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EFFECTS OF STRYCHNOPENTAMINE ON CELLS CULTURED IN VITRO

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SUMMARY

This paper describes the powerful cytotoxic action exerted by strychnopentamine (SP), a dimeric indole alkaloid extracted from *Strychnos usambarensis* Gilg, on B16 melanoma cells and on non-cancer human fibroblasts cultured in vitro. SP strongly inhibits cell proliferation and induces cell death at a relatively low concentration (< 1 μg/ml) after 72 h of treatment in the two lines. Incorporation of [^{3}H]thymidine and [^{3}H]leucine by B16 cells significantly decreases after only 1 h of treatment at 0.5 μg/ml. SP induces the formation of dense lamellar bodies and vacuolization in the cytoplasm, intense blebbing at the cell surface and various cytological alterations leading to cell death.

Key words: Strychnopentamine – Dimeric indole alkaloid – B16 melanoma – Non-cancer fibroblasts – Cytotoxicity

INTRODUCTION

Extensive studies performed in our Laboratory on the constituents of African *Strychnos* species have led to the isolation and structure determination of numerous indole alkaloids. A preliminary screening by a microtest was realized on the 46 indole alkaloids available in our laboratory with the view of detecting eventual cytotoxic properties [1]. Among them, strychnopentamine (SP) was the

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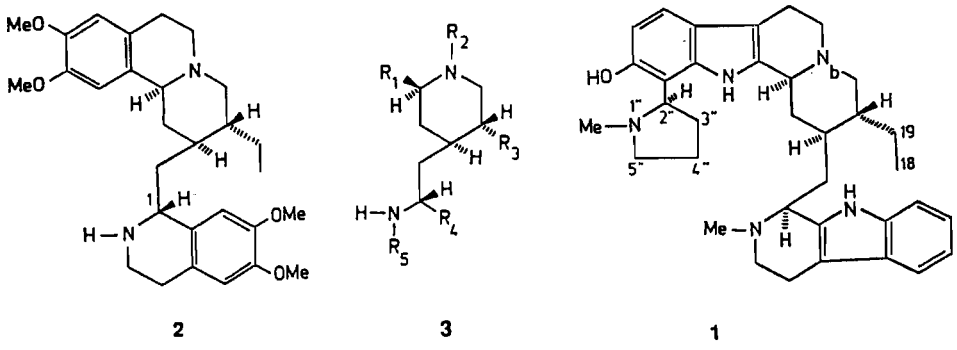


2 3 1

Fig. 1. Structural requirements for an emetine-like inhibition of protein synthesis (3) as compared to emetine (2) and strychnopentamine (1) [5].

most active compound [2]. Strychnopentamine (Fig. 1(1)), isolated from the leaves and stembark of *Strychnos usambarensis* Gilg [3 – 4] is a dimeric indole alkaloid, the structure of which can be related to emetine (Fig. 1(2)), which exerts a well known anticancer activity. Emetine can inhibit protein synthesis by acting on the 40S-ribosomal subunit and on peptide chain elongation; in fact, it prevents ribosomal translocation along mRNA. Unfortunately, emetine has severe side effects, especially at the neuromuscular and cardiovascular levels [5].

Grollman established the molecular structure requirements for inducing inhibition of protein synthesis by emetine analogues [5]. These requirements are: the (R) configuration at C-1′ and the secondary nitrogen atom at the 2′-position (Fig. 1(3)). In strychnopentamine, the carbon atom corresponding to C-1′ has the (S) configuration and the nitrogen is substituted by a methyl group (Fig. 1(2)). More recently, Gupta and coworkers [6] explained the activity of emetine and analogues by the possibility, for these molecules, to take a planar conformation with their two aromatic rings in the same plane and a nucleophilic element, such as a nitrogen atom, at a certain distance. The crystallographic data and the chemical shifts of several carbon atoms of SP showed that this molecule cannot take the required planar configuration [7 – 8]. Furthermore, SP has interesting in vitro antimalaric properties on *P. falciparum* at a concentration of 0.09 μg/ml, whereas chloroquine is active at 0.2 μg/ml on this strain [9]. In the present work, we analyse more precisely and with several methods the effects of SP in actively proliferating mammalian cells in vitro.

