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Antimicrobial and toxicological profile of the new biocide Akacid plus®

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***Objectives*: Akacid plus® is a new member of the polymeric guanidine family of disinfectants. It was especially developed to enhance the antimicrobial activity of this class with significantly less toxicity. The *in vitro* activity of Akacid plus® compared with chlorhexidine digluconate and mupirocin was tested against a total of 369 recent clinical isolates.**

***Methods*: The organisms tested by CLSI reference methods included the following: *Staphylococcus aureus* (98), *Staphylococcus epidermidis* (9), *Bacillus* spp. (2), *Enterococcus faecalis* (32), *Klebsiella* spp. (45), *Enterobacter* spp. (20), *Escherichia coli* (65), *Salmonella* spp. (6), *Shigella* spp. (2), *Yersinia enterocolitica* (1), *Acinetobacter* spp. (4), *Proteus* spp. (7), *Pseudomonas aeruginosa* (59), *Stenotrophomonas maltophilia* (4), *Candida* spp. (10) and *Aspergillus* spp. (7). *In vitro* selection of resistance to Akacid plus® was carried out on 24 strains. Toxicological analyses were also performed.**

***Results*: All tested agents were more effective against *Staphylococcus* spp. and *Bacillus* spp. than against *E. faecalis* and Gram-negative bacteria. The MIC_{90}s of chlorhexidine and mupirocin showed a 4-fold and 32-fold increase for methicillin-resistant *S. aureus* in comparison with methicillin-susceptible strains, while MIC values of Akacid plus® were similar for antibiotic-susceptible and multiresistant strains. Bactericidal action of Akacid plus® was observed at 1–2× MIC. The *in vitro* selection of resistance test showed no increase in MIC values of Akacid plus® for any isolate after 30 passages. In addition, Akacid plus® showed low oral and dermal toxicity.**

***Conclusions*: These preliminary results demonstrate the broad antimicrobial properties of Akacid plus®, which makes it a promising tool for topical application in the prophylaxis and treatment of bacterial and fungal infections.**

Keywords: bactericidal, resistance, toxicity

Introduction

The discovery and application of antimicrobial chemotherapy and the use of biocides in the form of antiseptics and disinfectants, particularly in the latter half of the twentieth century, allowed control over most infectious diseases. The emergence of bacterial resistance to antimicrobial agents began shortly after their introduction to clinical practice and has developed rapidly and increasingly throughout the 1990s.[1]

Biocides are clearly different from antibiotics in their mode of action, in their condition of use and in their respective acquired and intrinsic mechanisms by which bacteria resist their toxic effects, and they often display non-specific killing. In the face of multiresistant infectious-disease organisms that are difficult and, sometimes, impossible to treat, the search for new agents that do not select for resistant clones becomes ever more important.[2]

However, this issue has been further complicated by the finding that, as for antibiotics, intensive exposure of hospital pathogens to biocides may result in the emergence of resistance to these agents. Evidence for reduced susceptibility to biocides from exposure to these agents has been both laboratory based[3] and observed in the field.[4]

Akacid plus® is a new member of the polymeric guanidine family of disinfectants. It was especially developed to enhance the antimicrobial activity of this class with significantly less

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toxicity. This paper evaluates the antimicrobial profile of Akacid plus® in comparison with chlorhexidine digluconate (due to its widespread use) and mupirocin [due to its topical use in the hospital setting against methicillin-resistant *Staphylococcus aureus* (MRSA)], its toxicity and the potential for induction of resistance to Akacid plus®.

Materials and methods

Bacteria

A total of 369 recent clinical isolates were tested from patients with documented infections in hospitals located in Austria. The distribution of species and strain counts was as follows: methicillin-susceptible *S. aureus* (MSSA) (36); MRSA (62); methicillin-resistant *Staphylococcus epidermidis* (MRSE) (9); vancomycin-susceptible *Enterococcus faecalis* (27); vancomycin-resistant *E. faecalis* (VRE) (5); *Klebsiella* spp. (45, 15.5% ESBLs); Enterobacter spp. (20); *Escherichia coli* (65, 13.8% ESBLs) *Salmonella* spp. (6), *Shigella* spp. (2); *Yersinia enterocolitica* (1); *Acinetobacter* spp. (4); *Proteus* spp. (7); *Pseudomonas aeruginosa* (59, 28.8% ESBLs); *Stenotrophomonas maltophilia* (4); *Candida* spp. (10); *Aspergillus* spp. (7). Identifications were performed using the API system. In addition, *Bacillus subtilis* (spore suspension for the inhibitor test, Merck) and *Bacillus anthracis* CH10 (anthrax spores Merck reg. no. G112/WET/ACT 36/47) were tested.

