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Antimicrobial Activities of *N*-Chloramines and Diazolidinyl Urea

CARLOS M. LLABRES AND DONALD G. AHEARN*

Laboratory for Microbial and Biochemical Sciences, Georgia State University, Atlanta, Georgia 30303

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A combination of MICs of an *N*-chloramine, a simple chlorinated amino acid, and diazolidinyl urea gave synergistic activity against bacteria, but not fungi. The two compounds at a higher concentration, 0.1 and 0.3%, respectively, gave synergistic inhibition of fungi; kill times were 1 h for *Trichophyton tonsurans*, 3 h for *Aspergillus niger* and *Fusarium moniliforme*, and 6 h for *Aspergillus fumigatus*.

N-Chloramines have been used as antimicrobial agents in such commercial products as laundry bleaches and industrial sanitizing compounds and in the disinfection of sewage (21). Their inhibitory activities are of a broad spectrum, including interference with both glucose oxidation and ionic exchange and the disruption of the cell membrane (9, 16). Recently Selk et al. (20) recommended *N*-chloramine compounds for use in topical drugs for humans as an alternative to chlorhexidine. Two *N*-chloramine compounds, a chlorinated simple amino acid (II-A) and a chlorinated half-ester of succinic acid (III-A), both at concentrations of 0.1% or less, reduced bacterial inocula of 10^4 cells per ml in buffered sodium acetate (pH 4.5) to nearly 0 in 15 min. These compounds were reported to have a lower toxicity and to be 10 times more efficient than chlorhexidine (20).

Imidazolidinyl urea compounds have been used as preservatives in cosmetic products such as mascaras and eyeliners (6). The antimicrobial properties of imidazolidinyl urea compounds are based on their proposed mechanism of protein alkylation of sulfhdryl groups and their ability to release formaldehyde (5). These compounds have been recommended as effective preservatives against pseudomonads (3). Berke and Rosen (3) reported that imidazolidinyl urea in combination with parabens gave synergistic inhibition of the growth of *Pseudomonas aeruginosa*. Elder (10) indicated that imidazolidinyl urea is safe when incorporated in cosmetics because of its low toxicity.

A recently developed compound, Germall II, 5[1,3 bis(hydroxymethyl)ureido]-1,3-bis(hydroxymethyl)imidazol-2,5(3H,5H)dione, designated as diazolidinyl urea (DZU) by the Cosmetic Toiletry and Fragrance Association, has been reported to be an active antimicrobial agent with no sensitizing effect on guinea pigs (3). Little information is available on the antifungal properties of the above compounds. Currently available ophthalmic solutions show relative inefficacy against fungi such as *Aspergillus fumigatus* and *Candida albicans* (18). Inhibitory compounds such as mercury compounds, organotins, arsenic compounds, and phenolic compounds can be highly toxic to the mucosa and epidermal tissues (4, 16). The need for nontoxic antimicrobial agents with activity against fungi has prompted our study of the antimicrobial effectiveness of the DZU compound in combinations with *N*-chloramines II-A and III-A.

MATERIALS AND METHODS

The formulae of DZU (lot GT-108; Sutton Laboratories Inc.), and II-A and III-A (IRx 1767 and IRx 1775, respectively; INTERx, a subsidiary of Merek & Co., Inc., Merck Sharp & Dohme) are indicated in Fig. 1.

Test organisms were *Staphylococcus epidermidis* ATCC 17917, *P. aeruginosa* ATCC 15442, *Serratia marcescens* ATCC 14041, *A. fumigatus* ATCC 10894, *Candida lipolytica* CDC 32750 (obtained from a cleanser containing chlorhexidine), *Fusarium solani* ATCC 81015, *Fusarium moniliforme* CU-1, and *Trichophyton tonsurans* GSU768.

All microorganisms were grown at room temperature (25 to 27°C). Molds were grown in 2-liter flasks containing 100 ml of potato dextrose agar (Difco Laboratories), for a period of 7 days. Yeasts were grown in Sabouraud dextrose broth for 24 to 48 h, and bacteria were grown in tryptic soy broth (Difco) for 24 h.

