ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1995, p. 339–342 Vol. 39, No. 2
0066-4804/95/$04.00+0

Amoebicidal Efficiencies of Various Diamidines against Two Strains of *Acanthamoeba polyphaga*

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Received 20 June 1994/Returned for modification 14 October 1994/Accepted 15 November 1994

The first medical cure of *Acanthamoeba* keratitis was obtained by use of propamidine isethionate. Since then, it has been the basic drug recommended for use in treatment. Because some *Acanthamoeba* strains have been reported to be resistant to propamidine and propamidine was found to be only weakly cysticidal, superior homologs such as butamidine, pentamidine, hexamidine, heptamidine, octamidine, and nonamidine were tested for their amoebicidal effects on two *Acanthamoeba* strains isolated from patients with keratitis. Trophozoicidal and cysticidal efficiencies were found to be increased from propamidine to nonamidine; i.e., when the alkyl chain connecting the two benzene rings in their molecular structures was elongated, in comparison with propamidine, hexamidine and octamidine were the most amoebicidal molecules. As a result of these data, a kinetic study carried out on propamidine, hexamidine, and octamidine demonstrated that the amoebicidal effects resulted from two events: the diffusion of molecules through the plasma membrane or the double wall of trophozoites or cysts, respectively, and the lethal effects of molecules on amoebic protoplasm. The diffusion kinetics were increased when the alkyl chain was elongated, i.e., with an increase in the lipophilic properties of molecules. In contrast, the lethal effect kinetics were found to be unchanged by this elongation, indicating that they originated from the cationic surface-active properties induced by the protonated amidine groups attached to each benzene ring, which themselves remained unchanged from one molecule to the other. These results strongly advocate the immediate replacement of propamidine by hexamidine in the medical treatment of *Acanthamoeba* keratitis; in France, 0.1% hexamidine eyedrops are available (Desomedine). The results also advocate clinical investigations on the efficiency and toxicity of octamidine, which appears to be the most amoebicidal diamidine in vitro.

Acanthamoeba keratitis is a serious and potentially devastating corneal infection generally seen in soft contact lens wearers. The responsible amoeba has been detected in the human cornea in two forms: as fragile trophozoites and as resistant dormant cysts. The medical treatment of this ocular infection remains very difficult because of the lack of antiamoebic drugs effective against cysts, which are responsible for frequent and serious recurrences.

Since the first medical cure reported in 1985 (16), propamidine isethionate, an antimicrobial agent belonging to the diamidine family, has been the basic drug recommended for use in treatment, which includes the use of topical neomycin sulfate and various imidazoles (2, 5, 7, 8, 15). Unfortunately, propamidine is poorly cysticidal and the resistance of some *Acanthamoeba* strains has been reported (6, 10). In the present study, superior homologs of propamidine were tested for their amoebicidal effects both on trophozoites and cysts with the intent of finding whether some of them are more amoebicidal and, especially, cysticidal than propamidine. At first, contact times required for 100% killing of trophozoites and cysts were assessed and then the trophozoicidal and cysticidal kinetics of the most effective compounds were evaluated and compared with those of propamidine isethionate, which was used as a reference.

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MATERIALS AND METHODS

Amoeba strains. Two amoeba strains isolated from patients with keratitis were used in the study. They were grown at 30°C on a monoxenic nonnutrient agar (MNA) medium made of 1.5% agar (Bacto Agar; Difco) aseptically spread over with a live *Escherichia coli* suspension. They were assigned to the genus *Acanthamoeba* on the basis of the presence of acanthopodia in slowly moving trophozoites, the use of metamitosis for nuclear division, and the presence of double-walled cysts with polygonal and weakly stellate endocysts. After silver staining and microscopic examination, these cysts exhibited the features of group II defined by Pussard and Pons (11) with an *Acanthamoeba polyphaga*-like morphology. Because the exact species were not yet identified when they were tested, they were distinguished by code letters as *Acanthamoeba* sp. strain Pi and *Acanthamoeba* sp. strain LE.