Active substances

A stock solution of Akacid plus®, a 3:1 mixture of poly-(hexamethylen-guanidinium-chloride) and poly-[2-(2-ethoxy)-ethoxyethyl)-guanidinium-chloride] (Ch. 1007, POC), as 25% aqueous solution was used and diluted with sterile distilled water to the desired concentrations. Chlorhexidine digluconate 20% (Sigma, St Louis, MO, USA) and mupirocin powder (Smith Kline Beecham, London, UK) were selected as reference substances.

Susceptibility testing

To assess the antimicrobial activity of Akacid plus® in comparison with chlorhexidine and mupirocin, MICs were determined using the CLSI broth microdilution method with Mueller–Hinton broth.[5] For fungal testing 3-(N-morpholino)propanesulfonic acid-buffered RPMI 1640 medium was used.[6,7] MIC endpoints were read as the lowest concentration of antimicrobial that totally inhibited macroscopically visible growth of the inoculum. Quality control was provided by the concurrent testing of ATCC strains. MBCs of Akacid plus® were determined by methods published by the CLSI.[8] All susceptibility tests were performed in duplicate.

Killing curves for Akacid plus® were carried out on *S. aureus* ATCC 29213 and *E. coli* ATCC 35218. Concentrations of Akacid plus® at 0.5×, 1×, 2× and 4× MIC were used and monitored at time point 0 and at 5 min, 30 min, 2 h, 6 h and 24 h. Three independent experiments were performed per strain.

In vitro selection of resistance

In vitro selection of resistance to Akacid plus® was carried out on 24 strains: MSSA (1), MRSA (2), MRSE (4), VRE (5), *Klebsiella* spp. (2), *E. coli* (4, 50% ESBLs), *P. aeruginosa* (4, 50% ESBLs) and *Acinetobacter* spp. (2 strains). The broth selection method described by Markopoulos *et al.*[9] was used for the experiments. Thirty passages of each test isolate were performed. All tests were performed in triplicate for each isolate. If the three replicates differed at the end of all cycles, the highest MIC was taken as the result.

Toxicological studies

The toxicological studies were performed at the Toxicology Department of ARC Seibersdorf Research GmbH (Seibersdorf, Austria). The approval numbers for the animal experiments are LF1-TVG-5/025-2002 and LF1-TVG-5/024-2002.

The acute toxic effects of Akacid plus® after a single peroral administration to rats were determined according to EU method B.1.[10] Initially the study was carried out with one group consisting of three female animals given a dose of 200 mg of active ingredient per kg of body weight. Based on these observation results the dose was increased to 2000 mg/kg of body weight. All rats were killed by inhalation of CO_2 on day 14 and subjected to a gross necropsy examination.

The acute toxic effects of Akacid plus® after a single dermal administration to rats were investigated according to EU method B.3.[11] Akacid plus® at a dose of 2000 mg/kg of body weight was administered once dermally on an area of $\sim 5 \times 6$ cm on the dorsal thoracal region of five male and five female CRL:CD(SD) BR Sprague Dawley rats from Charles River Wiga (Germany) and the duration of the exposure was 24 h. They were killed by inhalation of CO_2 after 14 days and subjected to a necropsy including a gross pathological examination.

To examine a possible irritation or corrosion by Akacid plus® following a single application to the intact skin of rabbits the EU method B.4[12] was performed. The test substance (1.5 g) was spread on cellulose patches in a size of about 2.5×2.5 cm and was applied to the intact skin of each of three female New Zealand White rabbits from Charles River Wiga. At the end of the exposure period (4 h) the dressings and the patches were removed. The skin was examined for erythema/eschar and oedema as well as for other local alterations 1, 24, 48 and 72 h after patch removal.

