[CANCER RESEARCH 46, 922-925, February 1986]

Effects of Luzopeptins on Protein B23 Translocation and Ribosomal RNA Synthesis in HeLa Cells

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ABSTRACT

Localization of protein B23 in HeLa cells after treatment with luzopeptin A and its analogues was studied using indirect immunofluorescence. Bright nucleolar fluorescence was observed in control HeLa cells. After treatment with luzopeptin A (50 ng/ml), luzopeptin B (500 ng/ml), or luzopeptin D (10 ng/ml) for 2 h, uniform nucleoplasmic rather than specific nucleolar fluorescence was observed. Luzopeptin C had no effect on protein B23 translocation.

Luzopeptin D, A, and B inhibited [^{3}H]uridine incorporation into the trichloroacetic acid insoluble fraction of HeLa cells with 50% inhibitory concentration values of 3.7 ± 1.1 (SD), 10.8 ± 2.1, and 122.0 ± 34.0 ng/ml, respectively. Less than 10% inhibition of [^{3}H]uridine incorporation was found with luzopeptin C (500 ng/ml and 2 h incubation). Ribosomal RNAs (28 and 18S) were isolated from HeLa cells treated with luzopeptin D (50 ng/ml; 2 h). They were then separated and analyzed in 1% agarose gel electrophoresis. There were 90.1 ± 1.38 and 95.0 ± 1.04% inhibition of [^{3}H]uridine incorporation into 28 and 18S ribosomal RNA, respectively.

The order of potency for the loss of nucleolar fluorescence and the concurrent increase in nucleoplasmic fluorescence was luzopeptin D> luzopeptin A> luzopeptin B≫ luzopeptin C, which correlates with the order of their 50% inhibitory concentration values for inhibition of [^{3}H]uridine incorporation. With 34–55% inhibition of RNA synthesis, both nuclear and nucleolar B23 immunofluorescence were observed. With 70–85% inhibition of RNA synthesis, a uniform nucleoplasmic fluorescence was observed. These results indicate that translocation of protein B23 as observed by indirect immunofluorescence may be a rapid and simple screening test for the selection of antitumor agents which inhibit ribosomal RNA synthesis.

INTRODUCTION

Luzopeptins A, B, C, and D (formally BBM-928) (1–8) are a family of actinoleukin-like antibiotics which contain two substituted quinoline chromophores linked by a cyclic decadepsipeptide. Luzopeptin A is a monoacetyl derivative of luzopeptin B and a diacetyl derivative of luzopeptin C. The luzopeptins exhibit antimicrobial activity against gram-positive bacteria (1). Leuzopeptin A is the most active against bacteria; D and B are less active, and C is the least active. These analogues are also active against various mouse tumors. Luzopeptin A is more potent than luzopeptin B, and luzopeptin C has no antitumor activity (1).

Previous studies by viscometry and fluorometry (2) suggest a bifunctional intercalation of luzopeptin A with DNA which involves both quinoline chromophores. Its DNA-binding properties may result in inhibition of DNA synthesis or RNA synthesis. Recent studies (9) suggest that luzopeptin C, which has no antitumor activity, is slightly more effective than luzopeptins B and A in bifunctional DNA intercalation and DNA-DNA intermolecular cross-linking. These results suggest that the antitumor activity of the luzopeptins may involve other actions in addition to interaction with DNA. This paper reports the effect of luzopeptins on the translocation of nucleolar phosphoprotein B23 from nucleoli into the nucleoplasm which, as noted earlier (10), correlates with the inhibition of RNA synthesis.

MATERIALS AND METHODS

Drugs. Luzopeptins A, B, C, and D were generous gifts from Dr. B. Long of the Bristol-Baylor Laboratories, Baylor College of Medicine, Houston, TX.

Radioactive Material. [^{3}H]Uridine (specific activity, 16 Ci/mmol) was purchased from ICN Chemical and Radioisotope Division.

