DEOXYSPERGUALIN, A POTENT ANTITUMOR AGENT: FURTHER STUDIES ON THE CYTOBIOLOGICAL MODE OF ACTION†

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Under otherwise identical conditions, deoxyspergualin preferentially inhibits the growth of the T-cell leukemia line L5178y; an effective dose for a 50% inhibition (ED_{50}) of 0.0007 μM was determined. A much weaker cytostatic activity was found for murine lymphocytes (ED_{50}: approximately 25 μM) and for CV-1 monkey kidney cells (ED_{50}: 16.3 μM). Deoxyspergualin causes biphasic and differential effects on DNA metabolism of murine T and B lymphocytes. At lower concentrations (0.3~5 μM) the [^{3}H]TdR incorporation into non-activated or lipopolysaccharide-activated lymphocytes is significantly stimulated by the compounds; this effect was not observed with lymphocyte cultures stimulated with concanavalin A. This change of TdR incorporation rates was found to parallel with the variations of DNA polymerase α activity. Deoxyspergualin causes an additive effect together with bleomycin and a significant synergistic cytostatic effect in combination with avarol and avarone. Moreover, it is reported that deoxyspergualin causes neither a selective inhibitory effect on DNA-, RNA- or protein synthesis nor an alteration of the intracellular distribution pattern of the Ro and La antigens. However, detailed enzymic studies revealed that deoxyspergualin reduces DNA polymerase α but not β activity in lymphocytes at the ED_{50} concentration of this compound. These results support previous documentations that deoxyspergualin is of potential clinical usefulness (a) in treatment of certain tumors and (b) in organ transplantation.

15-Deoxyspergualin is a derivative of the antitumor antibiotic spergualin[1,2], which was discovered in the culture filtrates of the bacterial strain BMG162-aF2. The pharmacological activities of deoxyspergualin and spergualin are characterized by the following properties; (a) strong antitumor activity *in vitro*[3] and *in vivo*[2], (b) moderate activity against a series of microorganisms[2], and (c) prolonged graft survival time in several transplantation models[4]. From these data it was concluded[5] that deoxyspergualin/spergualin combines strong antitumor (anti-leukemic) activity (at low doses of the compounds) with immunosuppressive action (at high doses of the compounds). The cyto-biological mode of action causing this obvious specific dualistic effect is not known.

In this paper we describe (a) the cell specific cytotoxic effect caused by deoxyspergualin *in vitro* as well as (b) further cyto-biological experiments, helping to understand the mode of action of the compound.

Materials and Methods

Materials

Materials were obtained as followes: Concanavalin A (Con A) (No. C 5275) and lipopolysaccharide (LPS) (No. L 4130) were obtained from Sigma, St Louis, MO, U.S.A.; [*methyl*-^{3}H]thymidine

† This paper is dedicated to Prof. Dr. HAMAO UMEZAWA.

(TdR) (specific activity 87 Ci/mmol), [^{3}H]dCTP (30 Ci/mmol), [^{3}H]uridine (8.4 Ci/mmol) and [^{3}H]phenylalanine (9.3 Ci/mmol) from the Radiochemical Centre, Amersham, UK.

15-Deoxyspergualin was obtained from Prof. Dr. H. UMEZAWA; it was synthesized as described[1]. Stock solutions were prepared in distilled water (pH 6.0). Bleomycin (clinical mixture, containing 55~70% A_2; 25~32% B_2; <7% A_2; and <1% B_4) was obtained from H. MACK, Illertissen, West Germany. Avarol and avarone were prepared as described, and stored in dimethyl sulfoxide[6].

Cell Culture

L5178y mouse lymphoma cells[7] were grown in EAGLE's minimum essential medium supplemented with 10% fetal calf serum in roller tubes[8,9]. For the dose response experiments, 5-ml cultures were initiated by inoculation of 5×10^3 cells/ml and were incubated at 37°C for 72 hours; the controls showed a generation time of 12.5 hours. The cell growth was estimated by cell count with a Cytocomp counter (128-channel counter; system Michaelis; Mainz, West Germany)[10]. In the absence of inhibitors, the cell concentration after a 72-hour incubation period was determined to be 2.8×10^5 cells/ml.

