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Relation between surface activity and antibacterial activity of amine-fluorides

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Abstract

Amine-fluoride molecules consist of two functional groups – the organic amine, which is a surface active agent and the fluoride ion. Both ions are known to have antibacterial properties. Studies have suggested that there is a positive correlation between the antibacterial properties and the surface activity of various organic amines. The purpose of our study was to determine the antibacterial activity of the two functional groups of amine-fluoride molecules and its relationship to their surface activity. In this study, minimal inhibitory/bacteriocidic concentration (MIC/MBC) were determined for *Streptococcus sobrinus* 6715 at a range of pH between 5 and 7. Surface activity measurements were conducted using a surface tensiometer. Amine-fluorides were found to have similar MIC values to that of chlorhexidine digluconate at all tested pH values, thus showing high antibacterial activity against *Streptococcus sobrinus* 6715. The MIC values of amine-fluorides and amine-chlorides are similar, and are about 100 times lower than the MIC value of NaF. No synergistic effect was found between the fluoride and the organic amine. No correlation was found between the antibacterial properties and the surface activity of amine-fluorides. Therefore, we conclude that the antibacterial activity of amine-fluorides derives mainly from the cationic nature of the organic amine, and not from its properties as a surface active agent.

Keywords: Amine-fluorides; Surface activity; Antibacterial activity

1. Introduction

Dental diseases are among the most widespread chronic disorders affecting mankind. Both dental caries and periodontal diseases are caused by oral bacteria. Eliminating cariogenic and periopathogenic bacteria is the first step towards preventing and healing dental diseases. Amine-fluorides have been used for more than 30 years as anti-cariogenic agents in a variety of oral hygiene products. Amine-fluorides reduce caries by inhibiting the acid produced by plaque bacteria (Capozzi et al., 1967), reducing enamel solubility (Mühlemann et al., 1957), preventing bacterial adhesion to teeth and affecting the vitality of bacteria (Shern et al., 1970). Most investigations of the antibacterial activity of amine-fluorides were limited to their effect on supragingival bacteria. Recently, it has

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been shown that amine-fluorides are also active against periodontal pathogens (subgingival bacteria). Amine-fluoride molecules consist of two functional groups – the organic amine which is surface active, and the fluoride ion (Fig. 1). Both ions are known to have antibacterial properties: the fluoride interferes with bacterial cell functions like glycolysis and macromolecules synthesis by inhibiting enolase, phosphoenolpyruvate phosphotransferase and H^+-ATPase (Hamilton and Bowden, 1988). The antibacterial mode of action of amine-fluorides has not been studied extensively; it is not clear what is the contribution of the organic amine and the fluoride to the total antibacterial activity of the amine-fluoride molecule. It has been demonstrated that the bactericidal activity of aliphatic monoamines and diamines depends on their surface activity (Blois and Swarbrick, 1972). This dependency has been established in several structure activity relationship studies which have correlated antibacterial activity with critical micelle concentration (CMC) and surface tension reduction (Bass et al., 1975; Murata et al., 1990). The purpose of our study was to determine the contribution of each of the two functional groups of the amine-fluoride molecule to its antibacterial activity, and the effect of pH on its antibacterial activity. The relationship between amine-fluoride's antibacterial properties and its surface activity was examined by comparing these activities with other dental antiseptics.

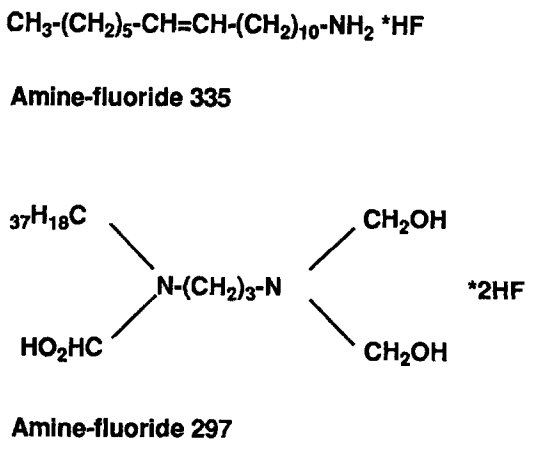

Fig. 1. Chemical structure of amine-fluoride 335 and amine-fluoride 297.

