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The Mucosal Toxicity of Different Benzalkonium Chloride Analogues Evaluated with an Alternative Test Using Slugs

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Purpose. The objective of this study was to evaluate the mucosal toxicity of different benzalkonium chloride (BAC) analogues using slugs as the alternative test organism.

Methods. The effect of different BAC analogues on the mucosal tissue of slugs was determined from the protein, lactate dehydrogenase, and alkaline phosphatase released from the foot mucosa after treatment. Additionally, mucus production and reduction in body weight of the slugs were measured. The eye irritation potency of the molecules was evaluated with the Bovine Corneal Opacity and Permeability (BCOP) assay. The antimicrobial activity of the different BAC analogues was also assessed.

Results. All BAC analogues induced severe damage to the mucosal epithelium of the slugs, and the irritation increased with decreasing alkyl chain length: BAC-C16 < BAC-C14 < BAC-C12 ~ BAC-mix. A similar ranking was obtained with the BCOP assay for eye irritation. The relative order of activities among the three BAC analogues was the same, *i.e.,* BAC-C14 ≥ BAC-C16 > BAC-C12. The BAC-C14 exhibited higher activity than the BAC-mix.

Conclusions. The toxicity and activity of BAC analogues depend on the alkyl chain length. The use of BAC-C14 as a conservative agent in pharmaceutical preparations instead of the BAC-mix should be considered.

KEY WORDS: mucosal toxicity; slug; BCOP assay; BAC analogues; antimicrobial activity.

INTRODUCTION

Benzalkonium chloride (BAC) is a quaternary ammonium chloride and is used as a preservative in several ophthalmic and nasal formulations. It is a monoalkyldimethylammonium chloride with one alkyl chain representing a mixture of alkyls from C_8H_{17} to $C_{18}H_{37}$. In pharmaceutical formulations BAC is predominantly $C_{12}H_{25}NC_9H_{13}Cl$, with smaller quantities of the C14 and C16 analogues (1). Several *in vitro* and *in vivo* studies have shown the unfavorable effects of BAC to different cell types (2–4). The effects of the different BAC analogues on mucosal tissue have so far not been compared. Analogue molecules with a different alkyl chain length can result in different degrees of toxicity. This was shown for phosphatidyl cholines (PC) with different acyl chain length. Didecanoylphosphatidyl choline induced hemolysis of red blood cells, whereas PCs with longer acyl chains did not have this effect (5). The same effect was reported for nonionic surfactants, those with the larger hydrophobic moiety being less hemolytic than those with the smaller chains (6).

Toxicity testing using vertebrates has been widely criticized for ethical reasons. The principal alternative to *in vivo* testing is *in vitro* testing. Many factors such as nervous control, systemic blood flow, reduced motility, and heterogeneous cell populations, however, are absent in simple cell culture models. In the workshop "The Three R's: The Way Forward," organized by the European Center for the Validation of Alternative Methods (Sheringham, Norfolk, UK, 1995) some replacement alternative methods and approaches were proposed, *e.g.,* the use of "lower" organisms with limited sentience and/or not protected by legislation controlling animal experiments, including invertebrates, plants, and microorganisms (7). We developed an alternative method for screening the mucosal irritating potential of chemicals using naked snails (slugs) as test organisms (8). The objective of this study was to evaluate the effect of different BAC analogues on mucosal tissue using the slug *Arion lusitanicus* as a model organism. The body wall of the slugs consists of a single-layered epithelium containing ciliated cells, cells with microvilli, and mucus secreting cells. Mucus secretion is necessary for the locomotion and the prevention of dehydration of the slug. In this study the effect of different BAC analogues on the mucosal tissue was determined from the release of protein, cytosolic lactate dehydrogenase, and membrane-bound alkaline phosphatase from the body wall of the slug after treatment. Additionally, the mucus production and the reduction in body weight of the slugs were measured during the treatment period.

The eye irritating potency of the BAC analogues was also assessed with the Bovine Corneal Opacity and Permeability (BCOP) assay. This is an *in vitro* alternative to the *in vivo* Draize eye irritation test (9). The test uses corneas from bovine eyes and measures the effect of the test substance on the opacity and permeability of the corneas (10). This *in vitro* model was shown to be a competent test system for the prediction of the (ocular) toxicity potential of chemicals and test formulations (11).