Mixed lymphocyte cultures were performed as described[10,11]. Spleen lymphocytes were prepared from NMRI mice. Macrophage-containing lymphocytes (2.5×10^6 cells) were placed into a final volume of 200 μl in microtiter plates and incubated for 72 hours in DULBECCO's minimal essential medium supplemented with 10% fetal calf serum. 18 hours prior to the end of the incubation 0.1 μCi of [^{3}H]TdR was added to each cup. Where indicated 2 μg/ml of Con A or 20 μg/ml of LPS were added to the cultures. Incorporation of [^{3}H]TdR was determined as described[10,11]. The compounds were added at time zero to the cultures.

CV-1 monkey kidney cells (ATCC CCL 70) were cultivated in monolayer cultures in growth medium supplemented with 10% fetal calf serum[12]. The cells were planted at a density of 5×10^4 cells per 60-mm plastic petri dish (Falcon Plastics) and incubated for 72 hours in the presence of the compounds in 5% CO_2[6]. At the end, the cell number was determined. The generation time was 20.4 hours; the cell concentration at the end of the incubation period was 2.4×10^5 cells/plate.

All cell culture experiments were performed in fetal calf serum which contained only very little amine oxidase. Applying the described assay procedure[9], the level of amine oxidase was determined to be less than 1×10^{-6} M H_2O_2 generated per 30 minutes.

Each value came from 10 parallel experiments. The ED_{50} concentrations (50% effective inhibitory dose) causing a 50% inhibition of cell growth (in the experiments with L5178y and CV-1 cells) or a 50% reduction of [^{3}H]TdR incorporation (in the studies with mixed murine lymphocytes) were estimated by logit regression[13].

The mathematical evaluation of the fractional inhibitory concentration index (FIC indexes) of deoxyspergualin given in combination with bleomycin, avarol or avarone were performed according to published equations[14] and experimental procedures[15]. FIC>1, antagonism; FIC=1, additive effects; FIC<1, suggestive of synergism; FIC<0.5, significant synergism. These studies were performed with L5178y cells (5,000 cells/ml as initial concentration; incubation period: 72 hours).

Incorporation of Nucleic Acid and Protein Precursors

For the determination of DNA, RNA and protein synthesis, 5-ml suspensions of exponentially growing L5178y cells at 100,000 cells/ml were treated for 24 hours with deoxyspergualin. The labeled precursors (10 μCi each/5-ml culture) were added 1 hour prior to harvest to the cultures. Samples of 1 ml were analyzed for cell concentration (results given in number of doubling steps[16]) and for acid-insoluble radioactivity[17].

Assay of DNA Polymerase Activities in Lymphocytes

The culture conditions for mixed lymphocytes were essentially as described above. DNA polymerase α and β activities in macrophage containing lymphocytes were determined exactly as described previously[18]. Briefly, the enzymes were extracted from 10^8 cells, 72 hours after addition of deoxyspergualin. Where indicated 2 μg Con A/ml or 20 μg LPS/ml were added to the cultures. The two DNA polymerases were separated by sucrose gradient centrifugation. Then the fractions from the gradient were collected and the enzyme activities were determined[18]. The DNA polymerase α mixture

consisted of (0.1 mM concentrations each) [^{3}H]dCTP (specific activity 30 cpm/pmol), dATP, dGTP, dTTP, 20 mM potassium phosphate buffer (pH 7.2), 1 mM 2-mercaptoethanol, 8 mM $MgCl_2$, and 0.5 A_{260} units of activated herring sperm DNA. The DNA polymerase β mixture was identical with the polymerase α mixture, except that 50 mM ammediol (pH 8.8), rather than potassium phosphate, was used as buffer. Before being mixed with the reaction mixture for DNA polymerase β, the enzyme fraction was preincubated at 0°C with 0.5 mM *N*-ethylmaleimide for 10 minutes. The activities are given as nmol of radioactive deoxyribonucleotide incorporated per hour.

Monoclonal Antibodies Against La and Ro Antigens

The preparation of monoclonal antibodies against homogeneous Ro and La antigens was described previously[19].