2. Materials and methods

2.1. Active compounds

The amine-fluorides investigated were (Fig. 1); N,N′,N′-tris(2-hydroxyethyl)-*N*-octadecyl-1,3-diaminopropane dihydrofluoride (AmF 297), 9-octadeceneamine hydrofluoride (AmF 335) and their corresponding amine-chlorides; AmCl 297 and AmCl 335 (GABA, Basel, Switzerland). Other active compounds tested and compared to amine-fluorides/chlorides were: cetylpyridinium chloride (CPC), NaF (Merck, Darmstadt, Germany), NaCl (Frutarom, Haifa, Israel), pluronic F-68 (BASF, Ludwigshafen, Germany), chlorhexidine diacetate (CHX acet.), chlorhexidine digluconate (CHX gluc.) and sodium lauryl sulphate (SLS) (Sigma, St. Louis, MO, USA).

2.2. Minimal inhibitory/bactericidal concentration (MIC/MBC)

AmF 297 and AmF 335 were the tested compounds. Amine-chlorides are composed of an organic amine identical to that in amine-fluorides but with a chloride substituting the fluoride, and thus were used to demonstrate the effect of the organic amine. NaF and NaCl were used as negative controls for the antibacterial activity of the fluoride and the chloride respectively. CHX acet. and CHX gluc. were both used as positive controls, since chlorhexidine is the most widely used antiseptic in dentistry. MIC and MBC were determined using the broth dilution method. Stock solution of each active compound in distilled water was prepared and filter sterilized immediately before use, using a 0.2 μm membrane filter (Schleicher and Schuell, Dassel, Germany). Each test tube contained 4 ml of (TSB) trypticase soy broth (Becton-Dickinson, Cockeysville, MD, USA) as bacterial growth media, and 0.2 ml of the active compound in different serial dilutions. Each tube

was inoculated with 0.1 ml containing 10^6 colony forming units (CFU) of *Streptococcus sobrinus* 6715. Controls were divided into two groups; one containing no active compound was inoculated with bacteria to determine maximal growth of bacteria, and the other containing the active compound without bacteria to check if turbidity occurred due to a chemical interaction between the active compound and TSB ingredients. The test tubes were incubated for 20 h (air supplemented with 5% CO_2) at 37°C. Bacterial growth in the test tubes was measured by spectrophotometer (LKB – 4052, Cambridge, UK) at 540 nm. The MIC value was determined as the lowest concentration in which the turbidity was $< 10\%$ of the control value. MIC was determined in the same way for *Actinomyces viscosus* (ATCC 43146), using BYG (brain-heart infusion, yeast and glucose) as the bacterial growth media. MBC was determined by plating 100 μl samples from all inoculated tubes with no visible growth (after 20 h of incubation) onto brain-heart agar plates. The plates were incubated for 48 h (air supplemented with 5% CO_2) at 37°C. Samples for control plates were drawn from the control test tubes. The MBC value was determined as the lowest concentration of the tested compound in which a reduction of 99.9% in CFU was observed, compared to the control value. The active compounds were tested in triplicate, and each triplicate was repeated at least twice. All experiments were conducted at pH = 7 unless stated differently. MIC values at different pH values (pH = 5, 6 or 7) were determined for AmF 297, AmF 335, AmCl 297, AmCl 335 and CHX gluc. The pH values of the media (TSB in buffer phosphate) were adjusted with HCl or NaOH prior to sterilization. Final pH values in each test tube were measured by a pH meter (Orion – 420A, Boston, MA, USA).

2.3. Isobologram

The isobologram method for testing antimicrobial combinations was used to determine the type of interaction (additivity, antagonism or synergism) between two active compounds (Barry, 1976). The effect of a combination of NaF with AmF 297 or NaF with AmCl 297 on bacterial growth was assayed: for each compound, MIC data was used to determine the appropriate concentration combinations. To construct the isobologram, the MIC for NaF was plotted on the horizontal scale and the MIC for AmF 297 or AmCl 297 alone was plotted on the vertical scale, using arithmetic scales intersecting at a zero value. Each concentration of NaF was combined with a range of concentrations of AmF 297 or AmCl 297. For each concentration of NaF plotted on the horizontal scale, an MIC of the vertical compound (AmF 297 or AmCl 297) was determined and plotted on the isobologram. In this way, a series of points was determined and joined by a curved line originating at the MIC of NaF and terminating at the MIC of AmF 297 or AmCl 297 alone. A straight line joining the values obtained with each individual drug represents an isobol that indicates an additive effect between the two compounds. Antagonism is indicated by an isobol that arcs upwards away from the coordinant, and an arcing towards the coordinant indicates a synergistic effect between the two compounds.

