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ANTIMICROBIAL AGENTS

Bactericidal activity of the new 4-quinolones DU-6859a and DV-7751a

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Summary. The bactericidal activity of two new 4-quinolones, DU-6859a and DV-7751a, was investigated against strains of *Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pneumoniae* and *Enterococcus faecalis*. DU-6859a and DV-7751a were more bactericidal than any 4-quinolone tested previously. Furthermore, DU-6859a was unique among 4-quinolones in being able to kill *Ent. faecalis* after incubation for only 3 h in nutrient broth. The bactericidal mechanisms of DV-7551a were similar to those of other 4-quinolones, but, uniquely, DU-6859a possessed additional bactericidal mechanisms against both *Str. pneumoniae* and *Ent. faecalis*. This may explain the unusually potent bactericidal activity of this agent against these species. These results show that DU-6859a is a unique extended-spectrum 4-quinolone, which should prove to be superior to established 4-quinolones.

Introduction

Preliminary studies have suggested that DU-6859a and DV-7751a may be significantly more active than currently available 4-quinolones against a wide range of bacteria.[1,2] To investigate this claim further, their bactericidal activity was studied against strains of *Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pneumoniae* and *Enterococcus faecalis*.

The bactericidal activity of 4-quinolones has been described with respect to three mechanisms of action, termed A, B and C. Mechanism A requires the bacteria to be undergoing multiplication and protein or RNA synthesis. It occurs with all 4-quinolones, except when high concentrations are used against *Pseudomonas aeruginosa*,[3] and is the sole bactericidal mechanism of older 4-quinolones, such as nalidixic and oxolinic acids.[4] Mechanism B is an additional mechanism of several modern 4-quinolones including ofloxacin, ciprofloxacin,[5] lomefloxacin,[6] levofloxacin,[7] fleroxacin, pefloxacin,[8] PD131628[9] and sparfloxacin.[10] Unlike mechanism A, mechanism B allows bactericidal activity against non-dividing bacteria and does not require active protein or RNA synthesis.[4] A related bactericidal mechanism, termed B_1, has been identified with clinafloxacin (PD127,391.)[11] Although it does not require active protein or RNA synthesis, mechanism B_1, is nevertheless lost against non-dividing bacteria.[11] Mechanism C, which has so far been found only with norfloxacin[12] and enoxacin,[6] does not require bacterial multiplication, but does need active protein and RNA synthesis.

Possession of additional mechanisms against one bacterial species does not guarantee their occurrence against others. For example, ciprofloxacin has mechanism B against *E. coli*,[5] but possesses only mechanism A against staphylococci.[13] Ofloxacin, on the other hand, exerts mechanism B against both *E. coli* and staphylococci,[5,13] as does levofloxacin.[7] All 4-quinolones tested so far possess only bactericidal mechanism A against *Str. pneumoniae*[14] and *Ent. faecalis*,[15] and these species represent a considerable weakness in the bactericidal spectrum of 4-quinolones. This is especially pertinent for *Ent. faecalis*, for which the rate of bactericidal activity of 4-quinolones is slower than for other gram-positive bacteria.[9,15] This study aimed to characterise the bactericidal mechanisms of DU-6859a and DV-7751a and to determine if they had any advantage over earlier agents against pneumococci and enterococci.

Materials and methods

Bacterial strains

E. coli KL16,[16] *S. epidermidis* SK360,[13] *S. aureus* E3T,[17] *Str. pneumoniae* C3LN4[18] and *Ent. faecalis*

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ATCC 19433[15] were used. These species and strains were chosen because of their previous use in determining the bactericidal activity and mechanisms of action of other 4-quinolones.

Antibacterial agents

DU-6859a and DV-7751a (Daiichi Pharmaceutical Co., Japan) were initially dissolved in 0·1 M NaOH at 10 mg/ml and immediately diluted with sterile distilled water. Chloramphenicol (Parke-Davis, Pontypool, Gwent) was prepared initially in methanol and diluted further in sterile distilled water.