Immunofluorescence Microscopy

CV-1 cells were grown for 72 hours on cover-slips in the presence of 0 or 16.3 μM (=ED_{50} concentration; Table 1) of deoxyspergualin. Then the cells were fixed and stained directly with fluorescein isothiocyanate coupled anti-Ro or anti-La monoclonal antibodies as described previously[19].

Results

Cytostatic Activity

It has been shown previously that the amine oxidase level present in serum used for *in vitro* culturing of cells can strongly influence the cytotoxic effect of deoxyspergualin[3]. Therefore we have performed all of our *in vitro* studies with fetal calf serum, which was determined to lack almost completely amine oxidase. Under identical incubation conditions deoxyspergualin strongly inhibited the proliferation of the T-lymphoma cell line L5178y (ED_{50}: 0.0007 μM); Table 1. CV-1 monkey kidney cells and mixed murine lymphocytes were far more resistant towards this antibiotic. This is in contrast to the experiments with bleomycin, which caused an inhibition of the different cell types at almost identical concentrations. Avarol and avarone inhibited primarily growth of L5178y cells and of lymphocytes, while CV-1 cells were almost insensitive towards these compounds.

Interesting was the finding that deoxyspergualin differentially influenced [^{3}H]TdR incorporation into DNA of murine T and B lymphocytes (Fig. 1). The baseline incorporation rates in the cultures were as follows; without mitogen, $0.69\pm0.1\times10^3$ dpm/10^6 cells per 18 hours and in the presence of 2 μg Con A/ml or 20 μg LPS/ml, $17.9\pm1.1\times10^3$ dpm or $14.8\pm1.0\times10^3$ dpm/10^6 cells per 18 hours, respectively. The Con A-activated lymphocytes showed a typical dose-response curve without any

Table 1. Inhibitory potencies of deoxyspergualin, bleomycin, avarol and avarone on L5178y mouse lymphoma cells, mixed murine lymphocytes and CV-1 cells.

The parameters for the estimation of the ED_{50} values were in the case of L5178y and CV-1 cells the cell count and for mixed murine lymphocytes the reduction of [^{3}H]TdR incorporation. The values represent the means of ten parallel experiments each; the means±SD are given.

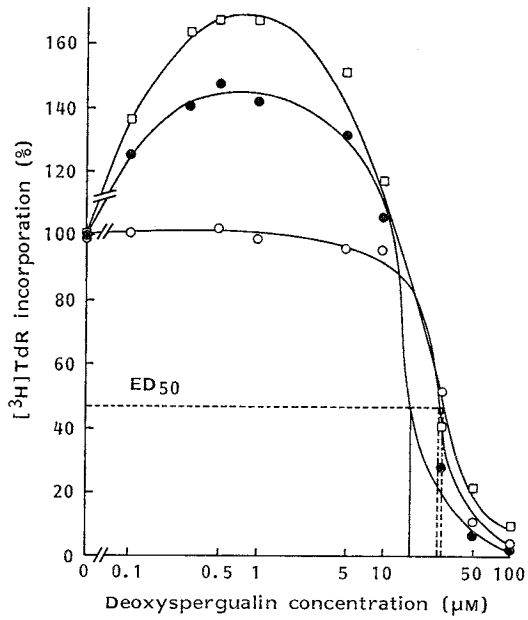
Compound	ED_{50} (μM)				
	L5178y cells	Mixed murine lymphocytes			CV-1 cells
		None	Con A	LPS	
15-Deoxy-spergualin	0.0007±0.0001	17.4±1.2	31.7±2.5	28.0±1.8	16.3±2.6
Bleomycin	0.94±0.06	0.4±0.1	1.4±0.1	0.6±0.1	0.7±0.1
Avarol	0.93±0.13	3.8±0.3	2.4±0.2	5.9±0.4	24.9±3.6
Avarone	0.62±0.11	2.9±0.2	1.9±0.2	4.3±0.3	19.7±3.4

stimulatory effect at sub-cytotoxic concentrations. However, the [^{3}H]TdR incorporation rates of both non-stimulated and LPS-stimulated lymphocytes were determined to be significantly augmented at a deoxyspergualin concentration within the range 0.3~5 μM. The maximal stimulation was measured at 0.5~1.0 μM with 173% (controls: 100%) for LPS-stimulated cells and with 150% for non-stimulated lymphocytes.