2.4. Surface activity

The surface tension (γ) was determined by the du Nouy ring method (Martin et al., 1983) in double distilled water at 25°C using a surface tensiometer (Fisher, Springfield, NJ, USA). For each tested compound a minimum of ten different concentrations was prepared, four replicates for each concentration were measured. The surface activity of each active compound is shown by a plot of surface tension versus log (active compound concentration). The CMC is determined as the intercept of the two linear segments. Π – the surface pressure is defined as the difference in dyne/cm between γ_0 (surface tension of pure water) and γ (surface tension of a selected concentration), $\Pi = \gamma_0 - \gamma$.

3. Results

The effect of the pH on the MIC values of AmF 297 and 335, AmCl 297 and 335 and CHX gluc. is shown in Table 1. For all tested compounds, the MIC values decrease with a decrease

Table 1
The effect of pH on MIC values (μmole/l) of the active compounds in phosphate buffered TSB for *Streptococcus sobrinus* 6715

Active compound	pH = 5	pH = 6	pH = 7
AmF 297	5	10	20
AmCl 297	5	10	20
AmF 335	10	20	200
AmCl 335	10	40	100
CHX gluc.	5	10	10

in the pH (from pH = 7 to pH = 5). Generally, the MIC values of all active compounds are similar to one another. A marked change in the MIC value occurs only for AmF 335 and AmCl 335 at pH = 7. The MIC and MBC found for AmF 297 and 335, AmCl 297 and 335, CHX acet. and gluc., NaF and NaCl are shown in Table 2. For *Actinomyces viscosus*, AmF 297, AmF 335, AmCl 297 and AmCl 335 have the same MIC value (5 μM), which is similar to the MIC value of CHX gluc. (2.5 μM). For *Streptococcus sobrinus* 6715, the MIC values of AmF 297 (5 μM), AmF 335 (20 μM), AmCl 297 (5 μM) and AmCl 335 (10 μM) were all similar to one another and to the MIC values of CHX acet. (8 μM) and CHX gluc. (5 μM). The MIC values of NaF (> 4000 μM) and NaCl (> 10 000 μM) are more than 100 times higher than the MIC values of the other tested compounds: AmF 297 and 335, AmCl 297 and 335 and CHX gluc. MBC for NaF and NaCl could not be determined because at saturation concentration of these agents, a reduction of 99.9% in CFU was not observed. For each of the other tested compounds, the MIC value was either equal to or half of the MBC value. The isobologram found for the combination of NaF and AmF 297 or AmCl 297 is illustrated in Fig. 2. The MIC values for AmF 297 and AmCl 297 do not differ significantly from the theoretical values of an additive effect. The relationship between surface activity and the MIC of the tested compounds is shown in Figs. 3 and 4. For each pair (AmF 297 and AmCl 297, AmF 335 and AmCl 335 or CHX gluc. and CHX acet.), the counter ion which is not surface active (the fluoride and chloride, or the acetate and gluconate) does not significantly affect the surface activity of the organic amine or chlorhexidine respectively (as can also be seen from their CMC values in Table 3). Table 3 compares the antibacterial activity, CMC and Π_{MIC} of several dental antiseptics. CHX is surface active at concentrations higher than those of all other active compounds. The MIC values for all compounds are similar to each other, with the exception of SLS, which is an anionic surface active agent with a lower MIC value. The MIC values for all compounds are much lower than their CMC values. Only AmF 297, AmCl 297 and AmF 297 + F-68 showed a significant reduction in the surface tension from γ_0 to γ_{MIC} relative to the other active compounds.

4. Discussion

The physiological pH of the oral cavity is between 6 and 7. After food consumption (especially carbohydrate rich diet), the pH drops to a value lower than 5 due to acid production from sugar fermentation by the oral bacteria. The results of our studies show that the antibacterial activity of AmF 335 increases as the pH decreases (from 7 to 5). The pKa of AmF 335 is 7.1. As the pH decreases, AmF 335 becomes more protonated and hence more active. AmF 297 and AmCl 297 have similar MIC values to that of CHX gluc. at all pH range tested; demonstrating high antibacterial activity against *Streptococcus sobrinus* 6715 independent of pH values. The MIC values found for NaF and CHX gluc. correspond to values found in the literature (Bradshaw et al., 1990; Drake et al., 1993). However, the MIC values for AmF 297 and AmF 335 reported in the literature (Kay and Wilson, 1988), are 10 times higher than those found in our experiment: this is probably due to the fact that serum was incorporated into their media, which can reduce the antibacterial activity of the compounds (Hennessey, 1973). Amine-fluoride and its corresponding hydrochloride salt have the same MIC values at all tested pH range (for both 297 and 335). Hermann and Mühlemann (1958) found same antiglycolitic effect for both AmF 297 and AmCl 297. Warner et al. (1976) found same MIC values for different