Chemicals. The chemical formulas and names of the experimental diamidines used in the study are given in Table 1. They are bipolar molecules displaying a lipophilic part consisting of two benzene rings connected by an alkyl chain of various lengths and a hydrophilic chain consisting of a protonated amidine group attached to each ring. Propamidine was provided by May and Baker (Dagenham, United Kingdom), pentamidine was provided by Roger Bellon (Neuilly, France), and hexamidine was provided by Chauvin-Blache (Montpellier, France), while the other diamidines were synthesized by one of us (J. C. Lancelot).

Diamidine solutions were prepared at a concentration five times that desired by dissolution in sterile phosphate-buffered saline (PBS; pH 7.2) and were stored at 4°C until they were used. In experiments, the concentration was adjusted to 100 μg/ml by dilution in a suitable volume of sterile PBS.

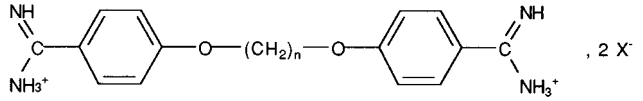
Trophozoite and cyst harvesting. The *Acanthamoeba* strains were grown at 30°C on MNA medium. Trophozoites were collected from 24- to 48-h cultures, and cysts were collected from 3-week cultures. The agar surfaces were flooded with 5 ml of PBS and were gently scraped with an inoculating loop. Trophozoites and cysts were harvested from the suspension by centrifugation at 350 × *g* for 10 min in a C 400-S4 centrifuge (Jouan-France). The supernatant was aspirated, and the sediment was washed twice in PBS in order to eliminate most of the bacteria.

The trophozoites or cysts in the resultant suspension were optically counted with a Malassez hemacytometer with a phase-contrast microscope, and the suspension was standardized to 10^5 trophozoites or cysts per 100 μl.

Procedure for determination of survival times. Experiments and their controls were carried out in 12-ml sterile glass test tubes.

Experimental tubes containing 200 μl of concentrated diamidine solution and

TABLE 1. Structures of experimental diamines used in the study



Name	*n*	X
Propamidine	3	Isethionate
Butamidine	4	Methanesulfonate
Pentamidine	5	Isethionate
Hexamidine	6	Isethionate
Heptamidine	7	Methanesulfonate
Octamidine	8	Methanesulfonate
Nonamidine	9	Methanesulfonate

700 μl of PBS were prepared; control tubes containing only 900 μl of PBS were also prepared. A 100-μl amount of standardized cyst or trophozoite suspension was pipetted into each tube. All of the tubes were sealed with Parafilm (American Can Co.) to prevent evaporation and were incubated at 30°C until they were processed. Every 30 min for 7 h for trophozoites and daily for 14 days for cysts, the viabilities of the organisms in one experimental tube and also in the corresponding control tube were checked by regrowing inocula from the tubes at 30°C on MNA medium. Before regrowth, the inhibitory effect of diamidine was neutralized by diluting the solution 1:1,000 with PBS; the degree of dilution was determined from the results of previous experiments.

The contents of both the experimental and the control tubes were diluted by adding 11 ml of sterile PBS, and the tubes were centrifuged at 350 × *g* for 10 min three times; 11 ml of supernatant was replaced with an equal volume of PBS in which the pellet was resuspended by shaking with a Vortex mixer. After discarding the last supernatant, the pellet was resuspended in the 1 ml of fluid remaining in the bottom of the tube. All of this material was spread onto a plate of MNA medium, and the plate was incubated at 30°C for 3 weeks. The plates were sealed with Parafilm to prevent the agar from drying out, and they were examined daily with an inverted microscope. If no trophozoites were observed at the end of 3 weeks, the inocula of trophozoites or cysts in the corresponding experimental tube were considered nonviable. Experiments and their controls were performed in quintuplicate.

Procedure for assessment of the amoebicidal kinetic effect. Sterile borosilicate flasks were used to assess the amoebicidal kinetic effects. Some flasks were used as inactivation vessels, and others were used as control vessels.