To rule out the possibility that the changes of the measured thymidine incorporation rates are due to possible changes in the thymidine pool sizes during incubation, additional enzymic studies were performed. The results revealed that in the absence of deoxyspergualin (Fig. 2) both Con A and LPS caused an increase of the DNA polymerase α activity in the lymphocytes. DNA polymerase β activity was found to be not influenced under these conditions. A quantitative comparison showed (Table 2) that LPS and Con A caused a 4.1-fold and 4.4-fold increase of DNA polymerase activity. Addition of 1 μM deoxyspergualin resulted in an increase of DNA polymerase α activity only in the controls (1.3-fold) and in LPS-treated lymphocytes (1.4-fold), but not in Con A-treated cells (Table 2). Hence, we have two independent series of experiments from which we conclude that deoxyspergualin caused a stimulation of DNA synthesis in LPS-stimulated and non-stimulated lymphocytes.

Fig. 1. Effect of deoxyspergualin on [^{3}H]TdR incorporation into murine mixed spleen lymphocyte cultures.

The lymphocytes were incubated in the absence (●) or the presence of 2 μg Con A/ml (○) or 20 μg LPS/ml (□). Addition of [^{3}H]TdR was 18 hours prior to the end of incubation. Deoxyspergualin was added at time zero. Means of five parallel experiments are presented; the SD was less than 8%. The values for a 50% inhibition of [^{3}H]-TdR incorporation of the corresponding dose-response experiments are given as vertical lines.



Influence on Synthesis of Macromolecules *In Vitro*

This set of experiments was performed with exponentially growing L5178y cells. The cells were incubated for 24 hours at different concentrations of deoxyspergualin; the radioactively labeled precursors were added 1 hour prior to harvest. Both cell growth and incorporation rate were determined (Table 3). The results revealed that almost parallel with the reduction of the growth rates (given in doubling steps per 24 hours) the incorporation rates of all three precursors decreased.

A more detailed analysis of the deoxyspergualin effect on the level of DNA polymerases revealed (Table 2) that this compound, at the respective ED_{50} concentrations (summarized in Table 1), caused a 1.7-fold reduction of DNA polymerase α activity in control lymphocytes and a 5.8-fold reduction or 3.9-fold reduction in LPS- or Con A-treated spleen cells; DNA polymerase β activity was not influenced at all. From these findings we conclude that at least one mode of action of deoxyspergualin is the inhibition of DNA polymerase α activity in the intact cell system.

Combination Studies of Deoxyspergualin with Bleomycin and Avarol

Given in combination the two polyamine containing compounds deoxyspergualin and bleomycin

Fig. 2. Alterations of DNA polymerase α and β levels in cells from mouse spleen in the absence or the presence of the mitogens Con A and LPS.

Mixed lymphocyte cultures were incubated in the absence (●) or the presence of 2 μg Con A/ml (○) or 20 μg LPS/ml (□) in the standard assay. Then the cells were harvested and homogenized. The cell free supernatant extracts were run through sucrose gradients. The resulting fractions were analyzed for DNA polymerase α and β activities. Details are given under "Methods". α and β mark the respective DNA polymerase.

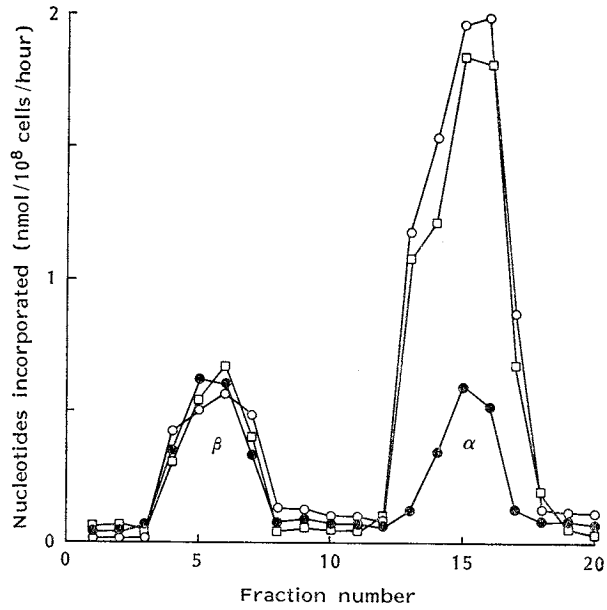


Table 2. DNA polymerase activities in cells from mouse spleen.