The antimicrobial activity of the different BAC analogues was also assessed.

MATERIALS AND METHODS

Chemicals

Benzalkonium chloride SigmaUltra (BAC-mix) (60–70% C12, 30–40% C14, and < 5% C16), benzyldimethylhexadecylammoniumchloride (BAC-C16), benzyldimethyltetradecylammoniumchloride (BAC-C14), and benzyldimethyldodecylammoniumbromide (BAC-C12) were purchased from Sigma (Bornem, Belgium). All other reagents were of analytical grade.

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Slugs

The animals were collected in the field and maintained in the laboratory (18–20°C) at a photoperiod of 12/12. The slugs were fed lettuce, cucumber, and commercial pig food.

Test Procedure

Slugs weighing between 3.5 g and 4.5 g were isolated from the cultures 4 days before the start of an experiment and placed in a vented plastic box lined with paper towel moistened with phosphate buffered saline and placed at 20°C.

All test substances were prepared in phosphate buffered saline (PBS, pH 7.4) at a 1% (w/v) concentration. Each experiment contained five negative control slugs (PBS) and five slugs for each treatment (different BAC analogues). To investigate the reproducibility of the test, the experiments were run in duplicate independently on a separate occasion. At the beginning of the experiment the slugs were placed individually on a membrane filter (cellulose acetate 0.45 μm, Sartorius AG, Germany) moistened with 2 ml of the test substance. After a 15-min incubation period the slugs were transferred to a fresh petri dish (90 mm) containing 1 ml PBS. After 45 min the PBS was collected with a micropipette and the slugs were transferred to a fresh petri dish containing 1 ml PBS. After 1 h the PBS was removed and the slugs were placed in a fresh petri dish containing 1 ml PBS for another hour. The samples were immediately analyzed for the presence of proteins, lactate dehydrogenase, and alkaline phosphatase released from the foot of the slug. The slugs were weighed separately before and after the 15 min during treatment. The change in weight caused by the treatment was calculated and expressed as % (w/w) of the body weight. The petri dishes containing the test medium were also weighed before and after the treatment. The weight of the mucus produced was calculated and expressed as % (w/w) of the body weight. During the night the slugs were placed in a petri dish with a membrane filter containing 2 ml PBS and some pig food. The petri dishes were placed in the dark at 20°C ± 0.5°C. This procedure was repeated during 5 successive days.

Analytical Procedures

Protein Content. The total protein concentration present in the PBS samples was determined with a NanoOrange™ protein quantitation kit (Molecular Probes, Leiden, The Netherlands) and expressed as μg/ml per g body weight. The NanoOrange reagent allows accurate detection of proteins in solutions at concentrations between 10 ng and 10 μg/ml. The fluorescence measurements were carried out on a fluorometer (Kontron instruments SFM 25, Van Hopplynus, Brussel, Belgium) using excitation/emission wavelengths of 485/590 nm. Bovine serum albumin was used as a standard.

Enzyme Activity. The enzyme activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was measured with an enzyme kit (DG 1340 K, Sigma Diagnostica, Belgium). The alkaline phosphatase activity (ALP, EC 3.1.3.1) in the samples was measured with an enzyme kit (DG 1245, Sigma Diagnostica, Belgium). The enzyme activity in the samples was expressed in U/L per g body weight. The LDH and ALP reagent measures the enzyme activity based on the optimized standard method recommended by the German Society for Clinical Chemistry (12). One unit of LDH activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol/L of NAD per minute under the conditions of the assay. One LDH unit gives a decrease in absorbance of 0.0002/min at 340 nm. One unit of ALP activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol/L of p-nitrophenol per minute under the conditions of the assay. One ALP unit gives an increase in absorbance of 0.0003/min at 405 nm. The enzyme activity measurements were conducted on a Cobas Mira Plus analyzer (ABX, Brussels, Belgium). The detection limit of the samples was 5 U/L.

Statistics

Statistically significant differences between the negative control (PBS) and the different treatments were determined using a one-way analysis of variance (ANOVA). For the mucus production, the sum of the amount of mucus produced during each contact period was calculated separately for each slug and this value was used for the statistical analysis. For each test organism the sum of the protein and enzyme release of all the samples was calculated and divided by the number of samples. These values were used for the statistical analysis. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If the variances were found not to be equal the data were transformed to their logarithm. To further compare the effects of the different treatments a multiple comparison among pairs of means was performed using a Scheffé test with $p < 0.05$ as significance level. For all the statistical analysis the computer program SPSS version 7.5 was used.