Determination of the bactericidal activities of DU-6859a and DV-7751a

Ten ml of Nutrient Broth No. 2 (Unipath Basingstoke, Hants) was inoculated with bacteria and then incubated overnight at 37°C to provide organisms for study. For *Str. pneumoniae* cultures, the nutrient broth was supplemented with laked horse blood (Unipath) 7%.

To study bactericidal activity, sterile double-strength nutrient broth first was dispensed in 5-ml volumes in sterile 1 oz bottles. The aqueous 4-quinolone and sterile distilled water were then added to give a final volume of 9·8 ml, and a drug concentration between 0·015 and 90 mg/l. When *Str. pneumoniae* was tested, laked horse blood was added to a final concentration of 7%. When required, aqueous chloramphenicol was also added to a final concentration of 20 mg/l or 2·5 mg/l for *Str. pneumoniae*. These concentrations of chloramphenicol were bacteriostatic (data not shown). The bottles were then warmed for at least 15 min in a waterbath at 37°C, after which 0·2 ml of overnight culture, containing *c.* $2{\cdot}5 \times 10^8$ cfu/ml, was added. Viable counts at time zero thus were *c.* 5×10^6 cfu/ml.

In some experiments PBS (NaCl 0·9% in 0·025 M sodium phosphate buffer, pH 7·4), replaced nutrient broth. In these cases the overnight cultures used were harvested by centrifugation at 4000 rpm for 15 min, washed, and then resuspended in 10 ml of sterile PBS to prevent nutrient carry-over. To prevent autolysis of *Str. pneumoniae*, horse serum (Unipath) 7% was added when this organism was studied.

All incubations were for 3 h at 37°C, except that *Ent. faecalis* cultures also were incubated for 6 and 24 h. Experiments were stopped by adding 10 ml of sterile ice-cold nutrient broth, after which the cells were harvested by centrifugation at 4000 rpm for 15 min, then resuspended in 10 ml of sterile nutrient broth at room temperature to prevent drug carry-over. Viable counts were determined by serial dilution in sterile nutrient broth at room temperature, and 0·1-ml amounts of the dilutions were spread on nutrient agar, supplemented with laked horse blood 7% for *Str. pneumoniae*. Owing to the strong bactericidal activity of DU-6859a against *E. coli*, a total volume of 1 ml was spread in three samples of 0·3 ml and one of 0·1 ml when this agent was tested at concentrations between 0·5 and 3 mg/l. All plates were incubated for 2 days at 37°C before the colonies were counted.

Results

A biphasic dose response occurred with DU-6859a against *E. coli*, with an optimum bactericidal concentration (OBC) of 0·9 mg/l (fig. 1). Most killing occurred in normal nutrient broth culture, but considerable killing was still achieved when a bacteriostatic concentration of the protein synthesis inhibitor chloramphenicol was added or when the bacteria were resuspended in PBS prior to exposure to the quinolone. Similar behaviour was seen for DV-7751a against *E. coli* and for both DU-6859a or DV-7751a against *S. aureus* or *S. epidermidis* (not shown).

The proportions of *E. coli*, *S. aureus*, *S. epidermidis* and *Str. pneumoniae* cells that survived exposure to DU-6859a or DV-7751a at OBC are shown in the table. Both drugs had OBCs of 0·9 mg/l for *E. coli*, but DV-7751a had lower OBCs than DU-6859a for staphylococci. The extent of killing by either DU-6859a or DV-7751a at OBC in nutrient broth was greater than with any 4-quinolone studied previously. In nutrient broth, DU-6859a was 58 times more bactericidal than DV-7751a against *E. coli* and 19 times more so against *S. epidermidis*, but both 4-quinolones had approximately equal bactericidal activity at OBC against *S. aureus*. However, DU-6859a was more bactericidal than DV-7751a against all three of these species in nutrient broth containing chloramphenicol, or in PBS.