Mixed lymphocyte cultures were prepared and incubated in the absence or presence of mitogens (20 μg LPS/ml or 2 μg Con A/ml) as well as in the absence or the presence of deoxyspergualin. DNA polymerase activities were determined after separation of the lymphocyte extracts by sucrose density gradient centrifugation (see Fig. 2). Further details are given under "Methods".

Lymphocyte cultures	15-Deoxyspergualin (μM)	DNA polymerase activity (nmol/10^8 cells/hour)	
		Form α	Form β
Saline control	0	1.7	1.9
	1.0	2.3	1.9
	17.4	1.0	2.0
LPS-treated	0	6.9	2.0
	1.0	9.7	1.9
	28.0	1.2	1.9
Con A-treated	0	7.4	2.1
	1.0	7.1	2.0
	31.7	1.9	2.1

inhibit L5178y cell growth in an additive or slightly suggestive synergistic manner (FIC values: 0.78~0.87); Table 4. However, if deoxyspergualin was added to the cultures in combination with avarol or avarone a strong synergistic inhibitory interaction was determined (FIC values: 0.29~0.42).

Effect on the Intracellular Distribution of the Ro and La Antigens

By applying direct immuno-cytochemical procedures, we recently described that in CV-1 cells (a)

Fig. 3. Single direct immunofluorescence microscopy of CV-1 cells with fluorescein isothiocyanate coupled-anti-La monoclonal antibody (a and b) or anti-Ro monoclonal antibody (c and d).
The cells were incubated with 0 (a and c) or 16.3 μM of deoxyspergualin (b and d).
Magnification: a and b, ×550; c and d, ×1,100.

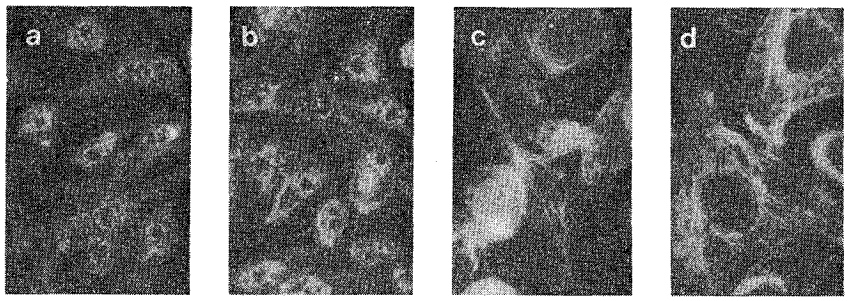


Table 3. Influence of deoxyspergualin on the synthesis of macromolecules in exponentially growing L5178y cells.
The experiments were performed as described under "Methods". The values represent means of five parallel experiments. The SD does not exceed 10%.

15-Deoxy-spergualin (μM)	Doubling steps	Incorporation into macromolecules/100,000 cells					
		[^{3}H]TdR		[^{3}H]Uridine		[^{3}H]Phenylalanine	
		dpm	%	dpm	%	dpm	%
0	1.77	203,429	100	10,428	100	6,952	100
0.0005	1.35	158,675	78	8,760	84	4,936	71
0.0010	0.94	124,092	61	7,195	69	4,032	58
0.0020	0.51	48,823	24	3,441	33	1,182	17

the La antigen is localized predominantly in the nucleus and (b) the Ro antigen resides primarily in the cytoplasm[18]. The staining pattern obtained with monoclonal antibodies against La is of the speckled type (Fig. 3a) and that against Ro of a fibrous type (Fig. 3c). These characteristic patterns were found to remain unchanged even after an incubation of the CV-1 cells for 72 hours in the presence of the ED_{50} concentration (=16.3 μM) of deoxyspergualin (Figs. 3b and 3d).

Table 4. Fractional inhibitory concentration indexes (FIC indexes) for deoxyspergualin in combination with bleomycin, avarol or avarone on L5178y cells.
The concentration ratios are based on X μM deoxyspergualin to Y μM of the second cytostatic agent.

