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THE MECHANISM OF ACTION OF DuP 721, A NEW ANTIBACTERIAL AGENT: EFFECTS ON MACROMOLECULAR SYNTHESIS

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Pulse labeling studies with Bacillus subtilis showed that DuP 721 inhibited protein synthesis. The IC_{50} of DuP 721 for protein synthesis was 0.25 μg/ml but it was > 32 μg/ml for RNA and DNA synthesis.

In cell-free systems, DuP 721 concentrations up to 100 μM did not inhibit peptide chain elongation reactions under conditions where chloramphenicol, tetracycline and hygromycin B inhibited these reactions. Furthermore, DuP 721 did not cause phenotypic suppression of nonsense mutations suggesting that DuP 721 did not inhibit peptide chain termination. Thus, the mechanism of action of DuP 721 is at a target preceeding chain elongation.

The oxazolidinones, a new and novel class of synthetic antibacterial agents, are bacteriostatic compounds whose spectrum of activity includes gram positive and anaerobic bacteria (1). These compounds are active against multiply resistant staphylococci and other gram positive bacteria bearing drug resistance markers. These findings suggest that the oxazolidinones may have an intracellular target which is distinct from that of other antibacterials. Because of these promising and unique properties, mechanism of action studies were conducted.

MATERIALS AND METHODS

Compounds: DuP 721, (S)-N-[3-[4-acetyl-phenyl]-2-oxooxazolidin-5-ylmethyl]acetamide), was synthesized by the Medicinal Chemistry Section, Pharmaceuticals and Biotechnology Research Division, DuPont Co., Wilmington, DE. [U-^{14}C]-L-phenylalanine (specific activity = 525 mCi/mmole) was purchased from Amersham, Arlington Heights, IL; [^{3}H]-L-lysine (specific activity = 92.4 Ci/mmole), [^{3}H]thymidine (specific activity = 108.5 Ci/mmole) and [^{3}H]uridine (specific activity = 28.5 Ci/mmole) were purchased from New England Nuclear Corp (NEN), Boston, MA. Nucleic acid polymers, Sephadex G25 and all other biochemicals were purchased from Sigma, St. Louis, MO unless otherwise indicated. Norfloxacin was a gift from Merck Sharpe and Dome Co.

Bacterial strains and media: B. subtilis ATCC 6633, was used in the pulse labeling studies and as the source of cell free extracts. All other strains were obtained from the Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio. The cultures were maintained on Difco medium #1 or grown at 32°C in the defined liquid medium described by Spizizen (2)

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supplemented with a trace elements solution (3) and 50 μM of each of 20 naturally occurring L-amino acids except lysine.

Sensitivity Determinations: Minimal inhibitory concentrations (MICs) were determined by a microtiter plate dilution technique (4). Disk diffusion assays were performed by placing a sensitivity disk containing 10 μg of DuP 721 on Difco medium #1 agar plates that had a thin layer of bacteria spread on them and measuring the zones of inhibition after 24 hr at 32°C.

Pulse Labeling Studies: A culture of B. subtilis was grown to an absorbance of 0.2/ml at 540 nm in supplemented Spizizens medium. Aliquots (140 μl) of the cell suspension were mixed with 5.0 μl solutions that contained graded concentrations of the test compounds dissolved in 10% dimethylsulfoxide and 5 μl of a labeling solution containing 2.5μCi of [^{3}H]thymidine, 0.5 μCi of [^{3}H]uridine or 1.25 μCi of [^{3}H]-L-lysine. The mixtures were incubated at 25°C for 5 min at which time the incorporation of isotope into macromolecules was stopped by the addition of 50 μl of 95% ethanol. Aliquots (100μl) were spotted on Whatman 3MM filter paper circles and processed as described by Eustice and Wilhelm (5) except that the hot TCA wash was omitted. Results are expressed as percent of control incorporation and were corrected for any isotope retained on the filter paper in the absence of cells. The IC_{50}s (concentration required to inhibit incorporation by 50%) were determined from plots of inhibitor concentration versus percent of control incorporation.