MATERIALS AND METHODS

Cell lines

B16 melanoma cells from a pigmented primitive tumor in a C57BL/6J mouse and Flow 2002 human embryonic fibroblasts obtained from Flow Laboratories were used.

Cultures on slides

B16 melanoma: 75 000 cells were seeded into Petri dishes (2 cm diameter) containing a slide (1.8 cm × 1.8 cm) and 2 ml of Minimum Eagle's Medium supplemented with 10% foetal calf serum (MEM-FCS-GIBCO) and 200 IU penicillin per ml. Flow 2002: 125 000 cells were seeded into Petri dishes (2 cm diameter) containing a slide (1.8 cm × 1.8 cm) and 2 ml of Basal Eagle's Medium supplemented with 10% foetal calf serum, 1% MEM nonessential amino acids (BEM-NEAA-FCS-GIBCO) and 200 IU penicillin per ml.

The experiments started after 24 h of incubation in this medium at 37°C, in an air-CO_2 5% incubator. After elimination of the nutrient medium, the cells were treated with 2 ml of the same medium but containing the required quantity of alkaloid (SP) under its acetate form (range of concentrations tested: from 0.4–5 μg/ml).

After treatment, the cells were fixed by a solution of absolute ethanol-acetone (1:1) for 24 h at 4°C and then absolute ethanol at 4°C. The cell DNA content was stained after hydrolysis by Feulgen reaction (Schiff reagent with *p*-rosaniline).

On these slides, we calculated the number (‰) of pycnotic and of mitotic cells, the percentages of the different phases of mitosis and the number (‰) of pigmented cells. For each condition, at least two slides were analysed and 4000 cells counted. The results were compared to control values by the statistical ANOVA test.

The DNA content of B16 melanoma cells was measured after Feulgen reaction by cytophotometric absorption in individual cells and with a Vickers M 86 microdensitometer. The reference mean diploid DNA value was measured in mouse lymphocytes stained together. Some of these slides were fixed with a formaldehyde solution containing calcium ions and the lipids were stained by fat red.

Cell counts, DNA, proteins and melanin measurements

B16 melanoma cells (350 000 or 2 000 000) were seeded into Petri dishes (6 cm diameter) containing 5 ml of medium (MEM-FCS 10%-penicillin 200 IU/ml), for respectively 72 h or 24 h of treatment by SP from 0.4–5 μg/ml.

The experiments started after 24 h of incubation as described above. After treatment, the cells were rinsed with PBS (phosphate buffer saline solution), trypsinated (trypsin 0.1% – EDTA 0.02%) and suspended in 1.5 ml PBS. In each case, the contents of two dishes were mixed in order to minimize possible differences due to seeding. One ml of this solution was used for cell counts (on a Thoma slide) or for DNA measurements. The remaining suspension was centrifuged at 1300 rev./min for 3 min, PBS was eliminated and 1.5 ml NaOH 1 N-DMSO 10% added for protein and melanin assays. For protein measurements, the method described by Bradford [10] was used and absorbance at 570 nm was obtained with a multiwell scanning spectrophotometer (Biotec Elisa 309 Reader); bovine serum albumin was used as a reference; 10 μl of the NaOH-DMSO solution neutralized with 10 μl HCl (1 N) were necessary. For melanin measurements, absorbance at 450 nm was measured with an Elisa reader. Four wells, each containing 200 μl of NaOH-DMSO solution were measured for each

set of experimental conditions. With purified melanin (Sigma) dissolved in the same solution, the linearity of absorbances was verified between 1 and 24 μg/ml. For DNA measurements, the fluorimetric method with bis-benzimidazole [11] was used after sonication of 900 μl of the cell suspension in PBS. Measurements were made with a Perkin-Elmer spectrometer. In each case, at least four dishes were used. The results are expressed as mean values.

