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Mechanism of Action of DuP 721: Inhibition of an Early Event during Initiation of Protein Synthesis

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The mode of action of DuP 721 was investigated. This compound was active primarily against gram-positive bacteria, including multiply resistant strains of staphylococci. Although inactive against wild-type *Escherichia coli*, DuP 721 did inhibit *E. coli* when the outer membrane was perturbed by genetic or chemical means. Pulse-labeling studies with *E. coli* PLB-3252, a membrane-defective strain, showed that DuP 721 inhibited amino acid incorporation into proteins. The 50% inhibitory concentration of DuP 721 for protein synthesis was 3.8 μg/ml, but it was >64 μg/ml for RNA and DNA syntheses. The direct addition of DuP 721 to cell-free systems did not inhibit any of the reactions of protein synthesis from chain initiation through chain elongation with either synthetic or natural mRNA as template. However, cell extracts prepared from DuP 721 growth-arrested cells were defective in initiation-dependent polypeptide synthesis directed by MS2 bacteriophage RNA. These cell-free extracts were not defective in polypeptide elongation or in fMet-tRNA$_f^{Met}$-dependent polypeptide synthesis stimulated by poly(G · U). We conclude, therefore, that DuP 721 exerts its primary action at a step preceding the interaction of fMet-tRNA$_f^{Met}$ and 30S ribosomal subunits with the initiator codon.

The 3-aryl-oxazolidinones, a new and novel class of synthetic antibacterial agents, are bacteriostatic compounds whose spectrum of activity primarily includes gram-positive and anaerobic bacteria (9). These compounds are active against multiply resistant staphylococci and other bacteria bearing drug resistance markers (3, 9). DuP 721 potently inhibited protein synthesis in *Bacillus subtilis*, but it did not inhibit polypeptide chain elongation programmed by synthetic mRNAs and was unlikely to inhibit chain termination (3). The present studies were conducted to determine whether DuP 721 inhibited the initiation of protein synthesis or elongation reactions with natural mRNA as template.

MATERIALS AND METHODS

Compounds. DuP 721 {(*S*)-*N*-[3-[4-acetylphenyl]-2-oxo-oxazolidin-5-ylmethyl]acetamide} (Fig. 1) was synthesized by the Medicinal Chemistry Section, Pharmaceuticals and Biotechnology Research Division, Du Pont Co., Wilmington, Del. L-[U-^{14}C]phenylalanine was purchased from the Amersham Corp., Arlington Heights, Ill., and all other isotopes were purchased from New England Nuclear Corp., Boston, Mass. Norfloxacin was a gift from Merck Sharp & Dohme, Rahway, N.J. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

Bacterial strains and media. *Escherichia coli* MRE 600 (ATCC 29417) and *B. subtilis* ATCC 6633 were obtained from the American Type Culture Collection, Rockville, Md. *E. coli* ESCH-1 and ESCH-K12 are laboratory strains with normal outer membrane porin protein F (OmpF). *E. coli* PLB-3252 with the OmpF-3252 mutation was obtained from S. Bensen, Princeton University, Princeton, N.J., through Rolf Menzel, Du Pont Co., and the detailed genotype is as described in reference 1. ESCH-140 is a laboratory strain of *E. coli* that is susceptible to many antibiotics which normally do not inhibit *E. coli* growth. Cultures were maintained on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) or grown at 37°C in the defined liquid medium described by Spizizen (10) and supplemented as described previously (3) when used for pulse-label studies or grown in Mueller-Hinton broth (GIBCO Laboratories, Grand Island, N.Y.).

Susceptibility determinations. MICs were determined by a microtiter plate dilution technique (8).

Pulse-labeling analysis. *E. coli* PLB-3252 was grown overnight in supplemented Spizizen medium. Cultures were diluted 1:20 with fresh medium and grown to mid-log phase (A_{540}, 0.2 per ml). Samples of 65 μl of the cell suspension were mixed with 5.0 μl of the antibacterial agents dissolved in media and incubated at 37°C for 5 min. Labeling was initiated by the addition of 5.0 μl of a solution containing 2.5 μCi of [^{3}H]thymidine, 0.5 μCi of [^{3}H]uridine, or 1.25 μCi of L-[^{3}H]lysine. The mixtures were incubated at 37°C for 5 min, after which the incorporation of isotope was stopped by the addition of 25 μl of 95% ethanol. Samples of 7 μl were removed for processing of labeled macromolecules as described by Eustice and Wilhelm (4) except that the hot trichloroacetic acid wash was omitted. Incorporation of isotope into cold trichloroacetic acid-precipitable material was measured in a Tri-Carb 2000CA liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.) with 3.0 ml of Econofluor-2. Results, expressed as percentage of uninhibited controls, were corrected for any isotope retained in the absence of cells. Absolute values for incorporation are given in the legends to the figures.