BCOP Assay

The BCOP assay was performed according to the methodology described by Vanparys *et al.* (11) and will be described briefly. Bovine eyes were excised in the slaughterhouse and used within 3 hours after killing. The eyes were immersed in Hanks' balanced salt solution. Three corneas were used per treatment. The corneas were dissected with a 2–3 mm rim of sclera and mounted in the cornea holders with the endothelial side downward. The anterior and posterior compartment of the cornea holder were filled with Minimal Essential Medium (MEM). After an incubation period of 1 h in a water bath at 32°C the MEM solution was removed from both compartments and replaced with fresh medium. The background opacity was measured with an opacitometer (OP-KIT, Electro Design, Riom, France). The opacitometer determines the difference in light transmission between a treated and a control cornea, and displays a numerical opacity value (arbitrary units, AU) (13). To start the experiment, the medium of the anterior compartment was replaced with the test substances. The BAC analogues and the positive control (imidazol) were applied at 20 (g/g) % to the corneas. For the negative controls 0.9% (w/v) NaCl was applied. After an incubation period of 10 min the test medium was removed and the epithelium was washed with MEM solution. The anterior compartment was filled with fresh MEM for another 2 h. Then the corneal opacity was measured. The permeability determination was performed immediately after the opacity readings. Therefore, the medium of the anterior compartment was replaced by 1 ml of a 0.5% (w/v) sodium fluorescein

solution for 90 min. Then the medium of the posterior compartment was removed and its optical density was determined with a spectrophotometer at 490 nm. An *in vitro* score (IVS) for the corneal injury was calculated with a formula that combines results from both corneal opacity and permeability (IVS = opacity plus 15 times optical density value) (13). The following classification system for the *in vitro* scores was established: ≤ 25.0: nonmild irritant; 25.1–55.0: moderate irritant; 55.1–80.0: severe irritant; and ≥ 80.1: very severe irritant (11,13).

Microbiological Procedures

Bacterial Strains, Media, and Growth Condition

Four bacterial test strains, *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 23741), *Pseudomonas aeruginosa* (ATCC 15441), and *Burkholderia cepacia* (ATCC 25416) (all from the American Type Culture Collection (ATCC), Manassa, VA) were used. They were subcultured overnight in tryptic soy broth (Difco Laboratories, Detroit, MI) at 37°C (*S. aureus, E. coli, P. aeruginosa)* or 30°C (*B. cepacia),* checked for purity and reidentified by standard techniques.

Antimicrobial Agents

BAC-mix, BAC-C16, BAC-C14, and BAC-C12 were tested. Working solutions were prepared starting from a 1% (w/v) aqueous stock solution.

Antimicrobial Susceptibility Assay

Susceptibility testing was carried out using a macrodilution assay (1). Prior to testing, the bacterial strains were grown overnight in Mueller-Hinton broth (Difco) at 37°C. The optical density of the cultures was adjusted spectrophotometrically (530 nm) to obtain 1.10^6 to 5.10^6 CFU/ ml. The inoculated tubes contained 10 ml broth and 0. 5 ml of the bacterial stock suspension.

Dilution series were prepared for each compound over the following concentration ranges: 0.048–100 μg/ml (*S. aureus* and *E. coli),* and 100–1000 μg/ml (*P. aeruginosa* and *B. cepacia*), respectively. In addition, a control solution without antibacterial was included in each run. The tubes were incubated at 37°C for 24 h. Each run was done in duplicate. The MIC was defined as the lowest concentration of the agents that inhibited visible growth relative to that of the control.