DV-7751a was most bactericidal against *Str.*

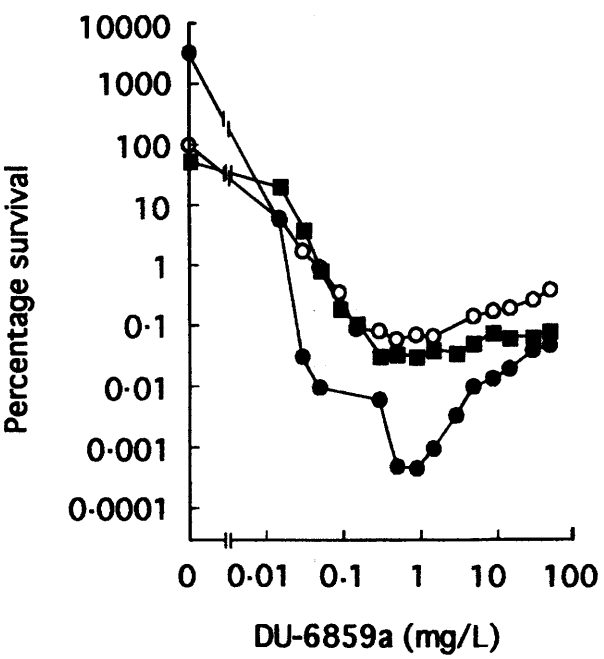

Fig. 1. Survival of *E. coli* KL16 after exposure to DU-6859a for 3 h in nutrient broth (●), phosphate-buffered saline (○) or nutrient broth containing chloramphenicol 20 mg/l (■) at 37°C.

Table. Survival rates of bacteria exposed to DU-6859a or DV-7751a at OBC for 3 h at 37°C

4-Quinolone	Organism (OBC, mg/l)	Percentage survival in		
		nutrient broth*	nutrient broth* plus chloramphenicol	PBS†
DU-6859a	*E. coli* (0·9)	0·00045	0·028	0·07
	S. aureus (3·0)	0·0043	0·09	0·18
	S. epidermidis (3·0)	0·0013	0·28	2·13
	Str. pneumoniae (0·9)	0·095	0·14	0·41
DV-7751a	*E. coli* (0·9)	0·026	0·70	1·29
	S. aureus (0·9)	0·0046	0·21	1·67
	S. epidermidis (1·5)	0·025	5·83	11·94
	Str. pneumoniae (1·5)	0·17	3·94	10·70

*For *Str. pneumoniae* laked horse blood 7% was added.
†For *Str. pneumoniae* horse serum 7% was added.