Bacteria and yeasts were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS; NaCl [8.0 g], Na_2HPO_4 [0.91 g], KCl [0.20 g], and KH_2PO_4 [0.12 g] in 1,000 ml of deionized water with the pH adjusted to 7.0); 20 ml of PBS was added to the flasks containing the fungal cultures. The flasks were swirled vigorously, and the resulting suspensions were decanted through a glass wool filter that retained most hyphal elements (less than 5.0% of inocula), but not the conidiospores. The conidiospore or cell suspensions used as challenge inocula were adjusted to give a final inoculum of 10^6 propagules per ml in PBS by comparison with established spectrophotometric curves. The

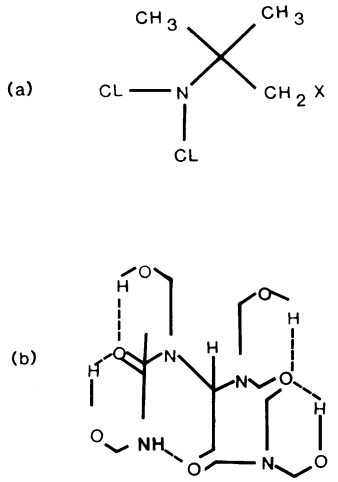

FIG. 1. (a) *N*-Chloramines: II-A, X = CO_2H; III-A, X = $O_2C(CH_2)_2CO_2H$. (b) DZU.

* Corresponding author.

TABLE 1. Survival of microorganisms in preservatives[a]

Preservative	Concn (%)	*P. aeruginosa*		*S. marcescens*		*S. epidermidis*		*C. lipolytica*		*A. fumigatus*	
		D-Value[b]	Kill time[c]	*D*-Value	Kill time	*D*-Value	Kill time	*D*-Value	Kill time	*D*-Value	Kill time
DZU	0.3	86 min	6 to 24 h[d]	67 min	6 to 24 h[d]	42 min	5 h	>24 h	>24 h	2 h	24 h
II-A	0.1	<1 min	<1 min	<1 min	1 min	<1 min	1 min	5 min	1 h	2 h	24 h
	0.01	48 min	4 h	11 min	1 h	13 min	1 h	—[e]	—	>24 h	>24 h
	0.005	90 min	>24 h	2 h	>24 h	20 min	2 h	>24 h	>24 h	>24 h	>24 h
III-A	0.1	1 min	30 min	7 min	30 min	>1 min	3 min	—	—	>24 h	>24 h
	0.05	6 min	1 h	7 min	30 min	16 min	1 h	—	—	>24 h	>24 h

[a] Solutions prepared in PBS (pH 7.0).
[b] Time required for a 90% reduction of the cell population (1 log reduction).
[c] Time required to reduce inoculum to <1 propagule per ml.
[d] Some cells survived the first measurement period, none at second.
[e] —, Not done.

actual inoculum density was established from colony counts after the inoculation of pour plates with the cell suspensions.

Compounds initially were evaluated for antimicrobial activity at pH 7.0 and at room temperature (25 to 27°C). The maximum concentrations tested were those recommended in the literature: DZU at 0.3% (3), III-A at 0.1%, and II-A at 0.1% (20). After the antimicrobial activity for each individual compound had been established, MICs of the compounds were combined and tested at various pHs with and without organic soil. The organic soil consisted of heat-killed cells of *Saccharomyces cerevisiae* ATCC 560, 10^6/ml, in fetal bovine serum added to give final concentrations from 1 to 5% (vol/vol).

The inhibitory compounds, dissolved in PBS (pH adjusted to 7.0), were inoculated and sampled at the indicated times, routinely, 0 time, 1 min, 15 min, 1 h, 3 h, 6 h, 24 h, 48 h, 72 h, 96 h, and 14 days and intermittently to 52 days. As a standard procedure the inoculated test solutions (10 ml in 125-ml Erlenmeyer flasks) were incubated in a water bath shaker (75 rpm) at 25 to 27°C. Samples (0.5 ml) were taken from the challenged solutions at the indicated times and were serially diluted from 10^{-1} to 10^{-5} in Dey-Engley neutralizing broth (Difco). Preliminary studies indicated that Dey-Engley broth gave better recovery of fungi than did Sabouraud broth; previously Dey-Engley broth was shown to be efficacious for bacteria (18). Duplicate pour plates of tryptic soy agar (10 ml/plate) (Difco) for bacteria and of Sabouraud dextrose agar (10 ml/plate) (Difco) for molds and yeasts were inoculated with 1.0 ml of each of the Dey-Engley broth dilutions. The dilutions in neutralizing broth were incubated on a roller drum (75 rpm) at 25 to 27°C, and the plates were incubated at 27°C for bacteria; 37°C was employed for the incubation of *A. fumigatus*, *A. niger*, and *F. moniliforme*. Developing colonies of bacteria and yeasts were enumerated at 24 h. All mold colonies were counted at 48 h and intermittently up to 14 days. The challenged solutions were sampled for up to 2 weeks after the recorded kill time, and both recovery broths and agar media were incubated for up to 2 weeks. The survival of all inocula was monitored in PBS without preservatives until kill times in preservatives were recorded.