Preparation of Extracts For Cell Free Protein Synthesis Studies: B. subtilis ATCC 6633 was grown to mid-log phase in 3.0 liters of Difco medium #1. Cells were collected by centrifugation at 5,000 x g for 10 min at 5°C. Cell-free extracts were prepared by grinding washed cell pellets with alumina using a mortar and pestle by the method of Wilhelm and Haselkorn (6) except that, instead of dialysing the extracts, the 30,000 x g supernatant liquid (S-30) was passed through a 1.8 cm x 25 cm column of Sephadex G25 equilibrated with 10 mM Tris HCl pH 7.6, 10 mM magnesium acetate, 30 mM KCl, 6 mM 2-mercaptoethanol and 20% glycerol.

Cell-free Protein Synthesis Assay: The assay for protein synthesis stimulated by the synthetic template poly[cytidylic-uridylic acid] (poly(CU)) was performed essentially as described by Eustice et al (7). The assay mixture (50μl) contained 60 mM Tris HCl (pH 7.6), 8.0 mM magnesium acetate, 60 mM NH_4Cl, 200 μg/ml of polymer, S-30 extract diluted to 20 A_{260}U/ml final concentration and 1.0 A_{260}U of E. coli tRNA per ml. All required aminoacids were added to 50 μM (with 10 μM of [^{14}C]phenylalanine). Polyamines and energy mix was used as described (7). After incubation at 30°C for 20 min, 40 μl aliquots were processed for radioactive phenylalanine incorporated into protein (5).

RESULTS

The effect of DuP 721 on macromolecular synthesis in B. subtilis was determined in pulse labeling experiments. DuP 721 substantially inhibited lysine incorporation, however, it had minimal effects on DNA and RNA synthesis (Figure 1). The control antibacterials, norfloxacin, rifampicin and chloramphenicol showed their expected specific inhibitory effects on RNA, DNA and protein synthesis. Cephaloridine, an inhibitor of cell wall synthesis, did not inhibit any of these processes (Figure 2). A comparison of the effects of DuP 721, chloramphenicol, erythromycin and tetracycline on [^{3}H]lysine incorporation is shown in Table 1. The IC_{50} of DuP 721 for protein synthesis was less than or equal to the MIC for B. subtilis. In contrast, the IC_{50}s of

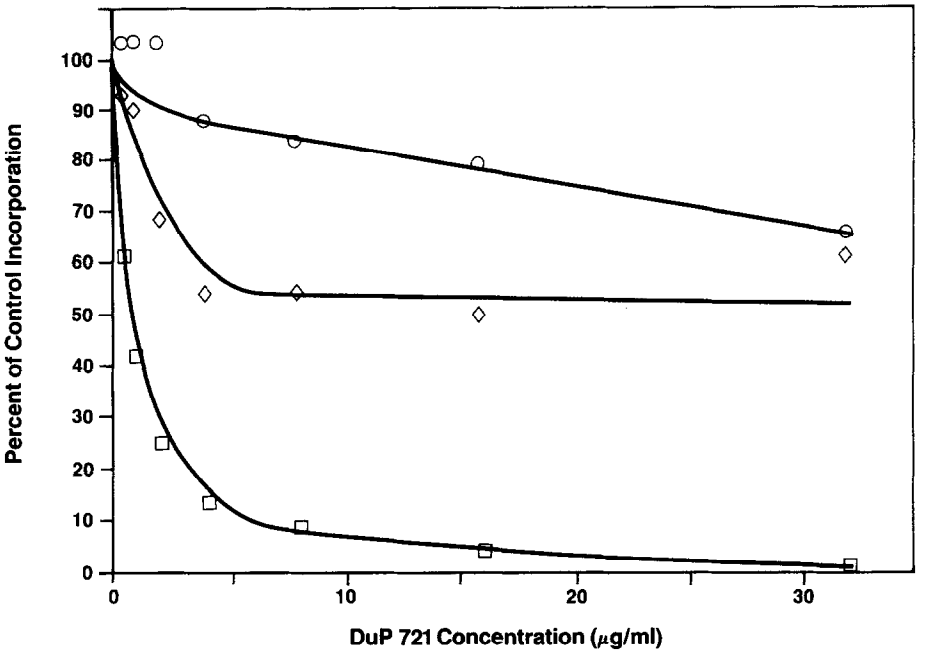

FIGURE 1. Effect of DuP 721 on protein, DNA and RNA synthesis in B. subtilis. Uninhibited controls incorporated 1.21 x 10^5 cpm of [^{3}H]lysine per 100 ul, 1.22 x 10^5 cpm of [^{3}H]uridine per 100 ul and 1.82 x 10^4 cpm of [^{3}H]thymidine per 100 ul. Symbols (□) lysine incorporation (protein synthesis), (○) uridine incorporation (RNA synthesis), (◇) thymidine incorporation (DNA synthesis). Drugs and isotopes were added at time zero and incubation was at 25° for 5 min.