The inactivation vessels contained 24 ml of concentrated diamidine solution and 96 ml of sterile PBS, while the control vessels contained only 120 ml of PBS. A 120-μl amount of a standardized trophozoite or cyst suspension was pipetted into all of the vessels, which were sealed with Parafilm and which were incubated at 30°C; their contents were kept mixed by magnetic stirring (200 rpm). At the beginning of incubation and then each hour over 8 h for trophozoites and twice a day at intervals of 8 h over 4 days for cysts, 13-ml samples were withdrawn and transferred from the inactivation vessels to sterile test tubes.

Concurrently, 13-ml samples were withdrawn from control vessels, but only at the beginnings and the ends of the experiments.

All of the samples were submitted to the previously described centrifugation-dilution cycles, with replacement of 12 ml of supernatant by an equal volume of PBS. After the last supernatant was discarded, the pellet was resuspended in 12 ml of PBS.

The resultant suspension was plated onto a set of 15 plates containing MNA medium, 5 of them were each seeded with 200 μl, 5 others were each seeded with 20 μl, and the last five were each seeded with 2 μl of the suspension. When the number of surviving trophozoites or cysts was expected to be very low (e.g., in samples withdrawn from inactivation reactors near the ends of the experiments), the plated volumes were increased 10-fold. All of the plates were sealed with Parafilm, incubated at 30°C for 3 weeks, and examined daily with an inverted microscope. From the number of positive cultures observed in each set, the most probable number of viable cysts or trophozoites was read on statistical tables corresponding to the plated volumes and published previously (1). Experiments and their controls were performed in quintuplicate.

RESULTS

The arithmetic means of the survival times of trophozoites and cysts found with each diamidine are listed in Tables 2 and 3, respectively.

Comparison of these means by the *t* test indicated that for both the trophozoites and cysts of each tested strain hexamidine, heptamidine, octamidine, and nonamidine were significantly more rapidly amoebicidal than propamidine ($\alpha < 0.01$), while butamidine and pentamidine were not ($\alpha > 0.1$). Likewise, octamidine was significantly more rapidly amoebicidal than heptamidine ($\alpha < 0.05$) and hexamidine ($\alpha < 0.01$), while heptamidine and nonamidine were never more amoebicidal than hexamidine ($\alpha > 0.1$) and octamidine ($\alpha > 0.05$), respectively. In accordance with these results, only the amoebicidal kinetics of octamidine and hexamidine were studied for comparison with that of propamidine, which was used as a reference.

TABLE 2. Survival times of *Acanthamoeba* sp. strain Pi and LE trophozoites in 0.1% diamidine solutions

Diamidine	Survival time (h)[a]			
	Pi		LE	
	Avg	SD	Avg	SD
Propamidine	7	1.26	9	1.26
Butamidine	8	2.52	8	1.41
Pentamidine	7	0.63	7	1.41
Hexamidine	5	0.63	4	1.09
Heptamidine	5	0	5	0.63
Octamidine	2	0.63	2	0.63
Nonamidine	3	1.26	3	0.63

[a] Averages and standard deviations were calculated from five experiments.

Amoebicidal kinetics curves are shown in Fig. 1. They result from semilogarithmic plots of the fraction N/N_0 of surviving trophozoites or cysts against contact time (with N_0 and N being the arithmetic mean most probable number of viable organisms [in quintuplicate] initially and at each withdrawal time, respectively).

For a given contact time, the mean surviving fractions recorded for propamidine, hexamidine, and octamidine were significantly different ($\alpha < 0.01$) except for cysts at the eighth hour and only between propamidine and hexamidine ($\alpha > 0.1$). Moreover, in controls no significant difference was found between the most probable number of viable forms initially and at the end of the experiments ($\alpha > 0.1$). The curves are not linear but consist of two segments, each with its own slope, indicating that the amoebicidal effects recorded in Tables 2 and 3 result from the development of two events with different kinetics. Curves of this type are encountered when delays in the diffusion of molecules are required to allow the lethal concentrations to be reached.