Preparation of extracts for cell-free protein synthesis studies. *E. coli* MRE 600 was grown to mid-log phase in 3.0 liters of Mueller-Hinton broth. Cells were collected by centrifugation at 5,000 × *g* for 10 min at 5°C. Cell extracts were prepared by grinding washed cell pellets with alumina, using a mortar and pestle as described previously (13). S-150 (supernatant liquid from a 150,000 × *g* centrifugation) was prepared by centrifugation of S-30 (supernatant from the 30,000 × *g* centrifugation) in a 70Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 2 h.

Preparation of high-salt-washed ribosomes and crude initiation factors. The partial purification of initiation factors from the high-salt wash of ribosomes was performed through the dialysis step after ammonium sulfate precipitation, as

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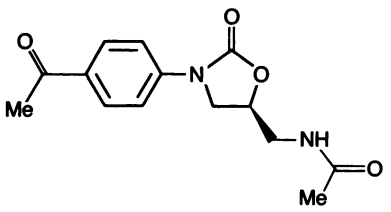

FIG. 1. Structure of DuP 721.

described by Dubnoff and Maitra (2). High-salt-washed ribosomes (ribosomal subunits) were prepared as described previously by Dubnoff and Maitra (2).

Aminoacylation of $tRNA_{f^{Met}}$ under formylating conditions. Aminoacylations were performed as described by Eustice and Wilhelm (5) except that the source of the synthetase and transformylase was as described by Dubnoff and Maitra (2). In addition, calcium leucovorin (calcium folinate) was added at 500 μg/ml, and *E. coli* $tRNA_{f^{Met}}$ was added at 4.0 A_{260} units/ml.

Purification of *E. coli* polysomes. Polysomes from *E. coli* MRE 600 were purified by the method of Girbes et al. (6).

Initiation reactions. Initiation reaction mixtures (100 μl) contained 100 mM Tris hydrochloride (pH 7.6), 80 mM NH_4Cl, 7.5 mM magnesium acetate, 8.0 mM dithiothreitol, 1.0 mM GTP, 240 pmol of *N*-formyl-[^{14}C]methionyl-$tRNA_{f^{Met}}$ (^{14}C-labeled fMet-tRNA), 200 μg of poly (G · U) per ml, 1,100 μg of initiation factors per ml, and 14 A_{260} units of high-salt-washed ribosomes per ml. The reaction mixture was incubated at 37°C for 10 min. Initiation complexes were recovered on type HA membrane filters (Millipore Corp., Bedford, Mass.) as described previously (5).

Elongation reactions. Reaction mixtures (50 μl) for protein synthesis programmed by synthetic templates contained 60 mM Tris hydrochloride (pH 7.6), 5.6 mM magnesium acetate, 60 mM NH_4Cl, 50 μM concentrations of each of the 19 naturally occurring L-amino acids except phenylalanine, 2.0 mM dithiothreitol, 1.0 A_{260} unit of *E. coli* tRNA per ml, 10 μM [^{14}C]phenylalanine (specific activity, 525 mCi/mmol), 17 mM KCl, 5.0 mM potassium phosphate (pH 7.2), 1.0 mM ATP, 0.5 mM GTP, 10 μg of pyruvate kinase per ml, 1.0 mM spermidine, 1.0 mM putrescine, 6.0 mM phosphoenolpyruvate, 15 A_{260} units of S-30 per ml, and 200 μg of poly(G · U_2) per ml. Reaction mixtures were incubated at 37°C for the times indicated, and 20-μl samples were processed for hot trichloroacetic acid-precipitable radioactivity as described before (4). When polysomes were translated, the magnesium acetate concentration was raised to 6.5 mM, [^{14}C]leucine was substituted for the [^{14}C]phenylalanine-labeled amino acid, and 2.0 A_{260} units of S-150 supernatant per ml was substituted for the S-30.