the control protein synthesis inhibitors were always greater than the MIC. Taken together, these results suggest that DuP 721 specifically inhibits protein synthesis. We, therefore, conducted studies to determine if strains bearing target level resistance to known protein synthesis inhibitors were cross resistant to DuP 721. To determine this, B. subtilis strains with well defined antibiotic resistance alleles mapping to ribosomal protein genes to the elongation factor Tu allele were studied. Determination of MICs in broth and zones of inhibition on agar plates clearly showed that there was no significant cross resistance between DuP 721 and streptomycin, erythromycin, spectinomycin, micrococcin, virginiamycin or thiostrepton (Table 2).

To determine the effects of DuP 721 at the ribosome level, DuP 721 was tested for the inhibition of polypeptide synthesis in a cell free system stimulated by the synthetic template, poly(CU). DuP 721 did not substantially inhibit polypeptide synthesis under conditions where known inhibitors, such as chloramphenicol, an inhibitor of peptide bond formation (9), tetracycline, an inhibitor of aminoacyl-tRNA binding (9) and hygromycin B, an inhibitor of peptidyl-tRNA translocation (9) all inhibited polypeptide synthesis (Figure 3).

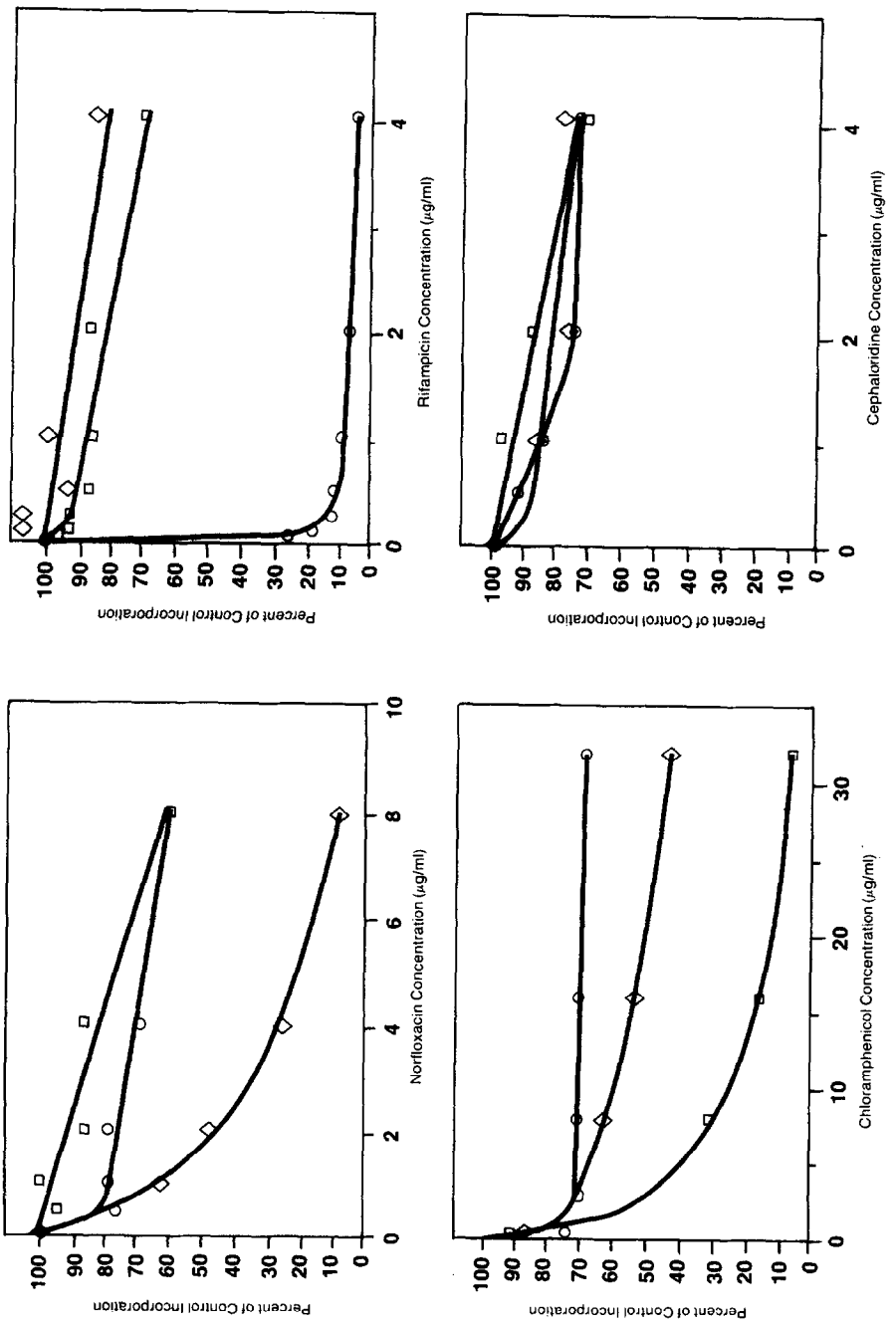

FIGURE 2. Effect of control drugs on the pulse label analysis with B. subtilis. Panel A: norfloxacin, panel B: rifampicin, panel C: chloramphenicol, panel D: cephaloridine. Symbols: (□) lysine incorporation (protein synthesis), (○) uridine incorporation (RNA synthesis), (◇) thymidine incorporation (DNA synthesis). Pulse label analysis was performed as described in Materials and Methods.