RESULTS

Toxicity of the Different BAC Analogues

Mucus Production and Reduction in Body Weight

Figure 1 illustrates the amount of mucus produced during a repeated contact period with the different BAC analogues. Slugs treated with PBS showed only a minor mucus production. The negative values are due to the handling of the petri dishes. The body weight of the negative control slugs fluctuated between 80 % and 100 % of the initial weight during the 5 days of treatment (Fig. 2). All BAC analogues induced a significant increase in total mucus secretion compared with the PBS slugs (Table I). The total amount of mucus produced increased with decreasing alkyl chain length. Mucus production resulted in a decrease of the body weight; after the fifth treatment with BAC-C12 and BAC-mix the body weight of the slugs was reduced to about 50% of the initial weight. Those molecules induced the highest mucus production. After the fifth contact period with BAC-C14 and BAC-C16 the body weight was decreased by 40% and 33%, respectively. ANOVA testing resulted in no significant difference ($p > 0.05$) for the total mucus production for the repeated experiments.

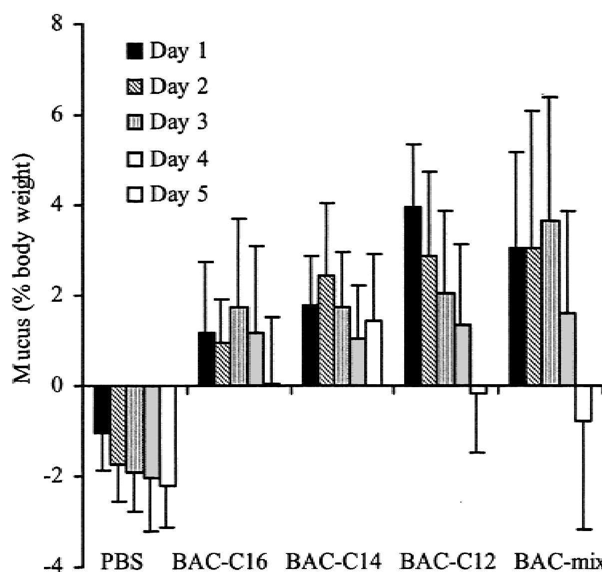

Fig. 1. Amount of mucus produced by the slugs during a 15-min contact period. Data are presented as the mean ± S.D. (n = 10) and expressed as percentage of the body weight.

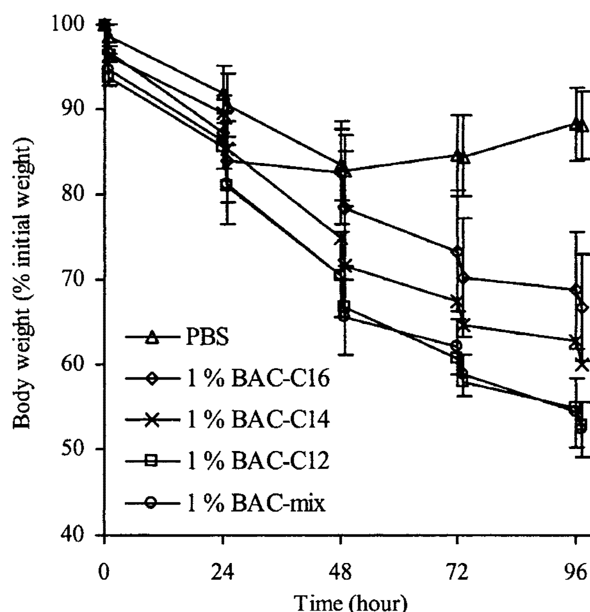

Fig. 2. Reduction in body weight caused by the different treatments. Data are presented as the mean (n = 10) and expressed as percentage of the initial body weight. S.D. bars are indicated for PBS, BAC-CI6, and BAC-mix.

Table I. Total Mucus Production for the 15-min Contact Period; Mean Protein, LDH, and ALP Released After Repeated Contact Periods for the Different Treatments

Treatment	Mucus[a]	Protein (μg/ml/g)	LDH (U/L/g)[b]	ALP (U/L/g)[b]
PBS (neg. control)	−8.99 ± 1.63	11 ± 6	—	—
BAC-C16 (1% w/v)	5.04 ± 4.60[c]	36 ± 28[c]	0.74 ± 0.96	0.09 ± 0.16
BAC-C14 (1% w/v)	7.74 ± 2.64[c]	65 ± 34[c]	2.54 ± 2.86	0.74 ± 0.65
BAC-C12 (1% w/v)	10.14 ± 3.49[c]	104 ± 40[c]	7.28 ± 4.95	1.41 ± 0.65
BAC-mix (1% w/v)	10.13 ± 5.73[c]	123 ± 30[c]	3.80 ± 2.34	1.48 ± 0.91

Note. Data are presented as the mean ± SD (n = 10).
[a] In % (w/w) of the initial body weight at the start of the contact period.
[b] Below the detection limit.
[c] Significantly different from the negative control slugs.