Preparation of cell extracts from DuP 721 growth-arrested cells. *E. coli* MRE 600 cultures (3 liters) were grown to 0.45 A_{540}unit/ml. DuP 721 was added to cultures at 64 μg/ml by the addition of 3.24 ml of a 20-mg/ml DuP 721 solution per liter in dimethyl sulfoxide, and incubation was continued for 20 min at 37°C with shaking. Cell-free extracts were made as described above except that all solutions contained 64 μg of DuP 721 per ml.

Translation of MS2 bacteriophage RNA. Reaction mixtures (50 μl) contained 40 mM Tris hydrochloride (pH 7.6), 4.0 mM magnesium acetate, 50 mM NH_4Cl, 2.0 mM dithiothreitol, 50 μM concentrations of all of the naturally occurring L-amino acids except lysine, 20 μM L-[^{3}H]lysine (specific activity adjusted to 830 mCi/mmol by addition of unlabeled L-lysine), 100 μm CTP, 1.5 mM calcium leucovorin, 5.0 mM potassium phosphate (pH 7.2), 1.0 mM ATP, 0.5 mM GTP, 10 μg of putrescine per ml, 6.0 mM phosphoenolpyruvate, and 15 A_{260} units of S-30 per ml. MS2 phage RNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was tested at 220 μg/ml, and incubation was at 37°C. At the times indicated in the figure legends, 20-μl samples were processed as described above for incorporation of L-[^{3}H]lysine into protein.

Initiation-dependent polypeptide synthesis with poly(G · U) as template. Reaction mixtures (50 μl) were prepared as described above for elongation reactions except that magnesium acetate was reduced to 3.0 mM and template-dependent incorporation of L-[^{14}C]phenylalanine was measured in the presence or absence of ^{14}C-labeled fMet-tRNA. Incorporation of L-[^{14}C]phenylalanine dependent on the presence of ^{14}C-labeled fMet-tRNA was determined by correcting for template-dependent incorporation of L-[^{14}C]phenylalanine in the absence of ^{14}C-labeled fMet-tRNA, as well as correcting for the template-dependent incorporation of ^{14}C-labeled fMet-tRNA in the presence of 10 μM unlabeled L-phenylalanine.

RESULTS

The MICs of DuP 721 and of reference antibiotics for *B. subtilis* and several strains of *E. coli* are shown in Table 1. *E. coli* PLB-3252 has a mutation in OmpF which renders this strain susceptible to antibiotics which do not normally inhibit the growth of these bacteria (1). Similarly, *E. coli* PLB-3252 also showed increased susceptibility to DuP 721 when compared with *E. coli* K-12. Other laboratory strains of *E. coli* showed some susceptibility to DuP 721 as well (Table 1). When *E. coli* ESCH-1 was treated with poly-L-lysine, the MIC of DuP 721 was reduced from 128 to 2 μg/ml (Table 2). Similarly, the perturbation of the outer membrane by poly-L-lysine resulted in an increased permeability to other antibacterial agents as well (Table 2) (11, 12). Subse-

TABLE 1. MICs of DuP 721 and other antibacterial agents for strains of *E. coli* and *B. subtilis*

Inhibitor	MIC (μg/ml) for:					
	B. subtilis 6633	*E. coli* strain				
		ESCH-1	PLB-3252	ESCH-K12	MRE 600	ESCH-140
DuP 721	0.25	128	32	>64	64	16
Novobiocin	NT[a]	128	32	>128	32	<1
Norfloxacin	0.06	0.03	<0.06	<0.06	1	<0.06
Rifampin	0.03	NT	0.25	>1	8	0.25
Erythromycin	0.125	NT	1	32	64	NT
Chloramphenicol	1	NT	1	4	32	1

[a] NT, Not tested.

TABLE 2. Effect of treatment with poly-L-lysine on susceptibility of *E. coli* ESCH-1 to DuP 721 and other antibacterial agents

Inhibitor	MIC (μg/ml)	
	No poly-L-lysine	Plus poly-L-lysine[a]
DuP 721	128	2
Cloxacillin	>128	<2
Novobiocin	128	<2
Erythromycin	32	<2

[a] The MIC of poly-L-lysine for *E. coli* was ≥2,000 μg/ml.

quent studies for further characterization of the mechanism of action of DuP 721 were carried out with *E. coli* MRE 600 or PLB-3252.