Incorporation of [^{3}H]TDR or [^{3}H]leucine

Treatment (72 h) by 0.4 μg/ml of SP. B16 melanoma cells were plated in Petri dishes and treated as above for 48 h. [^{3}H]TDR (2 Ci/ml, 5 Ci/mM, Amersham) was added to the culture medium (final concentration 0.19 μCi/ml). After 24 h of incubation at 37°C in an air-CO_2 incubator, the culture medium was eliminated and the cells rinsed with 3 × 2 ml PBS, trypsinated (trypsin 0.1% – EDTA 0.02%) and resuspended in 3 ml PBS. The contents of two dishes were also mixed here. After cell count (Thoma slide), the cell suspensions were centrifuged (3 min at 1300 rev./min) and the cells then resuspended in 500 μl NaOH 1 N. After 24 h, the solution was neutralized by 500 μl HCl 1 N and the radioactivity was measured in aliquots of 300 μl of these solutions to which 2 ml of scintillating liquid (Aquasol II) was added. The radioactivity was counted with a β-spectrometer (Packard-Tri-Carb-3255). At least four dishes were analysed in each case. The results are expressed as mean values.

Treatment (1 h) by SP from 0.5–20 μg/ml. B16 melanoma cells (50 000) were seeded into each well of a Nunc 96-well plate with 250 μl of medium (MEM-FCS) containing 10 μCi/ml [^{3}H]TDR (2 Ci/ml, 5 Ci/mM, Amersham) or 5 μCi/ml [^{3}H]Leucine (1 Ci/ml, 44 Ci/mM, Amersham) and the required quantity of SP in its acetate form.

The cells were then incubated for 1 h at 37°C in an air-CO_2 5% incubator and then collected on a filter (Skatron-11731) with a semiautomated cell harvester (Skatron-7019). The dry filters were then placed separately in a vial containing 3 ml of scintillating liquid (Lumac Lipoluma 1057). The radioactivity was then counted with a β-spectrometer. Eight wells were used for each condition. The results have been compared to control values by the statistical test of Mann-Whitney.

Electron microscope observations

Transmission electron microscopy on B16 melanoma cells. Cells (300 000) were seeded into Petri dishes (2 cm diameter) with 2 ml of medium and 5 000 000 cells were seeded into T_{75} flasks with 10 ml of medium (MEM-FCS-penicillin). After 24 h of incubation, the cells were treated as above (2 or 10 ml of medium containing the required quantity of SP). After treatment, the cells were fixed in situ in Petri dishes or, as pellets, after scratching and centrifugation of the cells, in the case of the T_{75} flasks. The cells were then dehydrated and the inclusion performed in Epon 812. Ultrathin sections were contrasted and observed with a Jeol 100CX II electron transmission microscope.

Scanning electron microscopy. B16 melanoma cells (100 000) were seeded into Petri dishes (2 cm diameter) containing a slide (1.8 cm × 1.8 cm) and 2 ml of me-

dium (MEM-FCS-penicillin). The treatment started after 40 h of incubation. After treatment (2 ml fresh medium containing 4 – 5 or 6 μg/ml SP), the cells were fixed with Osmium tetroxide, rinsed and then dehydrated.

The preparations were then covered with a 200 Å gold-palladium film by ion beam sputtering (Hummer I). The observations were performed with a Jeol-JSM-840A at 20 eV.

RESULTS

The number (‰) of mitotic cells and of pycnotic nuclei were calculated after the action of SP on cell populations cultured on cover-slips. No differences were observed concerning the phases of the mitosis. No significant effect was noted on the number of pigmented cells. As shown in Table I, SP, after 72 h of treatment, is cytotoxic for B16 melanoma cells from 0.5 μg/ml on. It is a little less active on 2002 fibroblasts. SP appears to be about one tenth as cytotoxic than emetine but is more active than isoemetine which possesses the (S) configuration at C-1′. SP might thus have a different mode of action. So we decided to analyse in more detail the activity of this compound on living cells.