Table 2
MIC and MBC values for the active compounds in TSB

Active compound	MIC (μmole/l) *Streptococcus sobrinus* 6715	MBC (μmole/l) *Streptococcus sobrinus* 6715	MIC (μmole/l) *Actinomyces viscosus*
AmF 297	5	10	5
AmCl 297	5	5	5
AmF 335	20	20–40	5
AmCl 335	10	10–20	5
CHX gluc.	5	5	2.5
CHX acet.	8	8	ND*
NaF	>4000	ND*	ND*
NaCl	>10 000	ND*	ND*

*ND, not determined.

alkylamines hydrofluorides and hydrochlorides. MIC values found for AmF 297, AmF 335, AmCl 297, AmCl 335 and CHX gluc. are either equal to or half of the MBC value. Under our experimental conditions all these compounds were found to be bacteriocidic, as found for AmF 297 and AmF 335 by Kay and Wilson (1988). The MIC values of NaF and NaCl indicate that the fluoride is a slightly more active than the chloride, and that each of them is far less active than the organic amine. Both AmF 297 and AmCl 297 showed the same additive effect when combined with NaF, thus indicating no synergistic effect between the fluoride and the organic amine. Collectively our results indicate that the antibacterial activity of amine-fluorides derives mainly from the organic amine, and that the fluoride's contribution to the antibacterial activity of the amine-fluoride molecule is negligible. Certainly, fluoride can affect the growth of a wide range of oral bacteria in vitro, but usually at concentrations higher than those believed to be available in dental plaque. The MIC value of fluoride for the inhibition of growth of *Streptococcus mutans* reported by Hamilton and Bowden (1988) is 135 μg/ml. The concentration of fluoride at the MIC of AmF 335 is 1.3 μM, which equals 0.025 μg/ml. This concentration is 1000 less than the known MIC value of the fluoride which kills *Streptococcus mutans* in vitro.

It has been demonstrated that the bactericidal activity of aliphatic monoamines and diamines

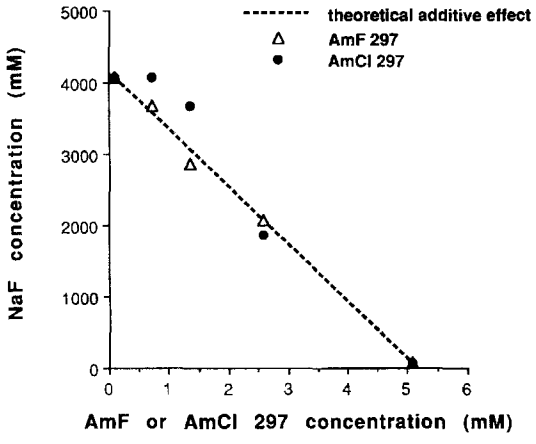

Fig. 2. MIC values of combinations of NaF and AmF 297 or NaF and AmCl 297 for *Streptococcus sobrinus* 6715.

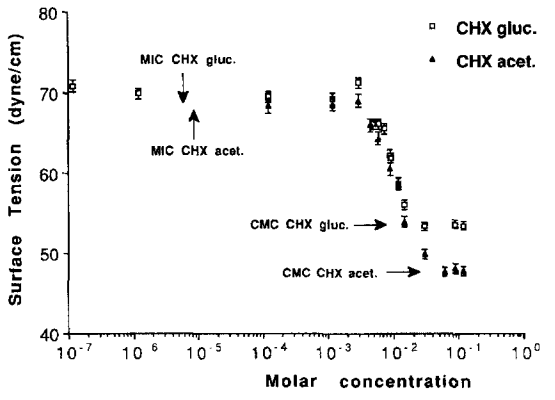

Fig. 3. Surface activity of CHX acet. and CHX gluc. including CMC and MIC values (vertical bars indicate the S.D.).