DuP 721 potently inhibited protein synthesis in *E. coli* PLB-3252 during pulse-labeling studies with L-[^{3}H]lysine (protein synthesis), [^{3}H]thymidine (DNA synthesis), or [^{3}H]uridine (RNA synthesis) (Fig. 2). The concentration required to inhibit 50% for protein synthesis was 3.8 μg/ml, but it was >64 μg/ml for both DNA and RNA syntheses. In parallel studies, drugs used as reference compounds, rifampin, chloramphenicol, and norfloxacin, showed their expected specificities for RNA, protein, and DNA syntheses, respectively.

To determine the effects of DuP 721 on protein synthesis in cell-free systems, cell extracts were prepared from *E. coli* MRE 600. DuP 721 did not inhibit 70S initiation complex formation with GUG initiation codons at concentrations up to 100 μM (27.6 μg/ml) under conditions in which the known inhibitors of initiation, aurin tricarboxylic acid and kasugamycin, were effective (Table 3). Similarly, DuP 721 did not inhibit this reaction when poly(A · U · G) or the trinucleotide ApUpG was used as template (data not shown). Furthermore, DuP 721 did not inhibit the formation of a peptide bond between fMet-tRNA and puromycin (data not shown).

To determine whether DuP 721 inhibited the elongation phase of protein synthesis, polysomes with natural mRNA purified from *E. coli* MRE 600 were used to measure nascent polypeptide chain elongation. DuP 721 was added at concentrations up to 1.0 mM (276 μg/ml). DuP 721 did not inhibit this phase of protein synthesis when measured with ribosomes that were already engaged in the elongation of peptides (polysomes) under conditions in which tetracycline and chloramphenicol effectively inhibited the process (Fig. 3).

TABLE 3. Effect of DuP 721 and other antibacterial agents on 70S initiation complex formation

Addition	Concn (μM)	^{14}C-labeled fMet-tRNA bound to Millipore HA membrane filters	
		pmol	%
None		6.0	100
DuP 721	10	5.5	91
	100	6.1	101
ATA[a]	10	4.5	75
	100	0	0
Kasugamycin	10	3.3	61
	100	2.3	38

[a] ATA, Aurin tricarboxylic acid.

To determine whether DuP 721 inhibited ribosome-dependent protein synthesis by interfering with a process that is not faithfully reproduced in cell-free systems, extracts were prepared from cells treated for 20 min with 64 μg of DuP 721 per ml. When these cell extracts were tested, the initiation-dependent incorporation of L-[^{3}H]lysine stimulated by natural mRNA (MS2 phage RNA) was found to be severely impaired (Fig. 4). This inhibition was not alleviated by dilution of the DuP 721 in the assay mixture to a concentration of 6.5 μg/ml. In addition, preliminary results showed that the addition of supernatant factors (S-150) from control S-30 did not overcome the impairment seen with MS2 phage template. In contrast, the same extracts impaired in the utilization of MS2 phage mRNA as template were fully active in the initiation-independent synthesis of polypeptides with poly(G · U$_2$) as template at 8.0 mM magnesium acetate (Fig. 5). Furthermore, S-150 prepared from these S-30 extracts efficiently supported the elongation of nascent polypeptides on polysomes (data not shown). Thus, extracts from DuP 721 growth-arrested cells were able to elongate polypeptides but were unable to utilize a natural mRNA for initiation.

To determine whether the initiation of protein synthesis was due to inhibition of a reaction before or after the

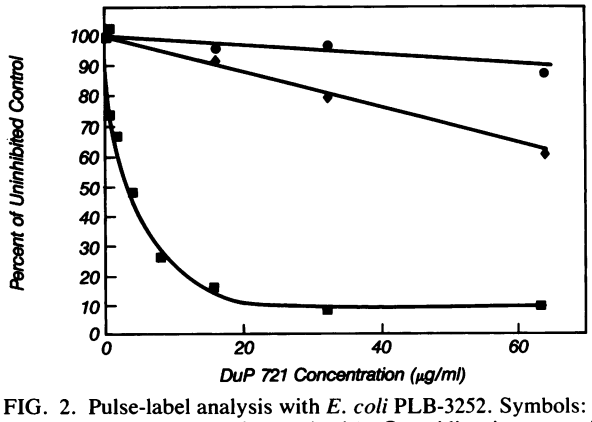

FIG. 2. Pulse-label analysis with *E. coli* PLB-3252. Symbols: ■, lysine incorporation (protein synthesis); ●, uridine incorporation (RNA synthesis); ◆, thymidine incorporation (DNA synthesis). Uninhibited controls incorporated 9.8 × 10^4 cpm of [^{3}H]lysine per 7.0 μl, 4.5 × 10^4 cpm of [^{3}H]uridine per 7.0 μl, and 9.9 × 10^3 cpm of [^{3}H]thymidine per 7.0 μl.