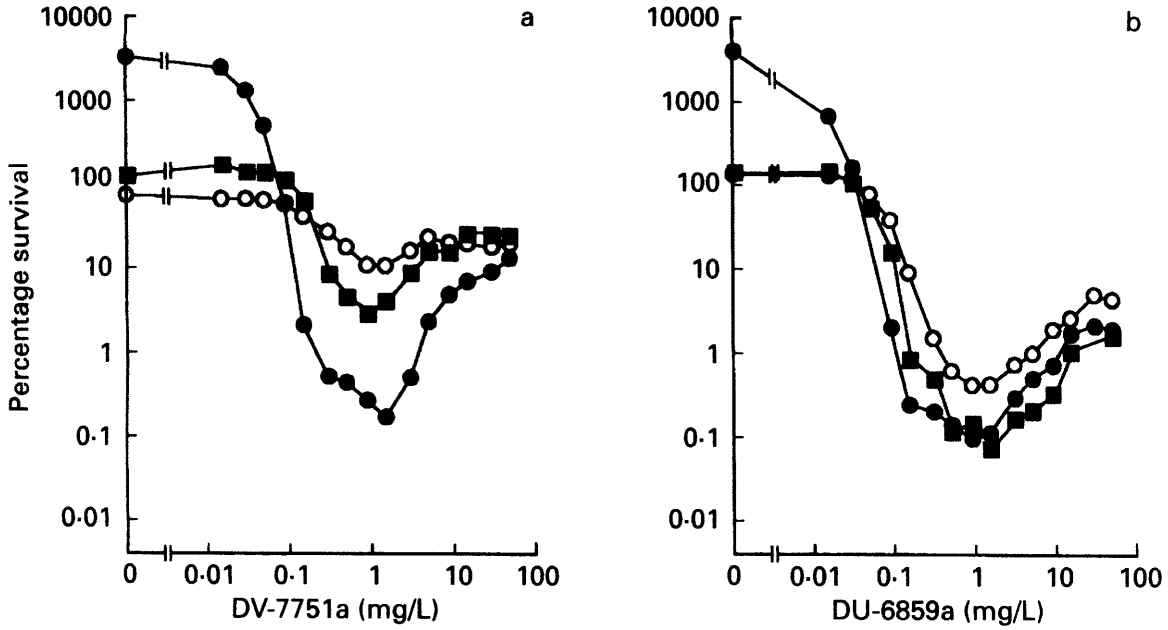

Fig. 2. Survival of *Str. pneumoniae* C3LN4 after exposure to: **a**, DV-7751a or **b**, DU-6859a for 3 h in nutrient broth (●), phosphate-buffered saline (○) or nutrient broth containing chloramphenicol 2·5 mg/l (■) at 37°C.

pneumoniae in blood broth and was less so when a bacteriostatic concentration of chloramphenicol was added, or when tested in PBS plus horse serum 7%. Nevertheless, some killing did occur under these latter conditions (fig. 2a). In contrast, DU-6859a was equally potent against pneumococci in blood broth whether or not chloramphenicol was added (fig. 2b), and was only slightly less bactericidal in PBS plus horse serum. This strong bactericidal activity of DU-6859a against non-dividing *Str. pneumoniae* is unique among 4-quinolone antibacterial agents.

Weak bactericidal activity was seen for DV-7751a against *Ent. faecalis* after incubation for 3 h in nutrient broth at 37°C, but more significant killing was seen only after incubation for 6 or 24 h (fig. 3a). By contrast, DU-6859a had good bactericidal activity against this organism within 3 h in nutrient broth at 37°C (fig. 3b), although still more killing was observed after 6 or 24 h. This rapid bactericidal activity of DU-6859a against *Ent. faecalis* is also unique among 4-quinolones. When bactericidal activity was tested against non-dividing enterococci in PBS, no significant killing occurred with DV-7751a up to 90 mg/L, even after 24 h (fig. 4a), nor, during exposure periods shorter than 24 h, in nutrient broth containing chloramphenicol. DU-6859a did not kill *Ent. faecalis* in PBS even after 24 h (fig. 4b); but, unlike with DV-7751a, bactericidal activity was seen in nutrient broth containing chloramphenicol, with 10, 1 and 0·01% survivors remaining after incubation for 3, 6 and 24 h, respectively. These results show that DU-6859a, unlike DV-7751a, possessed another bactericidal mechanism, in addition to mechanism A, against *Ent. faecalis*, and may explain the compound's rapid bactericidal rate against this species.