D-Values were determined at room temperature (25 to 27°C) by procedures adopted from the literature (7, 18, 19) and Food and Drug Administration guidelines (Microbiological guidelines for hydrophilic contact lenses, May 1983, unpublished). The *D*-values were calculated from the Stumbo equation [D = time/(log a − log b)], which estimates the time required for a 90% reduction of the microbial population (22). The kill time was defined as the time required to reduce the inoculum to less than 1 propagule per ml. Both plate counts and growth in the Dey-Engley broth dilutions were considered in the *D*-value calculations.

RESULTS

The relative susceptibilities of selected microorganisms to the inhibitory compounds are presented in Table 1. In PBS alone all inocula maintained essentially the same densities or showed slight increases for the indicated kill times. Fungi were more resistant than bacteria. *Candida lipolytica* survived in a solution of DZU at 0.3%; 10^5 to 10^6 cells per ml were recovered at 48 h. In II-A at 0.1% the inoculum of *C. lipolytica* was reduced to less than 1 propagule per ml within 1 h. At these same concentrations, 10^3 to 10^4 propagules of

TABLE 2. Effect of pH and organic soil on inhibition of microorganisms by a combination of preservatives[a]

Organism	With PBS		With organic soil[b]					
			pH 7.0		pH 4.5		pH 9.0	
	D-Value[c]	Kill time[d]	*D*-Value	Kill time	*D*-Value	Kill time	D-Value	Kill time
S. marcescens	23 min	3 h	16 min	3 h	9 min	3 h	37 min	4 h
P. aeruginosa	11 min	1 h	48 min	3 h	3 min	1 h	72 min	24 h
C. lipolutica	—[e]	—	22 min	1 h	—	—	—	—
A. fumigatus	>24 h	>24 h	>24 h	>24 h	—	—	—	—

[a] DZU (0.015%) plus II-A (0.005%) in PBS (original inoculum level of 10^6 propagules per ml).
[b] Organic soil contained 5% fetal bovine serum and 10^6 heat-killed cells of *S. cerevislae* ATTC 560 per ml.
[c] Time required for 1 log reduction.
[d] Time required to reduce inoculum to <1 propagule per ml.
[e] —, Not done.

TABLE 3. Survival of fungi in preservatives[a]

Preservative	Concn (%)	*A. fumigatus*		*A. niger*		*T. tonsurans*		*F. moniliforme*		*F. solani*	
		D-Value[b]	Kill time[c]	*D*-Value	Kill time	*D*-Value	Kill time	*D*-Value	Kill time	*D*-Value	Kill time
DZU	0.3	2 h	24 h	>24 h	>24 h	—[d]	—	—	—	>24 h	>24 h
II-A	0.1	2 h	24 h	3 h	24 h	—	—	3 h	24 h	3 h	24 h
DZU–II-A	0.3–0.1	40 min	>6 h	20 min	<3 h	1 min	<1 h	20 min	<3 h	40 min	<6 h

[a] Solutions were prepared in PBS (pH 7.0) and fortified with 10^6 heat-killed cells of *S. cerevisiae* ATCC 560 per ml in 5% fetal bovine serum.
[b] Time required to reduce inoculum by 90% (1 log reduction).
[c] Time required to reduce inoculum to <1 propagule per ml.
[d] —, Not done.

A. fumigatus were still viable at 6 h after treatment with either DZU or II-A. At any concentration tested, *N*-chloramine III-A was not effective against *A. fumigatus*.

The combination of II-A (0.05%) and DZU (0.015%) (both at dilutions below their recommended concentrations) gave approximately the same antimicrobial activity as that of III-A by itself (data not shown). Overall, II-A seemed to be the most active inhibitor for the organisms tested.