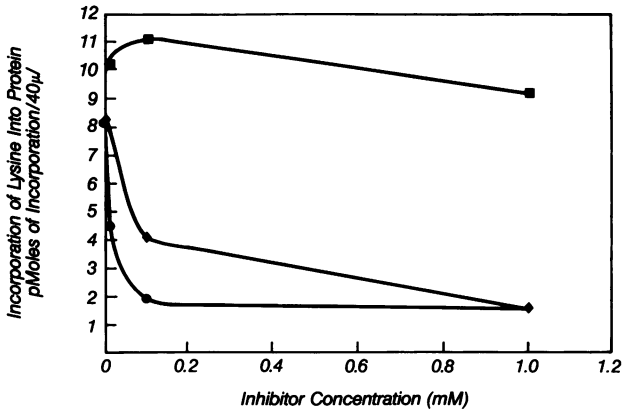

FIG. 3. Effect of DuP 721 on chain elongation with polysomes with natural mRNA. Symbols: ■, DuP 721; ◆, tetracycline; ●, chloramphenicol. Uninhibited controls incorporated 8.3 pmol of [^{14}C]leucine (6.3 × 10^3 cpm).

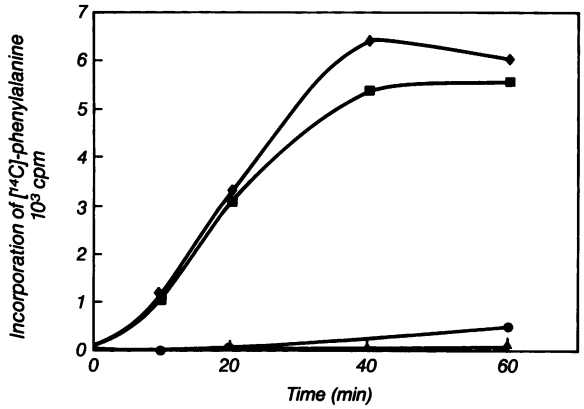

FIG. 4. Polypeptide synthesis with MS2 RNA by extracts from DuP 721-treated cells. Symbols: ■, control extract; ♦, control extract with 64 μg of DuP 721 per ml; ●, DuP 721-treated extract with 6.0 μg of DuP 721 per ml present after dilution of the DuP 721 in the assay mixture; ▲, DuP 721-treated extract with the DuP 721 concentration maintained at 64 μg/ml during the assay.

formation of the 70S initiation complex, a method for the measurement of initiation-dependent synthesis of polypeptides on mRNA lacking 3′ upstream sequences was developed. At low concentrations of magnesium ion (3.0 mM), poly(G · U)-dependent synthesis of polypeptides was substantially less than polypeptide synthesis in the presence of fMet-tRNA (Table 4). This result showed that substantial amounts of in vitro initiation were stimulated in these experiments by synthetic mRNA containing GUG codons but, otherwise, random trinucleotide sequences.

Extracts from DuP 721 growth-arrested cells were compared with extracts from cells grown in the absence of DuP 721 for activity in initiation-dependent polypeptide synthesis with poly(G · U) templates. Extracts from DuP 721 growth-arrested cells gave the same levels of incorporation as control extracts, indicating that extracts from DuP 721 growth-arrested cells could carry out the binding of fMet-tRNA to GUG codons and the subsequent enzymatic steps through chain elongation when the requirement for 3′ sequences upstream of the initiation codon was bypassed (Table 4).

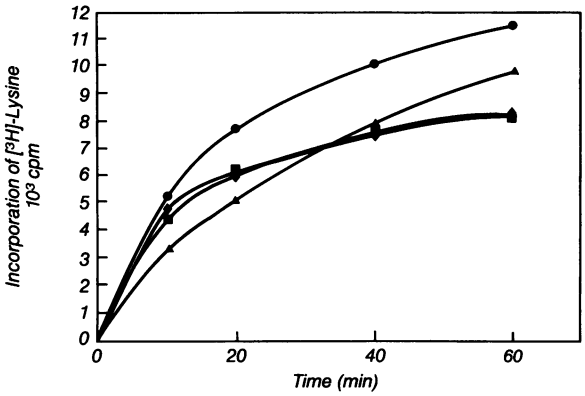

FIG. 5. Polypeptide synthesis with poly(G · U_2) by extracts from DuP 721-treated cells at 5.6 mM magnesium acetate. Symbols: ■, control extract; ♦, control extract with 64 μg/of DuP 721 per ml; ●, DuP 721-treated extract with 6.0 μg of DuP 721 per ml present after dilution of the DuP 721 in the assay mixture; ▲, DuP 721-treated extract with the DuP 721 concentration maintained at 64 μg/ml during the assay.

