (4). We selected serotype c, which is the most frequently isolated from humans (independently of age, country, or sampling site) (12), for our bactericidal studies. Various antibiotics which are active in vitro against *Streptococcus mutans* and other cariogenic gram-positive organisms have also been shown to reduce the development of dental caries in rats, when administered in food (9). However, there are few studies on the use of antibiotics for caries control in man (7, 13, 18). Other agents, such as CHX and other cationic compounds, are more extensively employed in various formulations (2, 3, 5, 6, 17).

TABLE 3. Inhibition of plaque formation by A-16686, CHX, BKC, and CPC

Streptococcus mutans strain	MIC (μg/ml) of:			
	A-16686	CHX	BKC	CPC
ATCC 25175	1.5	1.5	3.1	0.4
ATCC 27607	25	3.1	3.1	1.5
21 Typ P L650	12.5	6.25	3.1	1.5

TABLE 4. Activity of A-16686, CHX, BKC, and CPC against pregrown plaques exposed for 3 min

Streptococcus mutans strain	MIC (μg/ml) of:			
	A-16686	CHX	BKC	CPC
ATCC 25175	100	200	200	200
ATCC 27607	200	100	100	100
ATCC 27352	100	100	25	25
ATCC 27351	100	100	25	25
21-Typ P L650	100	50	50	50
Type a NCTC 10919	125	125	ND[a]	ND
Type c L659[b]	200	200	ND	ND
Type c L660[b]	60	60	ND	ND
Type c L661[b]	250	125	ND	ND
Type d L663[b]	60	60	ND	ND

[a] ND, Not determined.
[b] Clinical isolate.

These compounds showed good bactericidal activity by reducing cariogenic bacteria and good antiplaque activity (2, 15, 24, 25). For these reasons, CHX, BKC, and CPC were selected for comparison with A-16686.

A-16686 was as active as CHX, BKC, and CPC against most strains, confirming the bactericidal properties observed previously (20).

As expected from its mechanism of action (4), A-16686 was bactericidal for growing cells of *Streptococcus mutans*; its killing rate was very high. On preformed plaques, a more relevant model for in vivo conditions (24), A-16686 was able to penetrate through the bacterial plaque and inhibit the organisms involved in its formation. It also prevented plaque formation.

A potential hazard after local repeated use of antimicrobial agents is the selection of resistant bacteria. This would seem not to be a problem for A-16686, as no emergence of resistant mutants was observed after 22 serial transfers of various organisms (*Staphylococcus aureus* L1508, *Staphylococcus epidermidis* L1521, and *Streptococcus faecalis* L1331) in medium containing this antibiotic (R. Pallanza, R. Scotti, E. Randisi, and V. Arioli, submitted for publication); there is no reason to suppose that *Streptococcus mutans* behaves differently.

A-16686 is a white powder that gives stable water solutions. It is not absorbed by oral administration to rats (up to 2 g/kg of body weight). In preliminary experiments no effects were noticed on intact or abraded gingival mucosa of dogs or on intact oral mucosa and labial junction of rats, when A-16686 was administered in concentrated aqueous solution (20% [wt/vol]) five times a day for 4 days.

Based on these data, A-16686 appears to be a promising anticaries agent, because of the following properties: its spectrum of activity is limited to gram-positive bacteria (20), including the most cariogenic organisms; it does not select resistant mutants (Pallanza et al., submitted for publication), and there is no cross-resistance with clinically used antibiotics (20); and it has a rapid bactericidal effect. CHX and other cationic compounds share many of these attributes, but their use is limited by the fact that they stain the teeth and tongue (10, 17).

Studies in animals and in humans are now needed to assess whether the promising qualities of A-16686, shown in in vitro studies, are borne out in vivo.

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