Drug combination	Concentration ratio	FIC index
Deoxyspergualin: bleomycin	0.0004 : 1.0	0.87
	0.0007 : 1.0	0.78
	0.0009 : 1.0	0.82
Deoxyspergualin: avarol	0.0004 : 1.0	0.32
	0.0007 : 1.0	0.29
	0.0009 : 1.0	0.37
Deoxyspergualin: avarone	0.0004 : 0.5	0.42
	0.0007 : 0.5	0.39
	0.0009 : 0.5	0.41

Discussion

From the foregoing results it is obvious that deoxyspergualin causes a strong anti-leukemic activity *in vitro*, as determined with the L5178y mouse lymphoma cell line. The cytostatic activity against this cell line (ED_{50}: 0.0007 μM) is several thousand-fold higher compared to the one determined for murine normal lymphocyte cultures (ED_{50}: approximately 25 μM), under otherwise identical culture conditions. This finding was unexpected and requires further investigations. One possible reason for the obtained differential cytostatic activity could be a difference in the level of amine oxidase. This enzyme was

suggested to be necessary for "activation" spergualin/deoxyspergualin[3]; *e.g.* by converting the terminal amino group to the corresponding aldehyde derivative. The finding that lymphocytes are relatively insensitive towards deoxyspergualin supports previous data showing a lack of bone marrow toxicity by this compound[4].

Deoxyspergualin is a polyamine-like compound[1]. Hence it was interesting to determine its *in vitro* effect when it is co-administered with the polyamine containing cytostatic agent bleomycin. Using L5178y cell system we established that these two compounds affect cell growth in an additive manner. This finding might have some impact on future clinical trials. Although bleomycin, a DNA-degrading compound[20], and deoxyspergualin, an agent which does not change the integrity of DNA, have different modes of action on the molecular level, they cause only an additive inhibitory effect in the intact cell system. The inability of deoxyspergualin to induce breakage of an isolated covalently closed circular DNA (pBR322) was found in *in vitro* studies (unpublished). Moreover, we established deoxyspergualin not to cause frame-shift and point mutations in the *Salmonella* mutagenicity test system (unpublished). On the other hand the "T-lymphotropic" and anti-human T-lymphotropic retroviral agents avarol and avarone[10,21], which interfere with microtubule formation during mitotic events[22], were determined to act highly synergistically in combination with deoxyspergualin. Hence, the combination of deoxyspergualin with the antimitotic agent avarol for the treatment of leukemia *in vivo* is warranted; animal experiments with the L5178y mouse lymphoma system are currently being performed.

A further important result, presented in this paper, is the finding that the [^{3}H]TdR incorporation rate as well as the activity of DNA polymerase α of non-stimulated as well as LPS-stimulated mixed lymphocyte cultures is significantly enhanced in the presence of sub-cytostatic concentrations of deoxyspergualin. This finding is in contrast to the data obtained with Con A-stimulated lymphocytes, which do not show this effect. From these results we conclude that deoxyspergualin displays a biphasic effect on non-stimulated lymphocytes and B lymphocytes; activation of blastogenesis at lower doses of deoxyspergualin and suppression of this response at higher compound doses. On the other side, T lymphocytes are only monophasically affected (suppression of blastogenesis) by deoxyspergualin. Hence, these *in vitro* data support previous conclusions, that spergualin or its analogues might be potentially useful in organ transplantation[4].

The molecular mode of action of deoxyspergualin remains unclear. First, we established that it causes no specific effect on overall synthesis of macromolecules (DNA, RNA and protein) *in vitro*. However, detailed enzymic studies revealed that deoxyspergualin reduced DNA polymerase α activity in lymphocytes which had been treated with this compound at ED_{50} concentrations. In contrast, DNA polymerase β activity remained unchanged even under those concentrations of deoxyspergualin which caused a 50% reduction of [^{3}H]TdR incorporation rate. Furthermore, this cytostatic agent does not alter the intracellular distribution of the Ro and La antigens, which are frequently found in sera from patients suffering from systemic lupus erythematosus[18,23].

Acknowledgments

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