Mechanisms of zidovudine resistance in bacteria

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Summary. Unlike their parent strains, zidovudine-resistant derivatives of *Escherichia coli* KL16 and *Salmonella typhimurium* NCTC 5710 were found to be incapable of incorporating radiolabelled thymidine into their chromosomal DNA. Since incorporation was still prevented in the presence of EDTA, resistance to zidovudine was not associated with a permeability barrier, but appeared to result from the loss of thymidine kinase activity, required for the phosphorylation of zidovudine. *Pseudomonas aeruginosa*, which is intrinsically zidovudine-resistant, was also shown to be incapable of incorporating thymidine into its DNA, but *Staphylococcus epidermidis* SK360 and *Staph. aureus* E3T, which are also intrinsically zidovudine-resistant, possessed thymidine kinase activity. This suggests that two distinct mechanisms of resistance to zidovudine exist in bacteria. Zidovudine resistance did not appear to confer resistance to other antibacterial agents.

Introduction

Zidovudine is a thymidine analogue in which an azido group is substituted for the 3'-hydroxy group. It is active against human immunodeficiency virus (HIV) and is useful in the treatment of acquired immune deficiency syndrome (AIDS).^{1,2} Zidovudine also displays antibacterial activity against Enterobacteriaceae but not against *Pseudomonas aeruginosa* or staphylococci, which are intrinsically resistant to the drug (MICs > 500 mg/L).^{3,4} Both the antiviral and antibacterial activities of zidovudine are thought to be derived from the ability of the compound to act as a chain terminator after incorporation into viral or bacterial DNA. The azido group makes the formation of subsequent 5'–3' phosphodiester linkages impossible.^{1,5}

Escherichia coli and *Salmonella typhimurium* develop stable high-level zidovudine resistance after short-term exposure to the drug *in vitro* at concentrations up to 100 MIC.⁶ The mechanism of zidovudine resistance appears to involve the loss of thymidine kinase activity which is required to convert zidovudine to its active triphosphate form.^{3,6} This can be shown by the inability of thymidine to antagonise the action of trimethoprim in zidovudine-resistant mutants.⁶ Thymidine normally confers resistance to trimethoprim as the dihydrofolate reductase pathway can be bypassed,⁷ but this can happen only if the bacterium possesses thymidine kinase.⁸

This is only indirect evidence of thymidine kinase activity. Measuring the amount of radiolabelled thymidine incorporated into bacterial chromosomal DNA provides a direct method of assaying thymidine kinase activity as this enzyme is required to convert thymidine to thymidine monophosphate (fig. 1).⁸ In this investigation we studied the incorporation of radiolabelled thymidine into the DNA of *E. coli* KL16, *S. typhimurium* NCTC 5710 and zidovudine-resistant derivatives of these two strains, as well as *P. aeruginosa* and staphylococci, which are intrinsically resistant to the drug.

Materials and methods

Antimicrobial agents

Ampicillin (Beecham, UK), ciprofloxacin (Bayer, UK), gentamicin (Roussel, UK), tetracycline hydrochloride (Lederle, UK), trimethoprim lactate and zidovudine (Wellcome, UK) were dissolved in sterile distilled water. Lomefloxacin (Searle, UK) and ofloxacin (Hoechst, UK) were dissolved in 0.5 M NaOH and then made up to the appropriate concentration with sterile distilled water.

Bacterial strains

E. coli KL16,⁹ *S. typhimurium* NCTC 5710, *Staphylococcus aureus* E3T,¹⁰ *Staph. epidermidis* SK360¹¹ and *P. aeruginosa* CP (from Professor J. T. Smith) were used in this investigation. Zidovudine-resistant derivatives of *E. coli* KL16 and *S. typhimurium* NCTC 5710 were prepared by plating serial dilutions of bacteria that had been

exposed to zidovudine 100 mg/L, in nutrient broth overnight at 37°C, on to nutrient agar containing zidovudine 100 mg/L.

Minimum inhibitory concentration determinations

MICs were determined by the agar incorporation method on Nutrient Agar (Oxoid) or, in the case of trimethoprim, on Iso-Sensitest Agar (Oxoid) with an arithmetic dilution scheme that followed the progression: 1, 1.5, 2, 3, 4, 5 and 7.5. An inoculum of 10^4 cfu was used and the MIC was taken as the lowest concentration that inhibited visible growth after overnight incubation of the plates at 37°C.