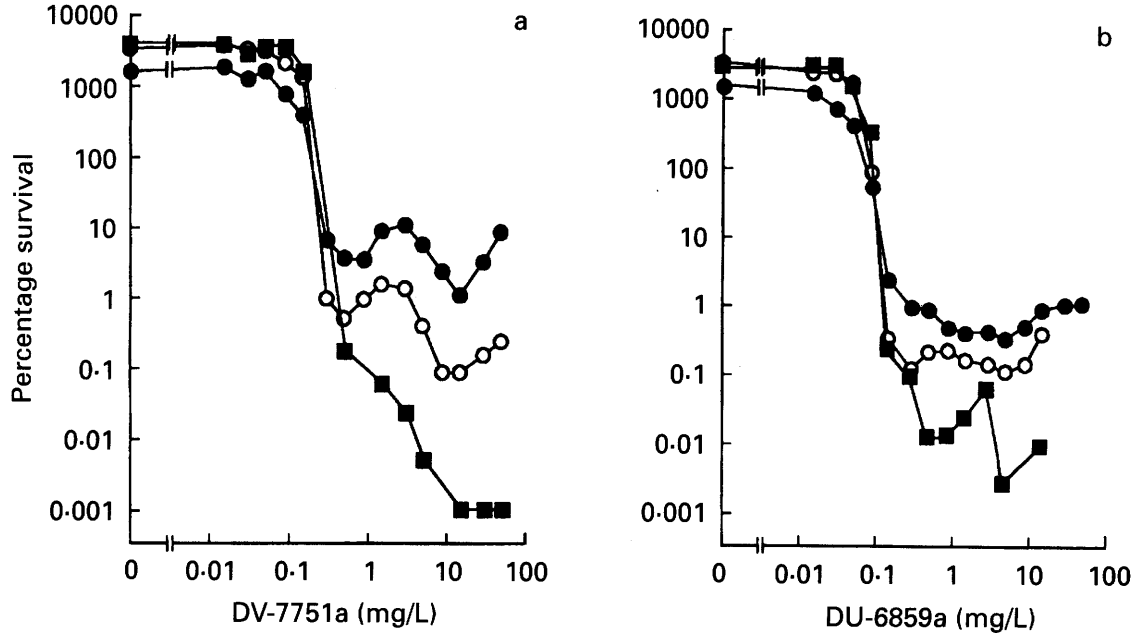

Fig. 3. Survival of *Ent. faecalis* ATCC 19433 after exposure to: **a**, DV-7751a or **b**, DU-6859a for 3 h (●), 6 h (○) or 24 h (■) in nutrient broth at 37°C.

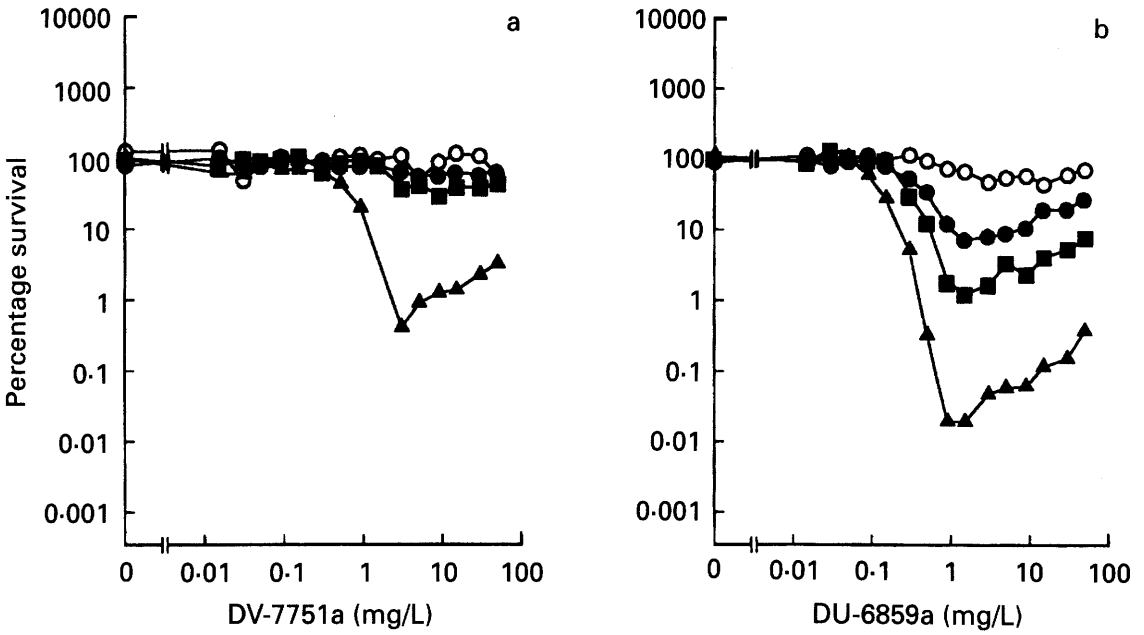

Fig. 4. Survival of *Ent. faecalis* ATCC 19433 after exposure to: **a** DV-7751a or **b**, DU-6859a for 24 h in phosphate-buffered saline (○), or 3 h (●), 6 h (■) or 24 h (▲) in nutrient broth containing chloramphenicol 20 mg/l at 37°C.