Results

Antimicrobial activity

Table 1 illustrates the activity of Akacid plus® in comparison with chlorhexidine digluconate and mupirocin against ATCC strains and clinical bacterial and fungal isolates. MIC values of chlorhexidine digluconate and mupirocin were comparable to the results obtained by other studies.[4] Akacid plus® showed good activity against staphylococci with MICs of 0.06–0.5 mg/L, regardless of their susceptibility to oxacillin. The MIC_{90}s of chlorhexidine and mupirocin showed a 4-fold (0.5 to 2 mg/L) and 32-fold (0.25 to 8 mg/L) increase for MRSA in comparison with methicillin-susceptible strains. All tested agents achieved lesser activity against *E. faecalis* (2–128 mg/L), but no difference in the MIC values was detected for vancomycin-susceptible *E. faecalis* and VRE. Potent activity was also observed regarding inhibition of spore germination of *B. subtilis* and *B. anthracis*. All tested substances were less active against Gram-negative bacteria. The testing of clinically relevant fungal species of *Candida* and *Aspergillus* furthermore proved the antifungal efficacy of Akacid plus® and confirmed that of chlorhexidine.

Ten strains, including CLSI quality control strains and clinical isolates of *S. aureus*, *E. faecalis*, *S. pneumoniae*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* were tested to compare Akacid plus® MIC and MBC results. MBC values of Akacid plus® were observed at 1–2× MIC. Killing curves were also carried out using Akacid plus® concentrations at 0.5×, 1×, 2× and 4× the measured organism MIC. Killing curves for *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 (inoculum 10^6 cfu/mL) are given in Figure 1

Table 1. MICs of Akacid plus® (AP), chlorhexidine digluconate (CHG) and mupirocin (MUP) for clinical strains of bacteria (352), fungi (17) and spores (2)

Species (no. of strains tested)	MIC	(mg/L) AP	CHG	MUP
MSSA (36)	range	0.06–0.5	0.06–1	0.06–1
	MIC_{50}	0.125	0.25	0.125
	MIC_{90}	0.25	0.5	0.25
MRSA (62)	range	0.06–0.5	0.5–2	0.06–>256
	MIC_{50}	0.125	2	0.125
	MIC_{90}	0.25	2	8
MRSE (9)	range	0.06–0.25	0.5–2	0.25–0.5
E. faecalis (27)	range	2–16	2–16	32–128
	MIC_{50}	8	8	64
	MIC_{90}	16	8	64
VRE (5)	range	4–16	4–16	32–64
Spores of *B. subtilis* (1)	range	0.125	1	1
Spores of *B. anthracis* (1)	range	0.125	1	1
E. coli (65)	range	1–8	2–8	128–256
	MIC_{50}	2	2	128
	MIC_{90}	4	8	256
Klebsiella spp. (45)[a]	range	1–8	4–32	32–>256
	MIC_{50}	2	8	256
	MIC_{90}	8	16	>256
Enterobacter spp. (20)[b]	range	1–8	8–32	128–>256
	MIC_{50}	2	8	256
	MIC_{90}	8	32	>256
P. aeruginosa (59)	range	4–32	8–32	32–>256
	MIC_{50}	8	16	>256
	MIC_{90}	32	32	>256
Proteus spp. (7)[c]	range	4–32	8–64	256–>256
Salmonella spp. (6)[d]	range	1–2	2–4	128–256
Shigella spp. (2)[e]	range	2–4	1–2	128–256
Y. enterocolitica (1)	range	2	32	256
Acinetobacter spp. (4)[f]	range	1–8	2–32	32–>256
S. maltophilia (4)	range	8–32	16–32	256–>256
Candida spp. (10)[g]	range	0.125–4	1–16	32–256
Aspergillus spp. (7)[h]	range	1–16	8–64	64–>256

[a]Includes *Klebsiella pneumoniae*, *Klebsiella oxytoca*.
[b]Includes *Enterobacter aerogenes*, *Enterobacter cloacae*.
[c]Includes *Proteus mirabilis*, *Proteus vulgaris*.
[d]Includes *Salmonella enteritidis*, *Salmonella typhimurium*.
[e]Includes *Shigella sonnei*, *Shigella flexneri*.
[f]Includes *Acinetobacter baumannii*, *Acinetobacter lwoffii*.
[g]Includes *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*.
[h]Includes *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*.