Chemicals and Culture Materials. Minimum essential medium (Eagle's), fetal calf serum, glutamine, and penicillin-streptomycin solutions were purchased from Grand Island Biological Co., Grand Island, NY. EM grade formaldehyde solutions were purchased from Polysciences, Inc., Warrington, PA. Other chemicals were of reagent grade.

Cells. HeLa S-3 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum, glutamine, and antibiotics in a 5% CO_2 humidified incubator at 37°C. For immunofluorescence studies, cells were grown on slides in a Petri dish.

Antibodies. The monoclonal antibody to protein B23 (37/5.1) was produced by *in vitro* fusion techniques (11). Antibodies were collected from the hybridoma cell culture medium and purified by ammonium sulfate precipitation.

Immunofluorescence. HeLa cells were fixed in 2% EM grade formaldehyde in PBS[2] for 20 min at room temperature. The cells were permeabilized with acetone at −20°C for 3 min. After a wash with PBS, the fixed cells were incubated with the monoclonal antibody (diluted 1:16) at 37°C for 1 h. Then cells were washed 4 times for 10 min each in PBS and incubated with fluorescein conjugated, affinity purified, goat anti-mouse IgG (diluted in 1:20 with PBS) at 37°C for 35 min. The cells were then washed 4 times for 10 min each with PBS and mounted in 50% glycerol in PBS (pH 9).

[^{3}H]Uridine Incorporation Determination. HeLa cells (approximately, 1 × 10^6) were preincubated with luzopeptins for 30 min before [^{3}H]-uridine (2.5 μCi/ml) was added. They were further incubated at 37°C for various intervals. The cells were scraped from slides and collected in centrifuge tubes, washed with PBS, and precipitated with 1.0 ml of 10% trichloroacetic acid (0°C). The pellets were then washed 3 times with

Received 9/30/85; accepted 11/4/85.

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[1] These studies were supported by Cancer Research Center Grant CA-10893, P1, awarded by National Cancer Institute, Department of Health and Human Services, USPHS; the DeBakey Medical Foundation; the Davidson Fund; the Pauline Sterne Wolff Memorial Foundation; the H. Leland Kaplan Cancer Research Endowment; the Linda and Ronny Finger Cancer Research Endowment Fund; and the William S. Farish Fund.

[2] The abbreviations used are: PBS, phosphate buffered saline; IC_{50}, concentration of drug that produces 50% inhibition.

cold 10% trichloroacetic acid. The residues were solubilized in 1 N NaOH and the radioactivity of each sample was determined in a Packard liquid scintillation counter after 5.0 ml of Aquasol were added.

Protein Determination. Protein was determined by the Bio-Rad protein assay (12).

Characterization of RNA. Total cellular RNA was extracted by a modification of the procedure of Tavitian *et al.* (13). Approximately 3×10^6 cells were harvested and resuspended in 2 ml of 0.1 M sodium acetate, pH 5.1,:0.3% sodium lauryl sulfate. RNA was extracted with an equal volume of 90% distilled phenol at room temperature. The RNA in the aqueous layer was precipitated with 2 vol of 2.0% potassium acetate in ethanol and kept at −20°C for 2 h. The precipitate was collected by centrifugation, redissolved in H_2O, and analyzed by 1.0% agarose gel electrophoresis in buffer (0.089 M Tris base:0.089 M boric acid:0.002 M EDTA) (14). RNAs were stained with ethidium bromide and visualized with a UV lamp. The 28 and 18S RNA bands were excised from the gel and the [^{3}H]uridine radioactivities were determined in liquid scintillation counter after 10 ml of Aquasol were added.

RESULTS

Localization of Protein B23. The effects of luzopeptins A, B, C, and D on the localization of nucleolar phosphoprotein were examined in HeLa cells using indirect immunofluorescence. In control HeLa cells, bright nucleolar fluorescence but little or no nucleoplasmic fluorescence was observed after immunostaining with the anti-protein B23 antibody (Fig. 1*a*). After treatment with luzopeptin A (50 ng/ml; 2 h), luzopeptin B (500 ng/ml; 2 h), or luzopeptin D (10 ng/ml; 2 h), a uniform nucleoplasmic fluorescence was observed (Fig. 1*c*). When HeLa cells were treated with reduced amounts of luzopeptins A (10 ng/ml), or B (50 ng/ml) for 2 h, both nucleoplasmic and nucleolar fluorescence were observed (Fig. 1*b*). Luzopeptin C (500 ng/ml) did not alter protein B23 nucleolar localization after 4 h of treatment.