The combination of II-A (0.005%) and DZU (0.015%) at neutral and acid pHs was synergistic against *P. aeruginosa* and *S. marcescens* (Table 2). This synergy was indicated by up to a four- to fivefold increase in activity of the combination over that of the individual compounds at pH 7.0 (Tables 1 and 2). Even this enhanced activity was increased at pH 4.5. Alkaline pH appeared to lessen microbicidal activity. Synergy was indicated also for *C. lipolytica*, since neither DZU nor II-A at a concentration below 0.01% individually gave the 90% inhibition obtained at 24 h with the combination. Although the dilute combination did not reduce recoverable numbers of *A. fumigatus* within 24 h, the conidia did not germinate in the combination as they did in the PBS. Furthermore, recoverable colonies decreased gradually in number with time; no colonies were recovered at 7 weeks, whereas in the control flask of PBS, 10^4 cells per ml were obtained. Storage of the combination for 6 months at 5°C did not change the *D*-values for *S. marcescens*, but by 10 months inhibitory activity was reduced from >99.9% to ≤90% of a challenge inoculum after 24 h of exposure.

In the presence of organic enrichment, the combination of higher concentrations of DZU (0.3%) and II-A (0.1%) challenged with *A. fumigatus* yielded a threefold increase in inhibitory activity (based on reduction in *D*-value) over that observed with the individual compounds (Table 3). The combination gave a ninefold increase in activity for *A. niger* and *F. moniliforme* when compared with the activity of II-A by itself.

DISCUSSION

Based on its cidal properties, II-A was the most effective antimicrobial agent. Its activity was enhanced at an acid pH and decreased at pH 9.0. These effects of pH on activity are in agreement with the observations of Hedgecock (11), Kaminski et al. (13), and Odlaug (16). At pH 7.0 at concentrations of at least 0.01%, II-A was lethal for bacteria within 4 h, a kill time recommended by Orth (17) as necessary for preservatives suitable for cosmetic use. At a concentration of 0.1%, but not at 0.005%, an organic enrichment had no appreciable effect on microbicidal activity. Selk et al. (20) reported that the loss of activity of *N*-chloramine compounds in the presence of 5% serum was negligible when the concentration of the *N*-chloramine was 1,000 μg/ml or higher. The inhibitory activity of *N*-chloramine, although impressive for bacteria, was less for fungi than the action reported for other disinfectants such as iodine and hydrogen peroxide (18). DZU at 0.3% also produced lethal effects on bacteria within 4 h and was similar to II-A in effectiveness against *A. fumigatus*.

Berke and Rosen (3) reported that DZU at 0.3% in combination with 0.1% propylparaben and 0.2% methylparaben had a kill time for 24 h for *P. aeruginosa* (inoculum of 10^6 cells per ml). This activity was reported over a broad pH range. A similar kill time was obtained in the present evaluation without the addition of the parabens. Also, the addition of organic enrichment did not have a significant effect of the inhibitory activity of DZU. Lawrence and Block (14) implied that synergism is best attributed to enhanced inhibition resulting from the combination of two or more differing agents with differing modes of action. This concept and the concern for toxicity guided us in our study. The synergistic activity of nontoxic antimicrobial agents against gram-negative bacteria such as *P. aeruginosa* could be of significance in the preservation of cosmetics and medicinal preparations. *P. aeruginosa* has demonstrated a development of resistance to many single preservatives, e.g., mercuric acetate (8), benzalkonium chloride (2), formaldehyde (12), and chlorhexidine (15). Species of *Pseudomonas*, *Serratia*, and *Aspergillus* are known to contaminate cosmetics, and such contaminants may be associated with infections (19, 24).

Higher concentrations of II-A and DZU combined gave kill times of less than 6 h for all fungi, even in the presence of organic soil. The combination was especially effective against the dermatophyte *Trichophyton tonsurans* (the etiological agent of epidemic tinea capitis), which was erradicated in less than 1 h. Potential uses of the combination of II-A and DZU could include the disinfection of showers, athletic equipment, industrial equipment, and utensils. New disinfection treatments for habitats infested with *Histoplasma capsulatum* or *Cryptococcus neoformans* via avian and chiropteran vectors are also needed (1, 23). Currently, these habitats are treated with 5% Formalin, a compound that at concentrations of 6 to 15 ppm is known to be carcinogenic for rats (1). Further evaluation of the stability, levels of toxicity, and microbial properties of DZU and II-A as nontoxic and noncorrosive disinfectants is recommended.

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