(lower detection limit 5×10^1 cfu/mL). Akacid plus® at ≥2× MIC and 1× MIC eradicated *S. aureus* and *E. coli* within 2 and 5 h.

In vitro selection of resistance

For this test not only susceptible ATCC strains but also multiresistant clinical isolates of Gram-positive and Gram-negative organisms were used. There was no increase in MIC values of Akacid plus® for any isolate after 30 passages.

Toxicological studies

The oral and dermal LD_{50} of Akacid plus® in rats was found to be above 2000 mg of active ingredient/kg of body weight. After a single oral administration of Akacid plus® at a dose of 200 mg/kg of body weight to female rats, all animals survived and no abnormalities in life were revealed from day 1 until the end of the observation period on day 14. One female and one male rat died on account of the treatment with 2000 mg/kg. The necropsy revealed no pathological abnormalities with exception of animals no. 4 and no. 8. These rats showed light lungs, a flat liver and spleen, and light mucous membranes. After a single dermal administration of Akacid plus® at a dose of 2000 mg/kg of body weight all animals survived until the scheduled termination of the study and no toxic effects of the test substance were noted in life. Body weights and body weight gain were inconspicuous during the whole study in all rats, and all animals were normal at the terminal necropsy.

In the acute dermal irritation/corrosion study with rabbits, no general toxic effects of Akacid plus® were observed and all exposed skin sites were normal at each examination term.

Discussion

The present study demonstrates the broad antimicrobial profile of Akacid plus® in comparison with chlorhexidine, another of the family of cationic antimicrobials, and mupirocin, an antibiotic with high activity against Gram-positive pathogens. MIC values of chlorhexidine digluconate and mupirocin were comparable to the results obtained by other studies.[4] Previous studies by Irizarry *et al.*[13] and Suller and Russell.[14] detected MRSA strains to be less susceptible than MSSA strains to chlorhexidine, triclosan and quaternary ammonium compounds. Likewise, Kresken *et al.*[15] observed mupirocin resistance almost exclusively in methicillin-resistant strains of *Staphylococcus* spp. In the present work the MIC_{90}s of chlorhexidine and mupirocin showed a 4-fold and 32-fold increase for MRSA in comparison with methicillin-susceptible strains, while MIC values for Akacid plus® were similar for both MRSA and MSSA. Recently, we have evaluated bactericidal activity of Akacid plus® 0.1% after exposure for 5 min in basic quantitative suspension tests against quality control strains of *S. aureus*, *Enterococcus hirae*, *E. coli* and *P. aeruginosa*.[16] Additionally, we have shown potent activity of nebulized Akacid plus® 0.5% for eradication of antibiotic-susceptible and multiresistant *S. aureus*, *P. aeruginosa* and *E. coli* on hard surfaces.[17] In the absence of neutralizing solution and presence of Akacid plus® bacterial cells of *S. aureus* ATCC 29213 and *E. coli* 35218 were eliminated at 1× MIC within <5 h. A multiple of the MIC of Akacid plus® accelerated the eradication of the exposed bacteria.

The increasing use of biocides has also raised concerns about the development of biocide resistance. In the present study we were not able to induce bacterial resistance to Akacid plus®. Exposure of subinhibitory concentrations did not result in reduced susceptibility of *Staphylococcus* spp., *Klebsiella* spp., *E. coli*, *P. aeruginosa* and *Acinetobacter* spp. In contrast, Markopoulos *et al.*[9] showed significant increases in MICs of teicoplanin for *S. epidermidis* after broth and agar selection methods.

Up to now it was a well-accepted fact that biocidal activity comes at a price; that is to say that high activity equals high toxicity. Chlorhexidine, which is registered as a disinfectant and