Table 1 summarizes the effect of the luzopeptins on the localization of protein B23. The order of potency for the reduction of nucleolar and the concurrent increase in nucleoplasmic fluorescence was luzopeptin D> luzopeptin A> luzopeptin B≫ luzopeptin C.

Effects of Luzopeptins upon RNA Synthesis. Inhibition of RNA synthesis was studied with HeLa cells treated with luzopeptins. RNA synthesis was measured as the incorporation of [^{3}H]uridine into trichloroacetic acid-insoluble material. To estimate the relative potency of the luzopeptins in terms of RNA synthesis inhibition, the IC_{50} values were determined using a 2-h incubation interval. The IC_{50} values of luzopeptins D, A, and B were 3.7 ± 1.1 (SD), 10.8 ± 2.1, and 122.0 ± 34.0 ng/ml, respectively (Table 2). Luzopeptin C inhibited RNA synthesis less than 10% at a concentration of 500 ng/ml (Table 2). The order of IC_{50} values for inhibition of RNA synthesis correlated well with the order of the loss of nucleolar fluorescence and the increase of nucleoplasmic fluorescence (Table 2).

Correlation of RNA Synthesis and Localization of Protein B23. Table 3 shows the effects of luzopeptins on the RNA synthesis and the cellular localization of protein B23 at various drug concentrations after 2 h of incubation. Luzopeptins A (5 ng/ml), and B (50 ng/ml) inhibited RNA synthesis 34–50%; both nucleolar and nuclear fluorescence were observed at these concentration levels (see Fig. 1*b*). Luzopeptins A (50 ng/ml), B (500 ng/ml), and D (10 ng/ml) inhibited RNA synthesis 75% or more; these cells had only nucleoplasmic fluorescence (results