Incorporation of thymidine into bacterial DNA

Incorporation of thymidine into bacterial DNA was estimated by a modified version of the method of Schubach *et al.*¹² Bacteria were grown in 100 ml of DM medium,¹³ containing glucose 0.28%, inoculated with 10 ml of an overnight culture and shaken for 3 h at 37°C. The cells were then harvested by centrifugation for 35 min at 6000 *g*. The supernate was discarded and the pellet was resuspended in DM broth containing deoxyadenosine 200 mg/L and methyl ³H thymidine (TRK 120 Amersham, UK) 2 µCi/ml; this suspension was then shaken for a further 2 h at 37°C. After incubation, the culture was cooled for 10 min in ice; 1 ml of trichloroacetic acid (TCA) 50% and 1 ml of a thymidine 1 mg/L solution were then added and a precipitate was allowed to form at 0°C for 60 min. Duplicate samples of 1 in 10 dilutions of the precipitate were filtered through a glass fibre filter disk (Whatmann, UK). The filter disk was then washed with 5 ml of cold TCA 1% followed by 15 ml of boiling,

distilled water. This was followed by washes with 5 ml of NaCl 5% and 10 ml of acetic acid 5%. The filter disks were then dried overnight in a desiccator and the radioactivity was determined in a Packard Tri Carb liquid scintillation counter. The scintillation fluid used was OptiScint "Safe" (LKB).

Results and discussion

In contrast to their respective parent strains, all nine zidovudine-resistant derivatives of *S. typhimurium* NCTC 5710 and *E. coli* KL16 (MICs of zidovudine > 500 mg/L) were unable to grow on Iso-Sensitest agar containing thymidine 10 mg/L and trimethoprim 10 mg/L (table I). This result suggests that the resistant variants had lost thymidine kinase activity, as when their dihydrofolate reductase pathway was blocked by trimethoprim they were unable to use the alternative thymidine pathway to synthesise thymidine monophosphate (fig. 1). In contrast, *Staph. aureus* E3T and *Staph. epidermidis* SK360, which were resistant to zidovudine (MICs > 500 mg/L), were able to grow on Iso-Sensitest agar containing thymidine 10 mg/L and trimethoprim 10 mg/L, although both strains were susceptible to trimethoprim (table I). This suggests that the intrinsic zidovudine resistance of the staphylococci is not related to a lack of thymidine kinase activity as both strains were able to use thymidine in the medium to antagonise trimethoprim action.

The ability of zidovudine-resistant Enterobacteriaceae to grow on Iso-Sensitest agar containing

Table I. Growth of bacteria on Iso-Sensitest agar containing trimethoprim alone or in combination with thymidine

Strain	No drug	Tp (10 mg/l)	Tp (10 mg/l) + Thy (10 mg/l)	Thymidine kinase activity
<i>E. coli</i> KL16	+	—	+	+
<i>E. coli</i> KL16RA30	+	—	—	—
<i>E. coli</i> KL16RA31	+	—	—	—
<i>E. coli</i> KL16RA33	+	—	—	—
<i>E. coli</i> KL16RA35	+	—	—	—
<i>S. typhimurium</i> NCTC 5710	+	—	+	+
<i>S. typhimurium</i> 5710RA36	+	—	—	—
<i>S. typhimurium</i> 5710RA37	+	—	—	—
<i>S. typhimurium</i> 5710RA39	+	—	—	—
<i>S. typhimurium</i> 5710RA40	+	—	—	—
<i>S. typhimurium</i> 5710RA41	+	—	—	—
<i>Staph. aureus</i> E3T	+	—	+	+
<i>Staph. epidermidis</i> SK360	+	—	+	+

Tp, trimethoprim; Thy, thymidine; +, growth; —, no growth (after overnight incubation at 37°C).

All strains grew on Iso-Sensitest agar containing thymidine 10 mg/L.

Fig. 1. Incorporation of thymidine derivatives in bacterial DNA. DHF, dihydrofolate reductase; THF, tetrahydrofolate; Tp, trimethoprim.

thymidine and trimethoprim provides a rapid means of assessing whether resistance to the compound results from a loss of thymidine kinase activity.