TABLE 1

COMPARISON OF THE ABILITY OF DuP 721 AND OTHER ANTIBACTERIALS TO INHIBIT BACTERIAL GROWTH AND PROTEIN SYNTHESIS IN WHOLE CELLS (B. subtilis)

TEST AGENT	MIC[1] (μg/ml)	IC_{50} (μg/ml)
DuP 721	0.25-0.5	0.25
Norfloxacin	0.06	>8.0
Rifampicin	0.03	>4.0
Erythromycin	0.12	0.8
Chloramphenicol	1	1.75
Tetracycline	0.12	0.9
Cephaloridine	0.06	>4

[1]Determined in supplemented Spizizens Medium.

DISCUSSION

Pulse labeling studies with whole B. subtilis cells treated with DuP 721 demonstrated a potent and specific effect on the incorporation of lysine into protein. The IC_{50} for lysine incorporation was less than or equal to the MIC of DuP 721 for B. subtilis. This finding indicates that the inhibition of protein synthesis is the primary action of DuP 721.

TABLE 2

EFFECT OF DuP 721 ON THE GROWTH OF B. subtilis STRAINS WITH ANTIBIOTIC RESISTANCE MARKERS AT THE TARGET LEVEL

STRAIN	ANTIBIOTIC RESISTANCE	GENE SYMBOL	EFFECT Of DuP 721	
			MIC[1] (μg/ml)	ZONE OF INHIBITION (mm)
ATCC 6633	None (Parent)	-	0.5	28
BGSC 3A2	Chloramphenicol	rplA7	1.0	28
BGSC 3A3	Chloramphenicol	rpl011	1.0	33
BGSC 1A1	None (Parent)	-	0.5	27
BGSC 1A186	Kasugamycin	ksgB502	0.5	28
BGSC 1A187	Kasugamycin	ksgA618	0.5	27
BGSC 1A190	Kirromycin	tuf-7	0.5	27
BGSC 1A191	Streptomycin	rpsl1	0.5	27
BGSC 1A218	Erythromycin	rplV1	0.5	28
BGSC 1A219	Spectinomycin	rpsE2	0.5	29
BGSC 1A220	Micrococcin	rplC1	0.25	28
BGSC 1A416	Virginiamycin	virM	0.25	29
BGSC 1A417	Virginiamycin	virS	0.25	26
BGSC 1A475	Thiostrepton	rplK6	0.25	27

[1]Obtained using Difco Medium 1.