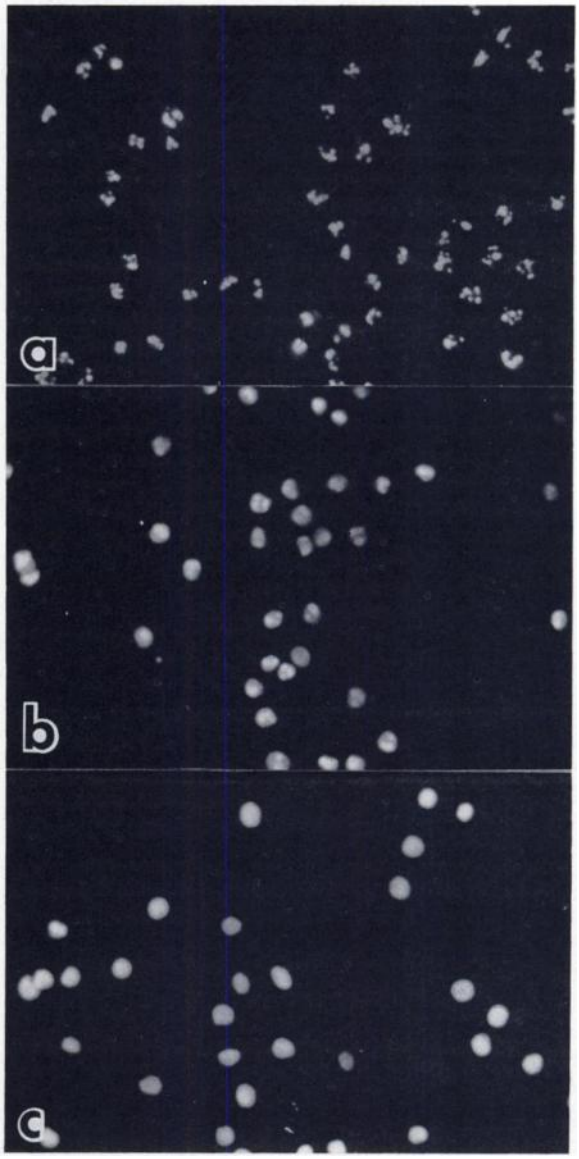

Fig. 1. Studies of the effect of luzopeptins on the cellular localization of protein B23. HeLa cells were grown on slides. Luzopeptin A or its analogue was added to cultures and incubated at 37°C for various times before the cells were fixed and immunostained by protein B23 antibody. *a*, control HeLa cells; only nucleolar fluorescence was observed; *b*, after treatment with luzopeptin A (10 ng/ml; 2 h) or luzopeptin B (50 ng/ml; 2 h), both nuclear and nucleolar fluorescence were observed; *c*, maximum effect after treatment with luzopeptin A (50 ng/ml; 2 h), luzopeptin B (500 ng/ml; 2 h), or luzopeptin D (10 ng/ml, 2 h) in which only nuclear fluorescence was observed.

are similar to those shown in Fig. 1*c*). Luzopeptin C (500 ng/ml) resulted in less than 10% of RNA synthesis inhibition; no change of protein B23 nucleolar localization was observed (see Fig. 1*a*). These results indicate that a direct relationship exists between luzopeptin-induced protein B23 "translocation" from the nucleolus to nucleoplasm and the inhibition of RNA synthesis.

Effects of Luzopeptin D on rRNA Synthesis. Inhibition of rRNA synthesis in HeLa cells was observed after treatment with luzopeptin D (50 ng/ml; 2 h) (Table 4). Total cellular RNA was isolated and then analyzed by 1.0% agarose gel electrophoresis (Fig. 2). Two major bands corresponding to 18 and 28S RNA were observed in both the control and the luzopeptin D-treated

Table 1

Effect of luzopeptins on the cellular localization of protein B23

HeLa cells were cultured on slides in minimum essential medium (Eagle's). Luzopeptin A or its analogues were added for 2 or 4 h before the cells were fixed and immunostained by protein B23 antibody. Viability of the cells was over 95% under these conditions.

	Immunofluorescence at following luzopeptin doses and times						
		10 ng/ml		50 ng/ml		500 ng/ml	
	0	2 h	4 h	2 h	4 h	2 h	4 h
Luzopeptin A	A[a]	B[b]	C[c]	C	C	C	C
Luzopeptin B	A	A	A	B	B	C	C
Luzopeptin C	A	A	A	A	A	A	A
Luzopeptin D	A	C	C	C	C	C	C

[a] A, over 90% of the cells showed bright nucleolar with little or no nuclear fluorescence (Fig. 1*a*). About 1200 cells were counted.
[b] B, over 90% of the cells showed both nuclear and nucleolar fluorescence (Fig. 1*b*). About 1200 cells were counted. This is an intermediate phenomenon between A and C.
[c] C, over 95% of the cells showed homogeneous nuclear fluorescence. No distinct nucleolar fluorescence was observed (Fig. 1*c*). About 1200 cells were counted.

Table 2

Summary of the activities of luzopeptins

HeLa cells cultured in minimum essential medium (Eagle's) were preincubated with various doses of luzopeptin A, B, C, or D (1–500 ng/ml) for 30 min before [^{3}H]uridine was added. Cultures were incubated at 37°C for 2 h and [^{3}H]-uridine incorporation was determined.

Luzopeptin	Dose of drug (ng/ml) that produces IC_{50} RNA synthesis inhibition	Translocation of protein B23 from nucleoli to nucleoplasm
A	10.8 ± 2.1[a]	++[b]
B	122.0 ± 34.0	+
C	Less than 10% inhibition at 500	No activity
D	3.7 ± 1.1	+++

[a] SD of 4 experiments.
[b] +++, most potent; +, least potent.