E. coli KL16 was able to incorporate ^3H thymidine into its DNA indicating that this strain possesses thymidine kinase activity. Conversely, the counts of ^3H thymidine/ 10^8 cfu were reduced 10-fold to 100-fold in the three zidovudine-resistant derivatives of *E. coli* KL16 (table II) suggesting that the zidovudine-resistant derivatives are deficient in thymidine kinase activity. All three zidovudine-resistant *S. typhimurium* strains investigated were similarly unable to incorporate radiolabelled thymidine into their DNA. *P. aeruginosa* CP which, like all *P. aeruginosa*, is intrinsically resistant to zidovudine (MIC > 500 mg/L), was also

found to be incapable of incorporating radiolabelled thymidine into its DNA (table II). To exclude the possibility that the resistance could be conferred by an impermeability mechanism, the ability of the strains to incorporate ^3H thymidine in the presence of 0.3 mM EDTA (edetic acid) was examined. The uptake of ^3H thymidine by *E. coli* KL16 in DM containing 0.3 mM EDTA was almost 10-fold greater than the uptake in DM alone (table II) indicating an increase in permeability following EDTA treatment. In contrast, the presence of 0.3 mM EDTA in the DM broth did not significantly increase the uptake of ^3H thymidine by either *E. coli* KL16RA35 or *P. aeruginosa* CP (table II) confirming that the inability of these strains to incorporate ^3H thymidine into their DNA derives from an absence of thymidine kinase activity rather than from a permeability barrier.

Both *Staph. aureus* E3T and *Staph. epidermidis* SK360 were able to incorporate ^3H thymidine into their DNA (table II) in confirmation of the result obtained in experiments with trimethoprim and thymidine which indicated that the intrinsic zidovudine resistance in *Staph. aureus* E3T and *Staph. epidermidis* SK360 did not result from a lack of thymidine kinase. EDTA could not be used to investigate impermeability as a possible cause of the lack of susceptibility to zidovudine in the staphylococci as this technique works only with gram-negative bacteria. Instead, the effect of zidovudine concentrations up to 100 mg/L on the incorporation of ^3H thymidine into bacterial DNA was determined. Reduction in the incorporation of ^3H thymidine into DNA by zidovudine would provide evidence that the drug was competing for the same uptake pathway as thymidine and,

Table II. Incorporation of ^3H thymidine into chromosomal DNA

Organism	MIC of zidovudine (mg/L)	Counts/ 10^8 bacteria	
		without EDTA	with EDTA
<i>E. coli</i> KL16	0.15	3250	29510
<i>E. coli</i> KL16RA30	> 500	156	NT
<i>E. coli</i> KL16RA31	> 500	267	NT
<i>E. coli</i> KL16RA35	> 500	36	48
<i>S. typhimurium</i> NCTC 5710	1	4835	NT
<i>S. typhimurium</i> 5710RA37	> 500	49	NT
<i>S. typhimurium</i> 5710RA39	> 500	30	NT
<i>S. typhimurium</i> 5710RA40	> 500	23	NT
<i>P. aeruginosa</i> CP	> 500	30	69
<i>Staph. aureus</i> E3T	> 500	6038	NT
<i>Staph. epidermidis</i> SK360	> 500	5285	NT

NT, not tested.

therefore, that impermeability was not the cause of the intrinsic resistance of the staphylococci to zidovudine.

High concentrations of zidovudine (> 10 mg/L) reduced by 80–90% the uptake of radiolabelled thymidine by both strains of staphylococci (fig. 2). Therefore, impermeability does not appear to be the cause of the insusceptibility to zidovudine. The inhibition of the uptake of extracellular thymidine by zidovudine will not completely inhibit bacterial multiplication because the dihydrofolate reductase pathway can be used to provide thymidine monophosphate (fig. 1).

A possible explanation for the intrinsic resistance of the staphylococci to zidovudine is that the compound is not a substrate for either staphylococcal thymidine kinase or DNA polymerase III and, therefore, the drug is not incorporated into the staphylococcal DNA.

Possible cross-resistance to other antibacterial agents was also determined. The MICs of ciprofloxacin, lomefloxacin, ofloxacin, ampicillin, gentamicin, tetracycline and trimethoprim for the zidovudine-resistant strains were identical with those for their parent strains.

In conclusion there appear to be at least two distinct mechanisms of zidovudine resistance: one involves a lack of thymidine kinase activity; for the second, found in the staphylococci, the mechanism

Fig. 2. Incorporation of ^3H thymidine by *Staph. aureus* E3T (●—●), *Staph. epidermidis* SK360 (○—○) and *E. coli* KL16 (□—□) in the presence of zidovudine.

of resistance remains unknown except that it does not seem to result from an impermeability barrier. Neither type of resistance appears to affect the susceptibility of the bacteria to other antibacterial agents. This is significant as we have recently reported the isolation of zidovudine-resistant *E. coli* in AIDS patients.¹⁴

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