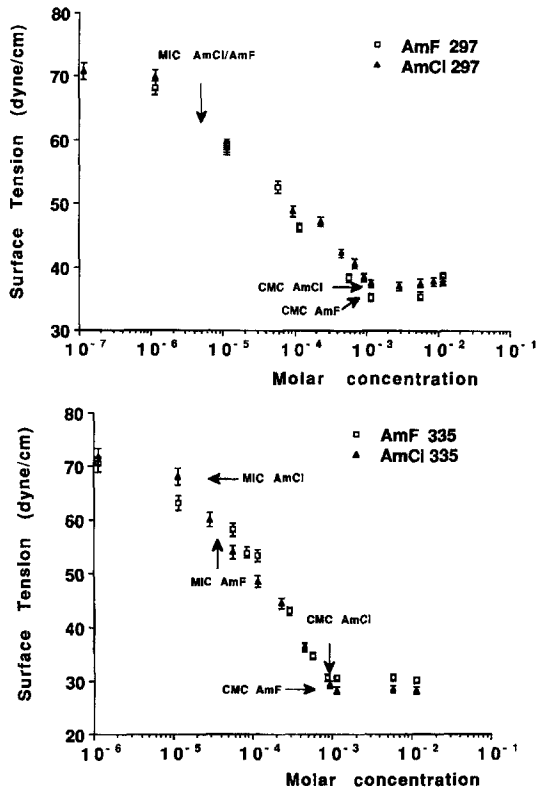

Fig. 4. Surface activity of (A) AmF 297 and AmCl 297 and (B) AmF 335 and AmCl 335 including CMC and MIC values (vertical bars indicate the S.D.).

depends on their surface activity (Blois and Swarbrick, 1972). Structure activity relationship studies of aliphatic monoamines have shown a positive correlation between their antibacterial activity and their CMC (Bass et al., 1975). CHX is surface active at concentrations higher than those of all other tested compounds. The CMC values found in our results for CHX acet. and CHX gluc. correspond to those found by Heard and Ashworth (1968) or Perrin and Witzke (1971). But their surface tension values (dyne/cm) were much lower than those found in our experiment, probably because of impurities which are known to lower the surface tension. Fisher et al. (1975) and Attwood and Natarajan (1980) could not find a CMC for CHX acet., but they did not test concentrations similar to or higher than the CMC found in our experiment. Powers et al. (1975) found a CMC value of 0.018 mM for AmCl 297, which is considerably lower than the CMC value found in our experiment. For all tested compounds the MIC value was at least 100 times lower than the CMC value. Therefore, we used a parameter which is a modification of the surface pressure – Π. This parameter, Π_{MIC} would give us more applicable information about the relationship between a compound's surface activity and its MIC. Π_{MIC} (dyne/cm) represents the reduction of the surface tension from the initial value of pure water to the surface tension found at the compound's MIC (Table 3). From all tested compounds, only AmF 297 and AmCl 297 significantly reduced the surface tension at MIC. F-68 is a nonionic surface active agent which was found to have no antibacterial activity (data not shown). When AmF 297 was tested with F-68, the MIC did not change, although there was a significant reduction in the surface tension value (from Π_{MIC} = 11.5 to Π_{MIC} = 23.7). Consequently, no obvious correlation between antibacterial activity and surface activity could be found.

The first stage in the mechanism of antibacterial activity is a rapid attraction and strong adsorption of the active compound to cell surface organelles. This stage is achieved through electrostatic interactions between charged groups in the active compound and on the bacterial cell wall (Denton, 1991). F-68 is a nonionic surface active agent which has no antibacterial properties. SLS is an anionic surface active agent with a higher MIC value than the MIC values of the other tested compounds. AmF's and AmCl's are all cationic surface active agents, CPC is a quaternary ammonium cationic surface active agent and CHX is a cationic bisguanidine. All tested cationic compounds have similar MIC values, although their surface activity differs. Hence we can conclude that the antibacterial activity of amine-fluorides derives mainly from the organic amine which is a cationic surface active agent. The cationic charged molecule adsorbs to negatively charged groups on bacterial cell wall. Baker et al. (1978) showed for ionic surface active agents that antibacterial activity does not always correlate with antiplaque activity. It may be postulated that without being protonated, the organic amine would not have antibacterial activity, however its surface activity

Table 3
MIC, CMC and Π_{MIC} values of the active compounds

Active compound	MIC (mmole/l)	CMC (mmole/l)	π_{MIC} (dyne/cm)
AmF 297	0.005	0.8	11.5
AmCl 297	0.005	1.0	10.0
AmF 335	0.020	0.7	5.5
AmCl 335	0.010	1.1	4.0
CHX gluc.	0.005	20.0	0.5
CHX acet.	0.008	40.0	1.0
SLS	0.400	7.0	2.5
CPC	0.0025	1.5	1.0
AmF 297 + F-68	0.005	ND*	23.7

*ND, not determined.

is important for its adsorption and retention on solid surfaces in the oral cavity.

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