TABLE 4. Initiation-dependent protein synthesis with the synthetic mRNA poly(G · U) by extracts from DuP 721 growth-arrested cells

Source of extract	DuP 721 concn during assay (μg/ml)	$fMet\text{-}tRNA_{f^{met}}$-dependent incorporation of [^{14}C]phenylamine into protein (pmol)[a]
Control cells	0	23.8
	64.0	20.3
DuP 721 growth-arrested cells	6.5[b]	21.9
	64.0	23.4

[a] Incorporation of [^{14}C]phenylalanine into protein in the presence of ^{14}C-labeled fMet-tRNA was determined as described in Materials and Methods. Initiation-dependent incorporation of [^{14}C]phenylalanine was determined by subtracting [^{14}C]phenylalanine incorporation (35.9 pmol) in the presence of ^{14}C-labeled fMet-tRNA (2.1 pmoles of ^{14}C-labeled fMet-tRNA incorporated) from [^{14}C]phenylalanine incorporation (12.1 pmol) without added ^{14}C-labeled fMet-tRNA.

[b] Concentration of DuP 721 after the S-30 extract was diluted in the assay mixture.

DISCUSSION

DuP 721 is primarily an inhibitor of gram-positive bacteria and not active on normal *E. coli* cells (9). Here we have shown that the outer membrane of *E. coli* confers the intrinsic resistance of *E. coli* to DuP 721. An OmpF mutant of *E. coli* showed the same pattern of inhibition of RNA, DNA, and protein syntheses when tested during pulse-labeling experiments as was found for *B. subtilis* (3). Thus, the potent inhibition of protein synthesis by DuP 721 in the OmpF mutant indicates that the intracellular target of DuP 721 is the same in both gram-negative and gram-positive bacteria.

When DuP 721 was tested in a cell-free protein-synthesizing system, no inhibition of polypeptide chain elongation was found with a synthetic mRNA template (3). In addition, it was indicated that DuP 721 did not inhibit polypeptide chain termination (3). Results presented here showed that DuP 721 did not inhibit polypeptide chain initiation when added to cell extracts or to partially purified systems. In addition, DuP 721, at concentrations as high as 1.0 mM, was shown not to inhibit polypeptide chain elongation in cell-free systems directed by purified polysomes with natural mRNA. A similar lack of effect of DuP 721 on polypeptide chain elongation was seen in cell-free systems with MS2 phage mRNA and synthetic templates (data not shown).

Thus, DuP 721 potently inhibited protein synthesis in whole cells, but when the compound was added to cell-free systems, none of the reactions of protein synthesis were found to be sensitive. To determine whether DuP 721 inhibited a phase of protein synthesis that was not faithfully reproduced in the cell-free systems, extracts from DuP 721 growth-arrested cells were tested for ability to support the elongation and initiation reactions. Extracts from DuP 721 growth-arrested cells were fully competent in supporting polypeptide chain elongation but were totally devoid of any ability to initiate protein synthesis on natural mRNA templates. Thus, DuP 721 requires the native intracellular environment to exert its inhibitory effect on protein synthesis, and this effect is exerted at or before the initiation step.

Since the poly(G · U) template (which lacks the upstream ribosome-binding sequences) was efficiently used by control

extracts for the fMet-tRNA-dependent synthesis of polypeptides, the normal requirements for a well-defined ribosome-binding site (7) were circumvented under the conditions used. Extracts from DuP 721 growth-arrested cells were also fully competent in supporting polypeptide synthesis in this system.

Since extracts from DuP 721 growth-arrested cells initiated protein synthesis with poly(G · U) as template but not with natural mRNA, DuP 721 must inhibit an early event in initiation, preceding the interaction among fMet-tRNA, the initiator codon, and the 30S subunit. Thus, the evidence suggests that DuP 721 may inhibit recognition of the 3′ upstream ribosome-binding sequence present in natural mRNAs.

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