Protein Release

Figure 3 illustrates the protein release after a repeated contact period with the BAC analogues. Slugs treated with PBS showed a low protein release. All BAC analogues induced a significant increase in protein release in comparison with the PBS slugs (Table I). Statistical comparison of the mean protein concentration between the repeated experiments resulted in no significant differences. The protein release profiles of the slugs treated with BAC-mix and BAC-C12 were comparable. BAC-C14 resulted in a lower protein release than that of the slugs treated with BAC-mix and BAC-C12. The mean protein concentration released from slugs treated with BAC-C16 was significantly lower ($p < 0.01$) than from those treated with BAC-mix and BAC-C12 (Table I).

Enzyme Release

The release of the cytosolic enzyme LDH and the membrane-bound ALP from the mucosa of the slugs is a measure of cell damage. For slugs treated with PBS the release of these enzymes was below the detection limit (Fig. 4 and Fig. 5). All BAC analogues induced LDH and ALP release, and the enzyme activity in the samples increased with repeated treatment. There were no significant differences for the mean LDH and mean ALP release between repeated experiments with the same BAC analogue. Multiple comparison suggested the following subsets for the mean LDH release (Scheffé test): (1) BAC-C16 and BAC-C14; (2) BAC-C14 and BAC-mix; (3) BAC-mix and BAC-C12. For ALP two homogeneous subsets were detected: (1) BAC-C16 and BAC-C14; (2) BAC-C14, BAC-C12, and BAC-mix.

BCOP Assay

Table II shows the opacity and permeability results obtained with the BCOP assay. The opacity of the corneas increased with decreasing alkyl chain length of the BAC analogues and ranged from moderate with BAC-C16 to very se-

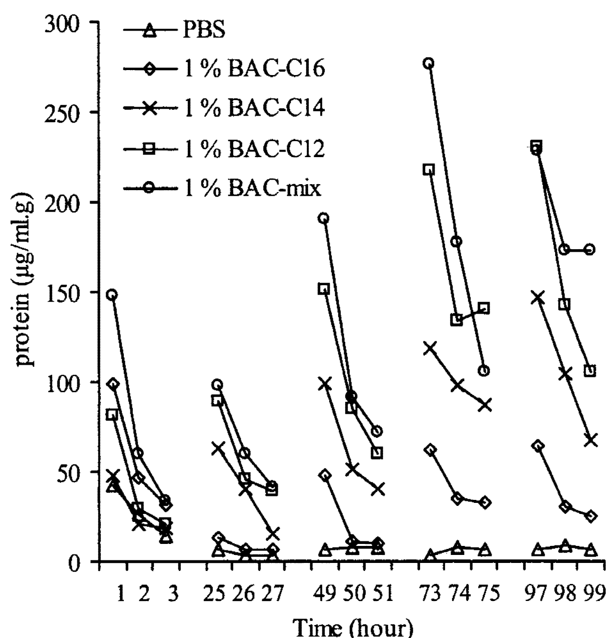

Fig. 3. Protein released from the mucosa of the slugs after a repeated contact period with the different treatments. Data are presented as mean values and expressed in μg/ml PBS per g body weight (n = 10).

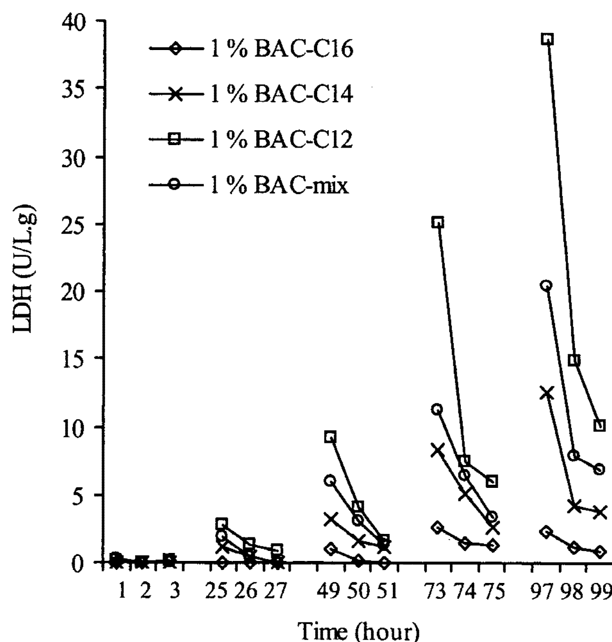

Fig. 4. LDH activity in the PBS samples after repeated contact periods with the different treatments. Data are presented as mean values and expressed in units/ml PBS per g body weight (n = 10).