Here, the slope and the length displayed by initial segments vary from one diamidine molecule to the other, indicating that the kinetics of diffusion are modulated in accordance with the

TABLE 3. Survival times of *Acanthamoeba* sp. strain Pi and LE cysts in 0.1% diamine solutions

Diamidine	Survival time (days)[a]			
	Pi		LE	
	Avg	SD	Avg	SD
Propamidine	6	0.63	7	1.09
Butamidine	7	1.26	7	0.63
Pentamidine	5	0.63	6	1.26
Hexamidine	3	0.63	3	0.63
Heptamidine	3	0.89	3	1.41
Octamidine	1	0	1	0
Nonamidine	2	0.63	2	1.09

[a] Averages and standard deviations were calculated from five experiments.

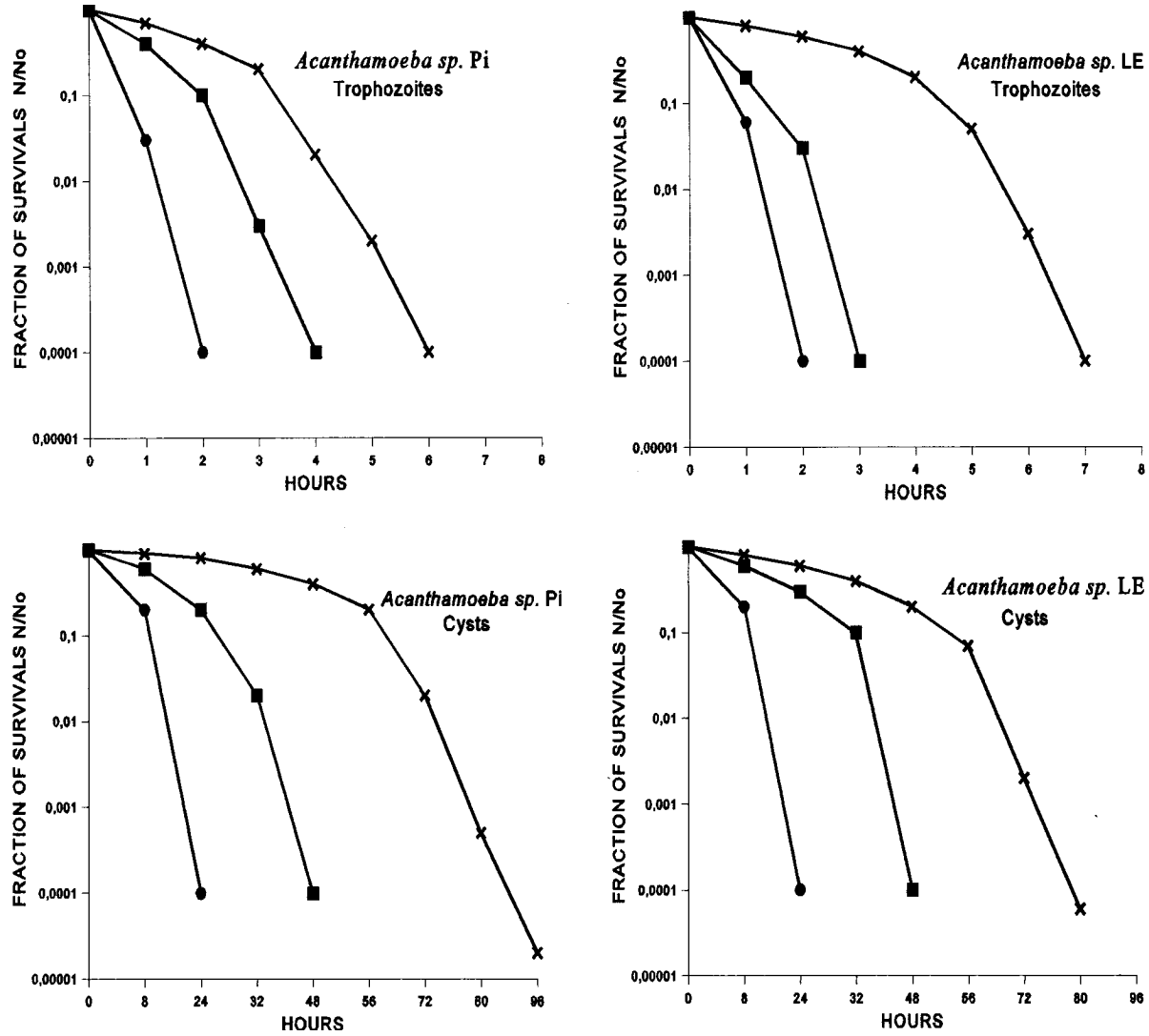


FIG. 1. Kinetic curves of amoebicidal effects on trophozoites and cysts of *Acanthamoeba* sp. strain Pi and *Acanthamoeba* sp. strain LE obtained with 0.1% propamidine (×), 0.1% hexamidine (■), and 0.1% octamidine (●). These curves are based on data derived from five experiments.

molecular structures of the diamidines. The slopes of the terminal segments, which are kept nearly parallel, indicate that the exponential kinetics of the typical lethal effects on amoebic protoplasms remain the same for each diamidine.

Consequently, it appears that, with regard to the amoebicidal effect of propamidine, the best efficiency, recorded with octamidine and hexamidine (Tables 2 and 3), is due to their higher diffusion kinetics rather than an increase in lethal effect kinetics.

DISCUSSION

Diamidines, a group of compounds of which propamidine, hexamidine, and octamidine are members, are well known for their antimicrobial effects resulting from the cationic surface-active properties generated from the bipolar structures of the molecules.

The lethal effects result from interactions of the protonated amidine groups attached to each benzene ring with the amphipathic lipids of the plasma membrane bilayer of amoebae, inducing structural changes that lead to modifications of cell permeability which could be responsible for the leakage of ions, water, and various biomolecules. Moreover, when these molecules have penetrated into the amoebic cytoplasm, coagulation and denaturation of cytoplasmic proteins and enzymes can occur because of the basicities of amidine groups.