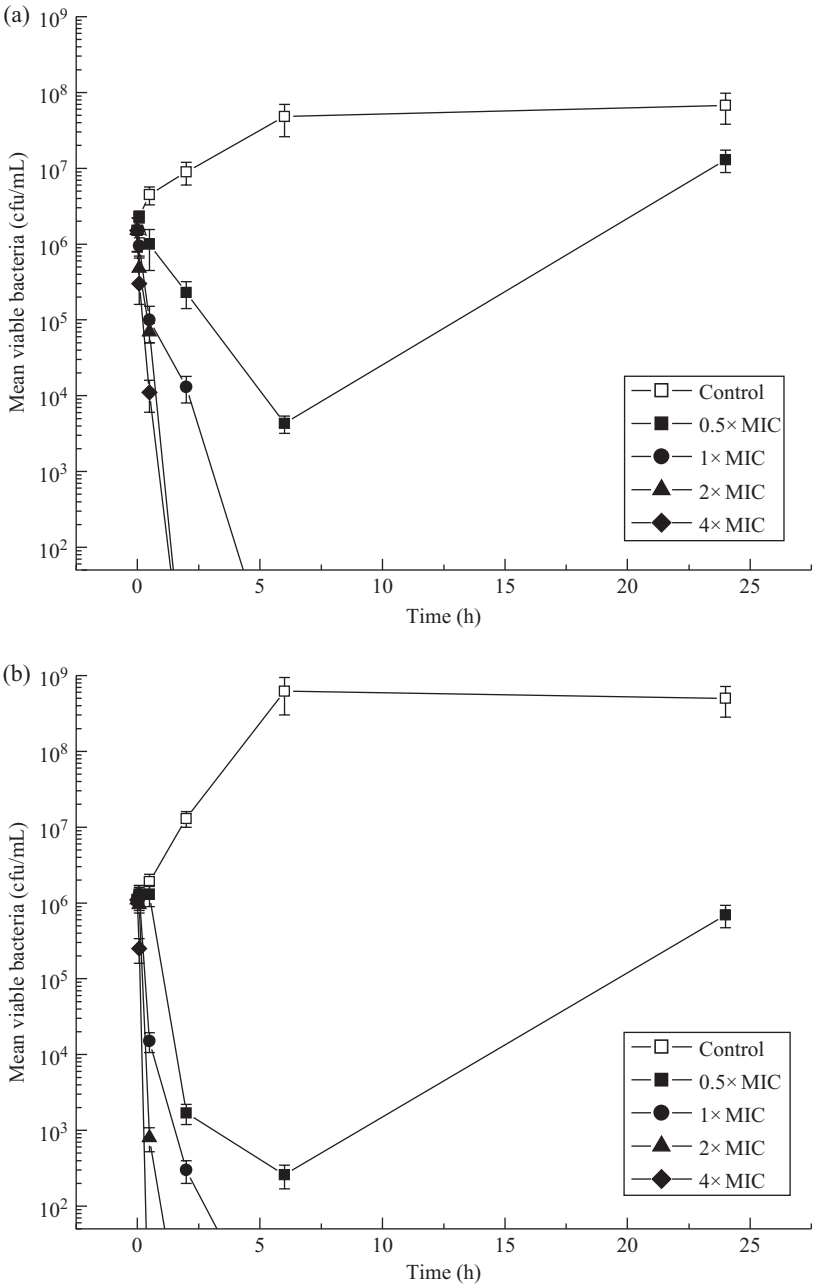

Figure 1. Time–killing curves for Akacid plus® versus *S. aureus* ATCC 29213 (MIC, 0.5 mg/L) and *E. coli* ATCC 35218 (MIC, 2 mg/L). Mean viable bacterial count (cfu/mL) of *S. aureus* (a) and *E. coli* (b) was evaluated in the presence and absence of Akacid plus® at 0.5×, 1×, 2× and 4× MIC at 5 min, 30 min, 2 h, 6 h and 24 h.

is used as a preservative in cosmetics and as a surgical rub, is irritating to the eyes. According to the results obtained in the toxicological studies, Akacid plus® showed a low acute oral and dermal toxicity with an LD_{50} > 2000 mg/kg of body weight (a concentration high above the therapeutic dose) and was not irritating to the skin. Further toxicity studies including acute eye toxicity, skin sensitization, mutagenicity and chronic exposure are needed to determine the complete toxicity profile of Akacid plus®.

The preliminary results of the present study demonstrate the broad antimicrobial properties, also against MRSA and ESBL-producing Gram-negatives, which make Akacid plus® a promising tool for topical application in the prophylaxis and treatment of bacterial and fungal infections. No difference in the MIC values between MSSA and MRSA was detected. Since the exact mechanism of action of Akacid plus® is not fully understood yet, further tests are underway to study the mode of action and full range of activity of this promising new substance.

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Transparency declarations

None to declare.

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