HeLa cells. The minor bands (about 15) might be degradation products or other species of RNAs. The patterns and intensities of these bands in both control and drug-treated RNAs were similar. There was a higher molecular weight band, which might be the 45S RNA or nonspecific aggregates, observed in the control but not in the drug-treated cells. The incorporation of [^{3}H]uridine into the 28 and 18S rRNA was measured. It was found that 90.1 ± 1.38% of 28S rRNA and 95.0 ± 1.04% of 18S rRNA synthesis were inhibited after treatment with luzopeptin D.

DISCUSSION

As shown in Table 2, the order of potency of luzopeptins for RNA synthesis inhibition and protein B23 translocation is luzopeptin D> A> B≫ C, which correlated with their antitumor activities against leukemia P388 cells as reported by Ohkuma *et al.* (1). Luzopeptin D is the most potent luzopeptin analogue. It is even more potent than actinomycin D (10) in its ability to inhibit RNA synthesis and in protein B23 translocation. Recent DNA binding studies by Huang and Crooke (9) indicated that the factors contributing to the differences in the antitumor activity of luzopeptins are not due to differences in direct interactions with DNA. The present studies suggest that two other effects of the antitumor action of luzopeptin are inhibition of rRNA synthesis and translocation of protein B23.

The cause-effect relationship of protein B23 translocation and inhibition of RNA synthesis is not known at present. Previous studies (10)[3] indicated that whenever the processing of 45S

Table 3

Correlation of RNA synthesis and location of protein B23

HeLa cells were cultured on slides. Doses of luzopeptin A or its analogues were added to the culture medium. The cellular incorporation of [^{3}H]uridine and the localization of protein B23 were then determined after 2 h of incubation. Viability of the cells as determined by trypan blue dye exclusion was over 95% under these conditions.

	Dose (ng/ml)	Inhibition of RNA synthesis (%)[a]	Immuno-fluorescence
Luzopeptin A	0	0[b]	A[c]
	5	34.0 ± 3.1[d]	B[e]
	10	49.5 ± 4.3	B
	25	59.5 ± 2.9	B
	50	84.3 ± 5.7	C[f]
Luzopeptin B	0	0	A
	10	15.1 ± 2.2	A
	50	45.2 ± 4.7	B
	500	75.3 ± 4.3	C
Luzopeptin C	0	0	A
	10	2.2 ± 1.9	A
	50	2.0 ± 2.2	A
	500	9.0 ± 1.7	A
Luzopeptin D	0	0	A
	10	78.8 ± 4.1	C
	50	92.9 ± 3.7	C

[a] Percentage of RNA synthesis inhibition was calculated as

$$100 \times \left(1 - \frac{\text{[}^3\text{H]uridine uptake in treated HeLa cells}}{\text{[}^3\text{H]uridine uptake in control HeLa cells}}\right)$$

[b] There were about 2.0×10^3 cpm [^{3}H]uridine incorporated/1.0 mg of protein in control HeLa cells.
[c] A, over 90% of the cells had bright nucleolar fluorescence with little or no nuclear fluorescence (Fig. 1*a*). About 1200 cells were counted.
[d] Mean ± SD of 3 experiments.
[e] B, over 90% of the cells had both nuclear and nucleolar fluorescence (Fig. 1*b*). About 1200 cells were counted. This is an intermediate phenomenon between A and C.
[f] C, over 95% of the cells had homogeneous nuclear fluorescence but no distinct nucleolar fluorescence was observed (Fig. 1*c*). About 1200 cells were counted.

Table 4

Effect of luzopeptin D on inhibition of rRNA synthesis

HeLa cells were preincubated with luzopeptin D (50 ng/ml) for 30 min before [^{3}H]uridine was added. Cultures were incubated at 37°C for 2 h. RNA was isolated and analyzed in 1.0% agarose gel (see Fig. 2). The 28 and 18S bands were sliced out. They were solubilized in H_2O (60°C) and the radioactivity of each sample was determined in a Packard liquid scintillation counter after 10.0 ml of Aquasol were added.