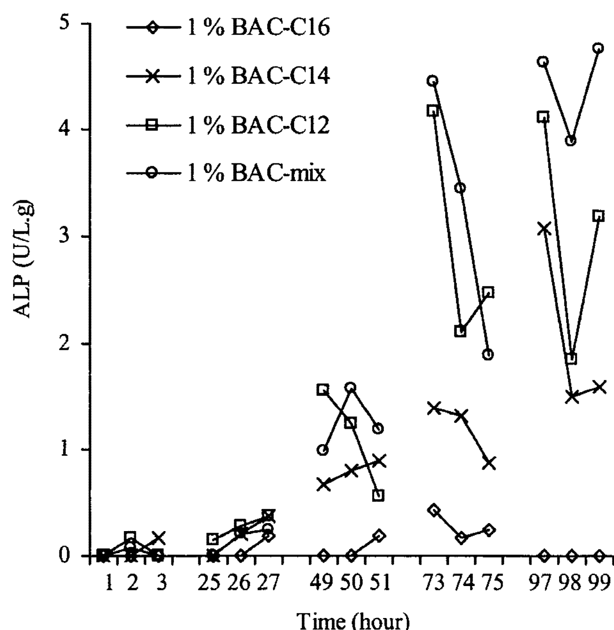

Fig. 5. ALP activity in the PBS samples after repeated contact periods with the different treatments. Data are presented as mean values and expressed in units/ml PBS per g body weight (n = 10).

vere with BAC-C12. The effect on the permeability was comparable for all the BAC analogues and resulted in a high value compared with the control eyes (0.9% NaCl), indicating a strong toxic effect. The *in vitro* score, which combines opacity and permeability effects, classified all the BAC analogues as severe eye-irritating compounds. The corneal injury increased from BAC-C16 < BAC-C14 < BAC-mix < BAC-C12.

Antimicrobial Activity

The antibacterial activities of the BAC analogues with different alkyl chain lengths are listed in Table III. Of these, BAC-C14 showed the highest activity against the four test strains, yielding MICs consistently lower than those for the BAC-mix. The BAC-C12 constituent was the least active against all strains except *B. cepacia*. For a given compound, widely divergent MICs were obtained, ranging from values as low as 0.2 μg/ml (*S. aureus*) to 1000 μg/ml (*B. cepacia*).

DISCUSSION

The body wall of slugs is particularly vulnerable to mechanical and chemical damage, and mucus secretions serve to lubricate and to protect the skin against damage (14). Slugs treated with irritating substances produce mucus to protect the body wall, thereby reducing the body weight. All BAC analogues induced severe irritation to the mucosal epithelium of the slugs. This was shown by the increased mucus production during the 15 min contact period followed by the reduction in body weight. The mucus production stopped immediately when the slugs were removed from the test substance. The severe membrane-damaging effects of the BAC analogues were demonstrated by the increased protein, LDH, and ALP released from the body wall. This membrane damage resulted in a further reduction of the body weight of the slugs. This is in agreement with published data using other models. The nasal mucosa of rats treated with BAC concentrations of 0.01% and higher showed minor to major morphological changes (3,15). The mucociliary transport rate of rabbit tracheas *in vivo* was significantly decreased by a treatment with 0.05% BAC (16). The adverse effects of BAC on the ciliary beat frequency was demonstrated using chicken embryonal tracheas, human adenoid tissue, human nasal cytological biopsies, and cryopreserved human sphenoidal sinus mucosa (17–21). Human corneal epithelial cells were strongly affected after a treatment with BAC (4). Rabbit eyes treated several times with 0.02% BAC solution showed high chemical toxicity scores (22). None of those studies looked at the relation between the alkyl chain length of the BAC analogues and the irritating potential.