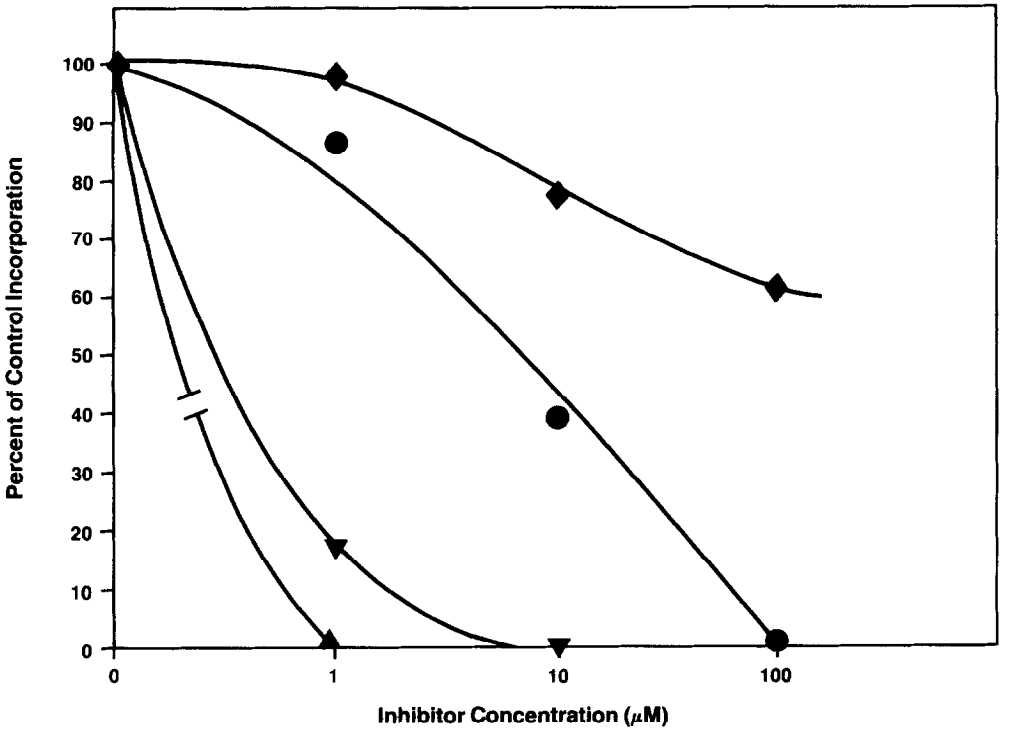

FIGURE 3. Effect of oxazolidinones on the synthesis of polypeptides in cell-free extracts directed by poly(CU). Uninhibited controls incorporated 1.1 pmoles of [^{14}C]phenylalanine. Symbols: (◆) DuP 721, (●) chloramphenicol, (▼): tetracycline, (▲) hygromycin B.

Further studies with whole cells showed that DuP 721 effectively inhibited the growth of B. subtilis strains carrying known target level resistance mutations to many other protein synthesis inhibitors. Among the strains tested were representatives that were resistant to inhibitors of peptidyl-tRNA translocation (erythromycin, spectinomycin and streptogramin), aminoacyl-tRNA binding (micrococcin, thiostrepton and streptomycin), peptide bond formation (chloramphenicol and kirromycin) and initiation (kasugamycin). Preliminary studies have shown that DuP 721 is not a likely inhibitor of polypeptide chain termination because it did not cause phenotypic suppression of suppressible nonsense mutations (data not shown).

Studies with cell-free protein synthesis systems directed by a synthetic template demonstrated that concentrations of DuP 721 in large excess over the MIC and IC_{50} values did not inhibit polypeptide chain elongation under conditions where control inhibitors were effective. These results suggest that DuP 721 has a mode of action different from that of other protein synthesis inhibiting antibiotics most of which act at the level of polypeptide chain elongation.

Thus, since DuP 721 did not inhibit polypeptide chain elongation or chain termination, the mode of action of DuP 721 must be the inhibition of either the initiation process or the recycling of ribosomal subunits following chain termination. Studies are in progress to determine if DuP 721 inhibits protein synthesis initiation in cell-free systems.

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