	[^{3}H]uridine incorporation into 28S rRNA			[^{3}H]uridine incorporation into 18S rRNA		
	cpm/absorbance at 260 nm		Inhibition of synthesis (%)	cpm/absorbance at 260 nm		Inhibition of synthesis (%)
Experiment	Control	Luzopeptin D		Control	Luzopeptin D	
1	3174	286	91.0	2028	86	95.8
2	3193	366	88.5	2562	152	93.8
3	2719	251	90.8	1758	84	95.3
Mean ± SD		90.1 ± 1.38			95.0 ± 1.04	

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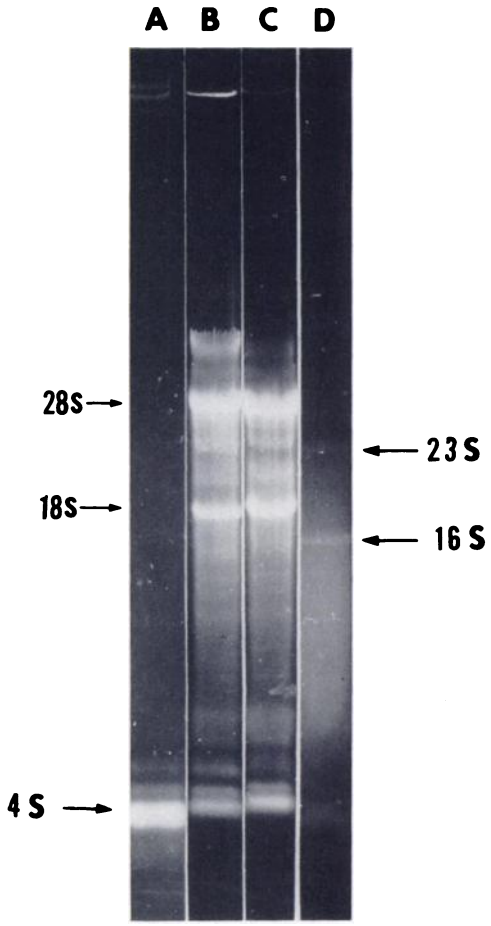

Fig. 2. Effect of luzopeptin D on the inhibition of rRNA synthesis. HeLa cells were preincubated with luzopeptin D (50 ng/ml) for 30 min before [^{3}H]uridine was added. Cultures were incubated at 37°C for 2 h before the cells were harvested. RNA was isolated and analyzed as described in "Materials and Methods." *Lane A*, 4S tRNA; *Lane B*, control; *Lane C*, luzopeptin D treated; *Lane D*, 23 and 16S rRNA from *Escherichia coli* (Boehringer Mannheim).

rRNA or pre-ribosomal ribonucleoprotein is inhibited by antibiotics (actinomycin D, toyocamycin, and high-dose α-amanitin), protein B23 translocated from the nucleolus to the nucleoplasm. Protein synthesis inhibitors (cycloheximide or puromycin) did not produce this translocation. Two protein B23 species were identified in nucleoli (15); only the lower molecular weight form was found predominantly in nucleoplasm. It is conceivable that protein B23 is involved in ribosome assembly and ribosome transportation from nucleoli to the nucleoplasm.

In this paper, we report that there is a correlation between the translocation of protein B23, RNA synthesis inhibition, and antitumor activity. These results indicate that translocation of protein B23, as observed by the indirect immunofluorescence method, may be a useful screening test for the selection of antitumor agents which inhibit RNA synthesis. This rapid indirect immunofluorescence method is sensitive, inexpensive, and simple. Since many important antitumor agents inhibit RNA synthesis, this method may be generally useful in screening novel antineoplastic agents.

ACKNOWLEDGMENTS

We wish to thank Dr. B. Long in the Bristol-Baylor Laboratories for providing the luzopeptins A, B, C, and D, Dr. R. Reddy and D. Henning for helping in the RNA analysis, and Drs. L. C. Yeoman, R. L. Ochs, and J. Freeman for reviewing the manuscript.

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