Discussion

The bactericidal activity and mechanisms of many 4-quinolones have been studied previously.[4–15] In this study, the bactericidal activities and mechanisms of action of two new 4-quinolones, DU-6859a and DV-7751a, were investigated against the same test strains used in these earlier studies. DV-7751a and DU-6859a, like many other 4-quinolones, displayed another bactericidal mechanism, in addition to mechanism A, against *E. coli*, *S. aureus* and *S. epidermidis*. This appeared to be mechanism B, because both drugs could kill bacteria in the absence of active protein or RNA synthesis and when the bacteria were not multiplying.

DV-7751a had very similar OBCs against *E. coli*, *S. aureus* and *S. epidermidis*. This is unusual among 4-quinolones, and has only been seen previously with sparfloxacin.[10] DU-6859a, on the other hand, was more like other 4-quinolones in that its OBC for *E. coli* was about three-fold lower than for either staphylococcal species. However, comparison of OBCs alone may not allow a fair assessment of DV-7751a and DU-6859a because both drugs were extremely bactericidal at even lower concentrations. This was especially marked for DU-6859a against *E. coli*, where a modification of the usual viable counting method was necessary to enumerate the few survivors.

OBCs of both new quinolones against *Str. pneumoniae* were considerably lower than those of

other 4-quinolones.[14] Moreover, both DV-7751a and DU-6859a were able to kill *Str. pneumoniae* in the absence of active protein or RNA synthesis, or when the bacteria were not multiplying, indicating the presence of mechanism B. All 4-quinolones previously tested showed only mechanism A against this species.[14] The occurrence of mechanism B may explain the greater bactericidal activity of these drugs for pneumococci and may be extremely useful in the treatment of infections, where the bacteria may not be dividing. Mechanism B was particularly strong with DU-6859a, where essentially the same extent of killing was seen against *Str. pneumoniae* in blood broth whether or not a bacteriostatic concentration of chloramphenicol was added. Possession of such a powerful mechanism B suggests that DU-6859a might be used in combination with those antibacterial agents that inhibit bacterial protein synthesis, whereas the bactericidal activity of other 4-quinolones may be seriously impaired by inhibitors of protein synthesis, as has been shown with levofloxacin.[19] Such combination therapy might be desirable to prevent the development of bacterial resistance.

Previous 4-quinolones have had very slow rates of bactericidal activity against *Ent. faecalis*[9,15] although, contrary to a recent claim,[20] they are not merely bacteriostatic against these organisms. In this study a slow rate of bactericidal activity was seen with DV-7751a, although reasonable killing occurred after exposure for 6 h, whereas other 4-quinolones require exposure for 24 h to act significantly.[9,15] Moreover, DV-7751a, like other 4-quinolones, possessed only bactericidal mechanism A against *Ent. faecalis*. DU-6859a, on the other hand, was bactericidal against *Ent. faecalis* within 3 h and had a bactericidal mechanism additional to mechanism A. This additional mechanism was neither B nor C because, although killing occurred when the bacteria were incapable of active protein or RNA synthesis, it was not achieved against non-multiplying bacteria. Thus, this additional mechanism appears to be mechanism B_1, which has been reported previously only for clinafloxacin against *E. coli*, *S. aureus* and *S. epidermidis*.[11]

To conclude, DV-7751a appears similar in its bactericidal activity and bactericidal mechanisms of action to other available fluoroquinolones, except for having enhanced anti-pneumococcal activity and very powerful bactericidal activity against *S. aureus*. It may perhaps acquire a special role against these species. Overall, however, DU-6859a was a more potent agent, and was more bactericidal than any other 4-quinolone investigated with these test bacteria. The bactericidal activity of DU-6859a was particularly powerful against *Str. pneumoniae* and *Ent. faecalis*, perhaps because of the occurrence of bactericidal mechanisms B or B_1 in addition to mechanism A. If brought into clinical use, DU-6859a would be the most active 4-quinolone available.

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