Nevertheless, such penetration across the amoebic cell membrane reflects the lipophilic properties that are conferred by the alkyl chain connecting the benzene rings.

It is interesting that, both for trophozoites and cysts, the initial segments were shorter and their slopes were steeper from propamidine to octamidine, indicating that the kinetics of diamidine diffusion are modulated by the length of the alkyl chain and increase as the propyl chain is progressively elongated to a hexyl chain and an octyl chain. This elongation increases the lipophilic properties of molecules and makes it easier for them to be incorporated into the lipid bilayer of the amoebic plasma membrane, allowing the threshold of the lethal concentration required to generate amoebicidal effects to be reached more rapidly.

Thus, it is understandable that the kinetics of diffusion recorded for cysts remain lower than those for trophozoites be-

cause molecules must pass through the double wall before being incorporated into the plasma membrane. Nevertheless, it is more difficult to understand why the increasing in lipophilic properties accelerates the diffusion through this double wall consisting mainly of cellulose (3, 9, 13, 14), a hydrophilic compound.

The exponential kinetics of the typical amoebicidal effects remain unchanged, confirming that they result from the properties of protonated amidine groups which themselves remain unchanged, whatever the experimental diamidine.

All of these data agree with the results of Saunders et al. (12), who found that propamidine isethionate clearly became cysticidal when it was combined with 30% dimethyl sulfoxide, which, according to those investigators, could act as a carrier.

The main practical application resulting from the present study could be the immediate replacement of propamidine isethionate (Brolene) by hexamidine in the medical treatment of *Acanthamoeba* keratitis. Indeed, 0.1% hexamidine isethionate eyedrops (Desomedine) are commercially available in France, and medical cure with hexamidine isethionate has been obtained in two patients with *Acanthamoeba* keratitis (4). Furthermore, these results advocate clinical investigations about the efficiency and toxicity of octamidine, which appears to be the most amoebicidal diamidine in vitro.

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