From the present study we can conclude that BAC analogues with a different alkyl chain length may possess different degrees of toxicity. The mucosal irritation in the slugs increased with decreasing alkyl chain length: BAC-C16 < BAC-C14 < BAC-C12 ~ BAC-mix. BAC-C12 and BAC-mix showed comparable effects on the mucosal surface of the slugs. This is consistent with the composition of BAC-mix that contains 60%–70% C12, 30%–40% C14, and less than 5% C16. These results were also confirmed with the *in vitro* BCOP assay in which the following rank order of increasing corneal injury was obtained: BAC-C16 <BAC-C14 < BAC-mix < BAC-C12. However, the concentrations used in both irritation assays are much higher than in pharmaceutical formulations. The aim of the tests was to screen for the irritation potential of the chemicals and to rank the chemicals into different irritation categories. So, the most important conclusion of the mucosal irritation test and the BCOP assay is that the toxicity of the BAC analogues depends on the alkyl chain length.

For the comparative assessment of the antibacterial activities of the BAC analogues with different alkyl chain lengths, four bacterial strains were tested. *S. aureus* and *E. coli* were chosen as representative susceptible Gram positive

Table II. Effect of the Different BAC Analogues on the Corneal Opacity, Permeability, and *In Vitro* Score (IVS = Opacity × 15 Times Permeability) in the BCOP Assay (Mean ± SD)

Treatment	Opacity (A.U.)	Permeability (O.D.)	IVS	n
NaCl 0.9% (w/v)	0.3 ± 0.6	0.005 ± 0.002	0.4 ± 0.6	3
Imidazol 20% (g/g)	86.7 ± 7.5	3.565 ± 0.717	140.1 ± 17.5	3
BAC-C16 20% (g/g)	43.0 ± 4.0	3.088 ± 1.110	89.4 ± 20.3	3
BAC-C14 20% (g/g)	65.4 ± 3.5	3.946 ± 0.407	124.6 ± 3.4	3
BAC-C12 20% (g/g)	148.7 ± 7.5	3.292 ± 0.445	198.1 ± 13.1	3
BAC-mix 20% (g/g)	94.4 ± 6.1	3.763 ± 1.098	150.8 ± 21.0	3

Table III. MICs of BAC Analogues Against *S. aureus, E. coli, P. aeruginosa,* and *B. cepacia*

Antimicrobial agent	MIC (μg/ml)			
	S. aureus	*E. coli*	*P. aeruginosa*	*B. cepacia*
BAC-C16	0.2	25	900	>1000
BAC-C14	0.2	15	500	350
BAC-C12	1.5	50	>1000	750
BAC-mix	0.45	27.5	750	500

and Gram negative species, respectively. *P. aeruginosa* and *B. cepacia* were included because of their reported resistance to quaternary ammonium compounds and their ecological significance, *i.e.,* their possible occurrence, particularly of *P. aeruginosa* in eye drops. The lowest MIC values were noted for *S. aureus,* which is consistent with the known higher activity of quaternary ammonium compounds against Gram positive bacteria (1). The MICs for *P. aeruginosa* and *B. cepacia* were several orders of magnitude higher than those for *S. aureus* and *E. coli,* indicating some degree of intrinsic resistance. However, the relative order of activities among the three BAC analogues was the same, irrespective of the test bacterium, *i.e.,* BAC-C14 ⩾ BAC-C16 > BAC-C12, except for *B. cepacia* where BAC-C12 was more active than BAC-C16. In addition, the BAC-C14 always exhibited higher activity than the BAC-mix.

From this study we might suggest further investigation of the use of BAC-C14 as a preservative agent in nasal and ophthalmic formulations. The mucosal irritation test using slugs answers the problem presented by the absence of some factors in simple cell culture systems. The *in vitro* models for evaluating the effect of chemicals on epithelial tissue may be too sensitive because of the lack of a protective mucus barrier. The use of slugs may give an alternative to the use of vertebrates. The different *in vivo* models are often invasive or are based on a subjective scoring system. The mucosal irritation test using slugs is a reproducible quantitative method that is easy to handle because the mucosal surface is located at the outside of the animal. This allows that rapid screening tests can be carried out using simpler toxicity end-points, which have the advantage of not requiring complex test equipment, sophisticated chemical analysis, and a long experimental time.

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