In a second experiment, cells were treated for 3 days with a culture medium containing 0.4 μg/ml SP. This concentration was selected because it induces a measurable decrease of the number of cells but not their complete destruction as this occurs at 0.5 μg/ml under the same experimental conditions (Table II). These experiments show that SP induces a decrease in the average total quantity of melanin, proteins and DNA per culture dish. Nevertheless, no decrease in the

TABLE I

EFFECTS OF SP ON CELLS CULTURED IN VITRO AFTER 72 h OF TREATMENT

M.I. mitotic index. P.I. pycnotic index. *Significant (ANOVA test – $P < 0.004$).

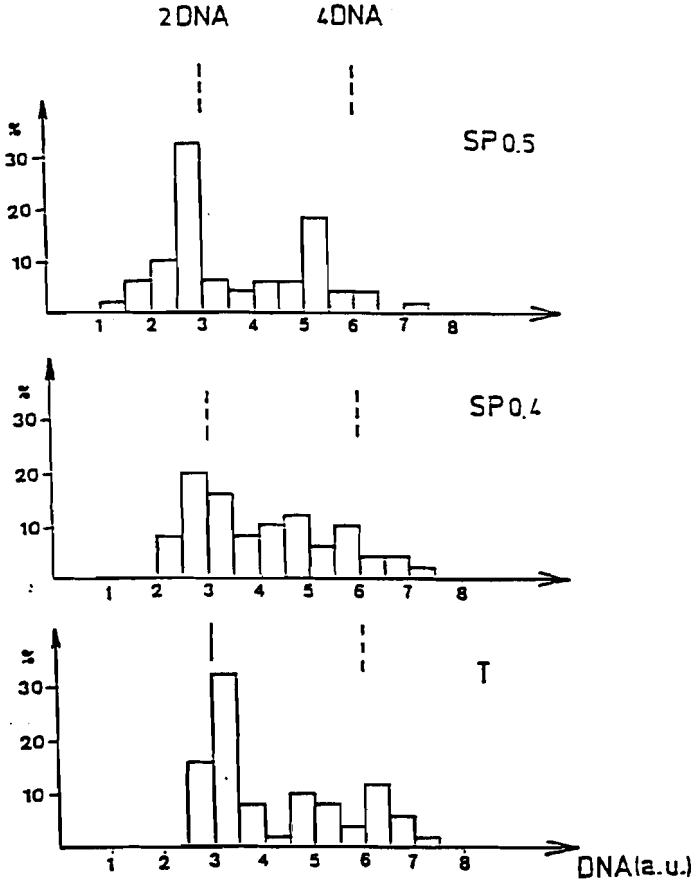
	Conc. (μg/ml)	B16		2002			
		M.I. (‰)	P.I. (‰)	M.I. (‰)	P.I. (‰)	M.I. (‰)	P.I. (‰)
Controls		28.9	19.3	8.3	8.1	10.8	2.5
Emetine	0.05	1*	455*	–	–	2.65*	21.5*
	0.1	0*	740.5*	–	–	1*	16.5*
	0.5	0*	792.5*	–	–	0*	62.5*
Isoemetine	1	29.25	30.6	–	–	11.65	3.3
	5	21.2	68.2	–	–	10.3	2.65
	10	0*	211.5*	–	–	–	–
SP	0.5	23.4	173.5*	6.25	10.5	–	–
	1	0*	1000*	0*	327.6*	0*	1000*
	5	0*	1000*	0*	1000*	0*	1000*

TABLE II

EFFECTS OF SP ON THE NUMBER OF B16 MELANOMA CELLS AND ON THEIR PROTEINS, MELANIN AND DNA CONTENTS

A_{mel}, absorbance due to melanin. All these results are expressed as percentages of control values considered as 100%.

	Treatment duration								
	72 h		24 h						
SP conc. (μg/ml):	0.4	0.5	1.5	2	2.5	3	3.5	4	4.5
No. of cells	75.3	0	115	75	63	17.8	10.5	5.2	0
$A_{mel}/10^6$ cells	131.3	0	99	143	127	240	254	170	0
μg proteins/10^6 cells	129.3	0	104	148	103	147	145	72	0
μg DNA/10^6 cells	97.5	0	74	86	95	176.5	161	216	0
A_{mel}/μg DNA	126	0	118	141	121	148	156	79	0
μg proteins/μg DNA	141.5	0	126	151	112	83	91	24	0



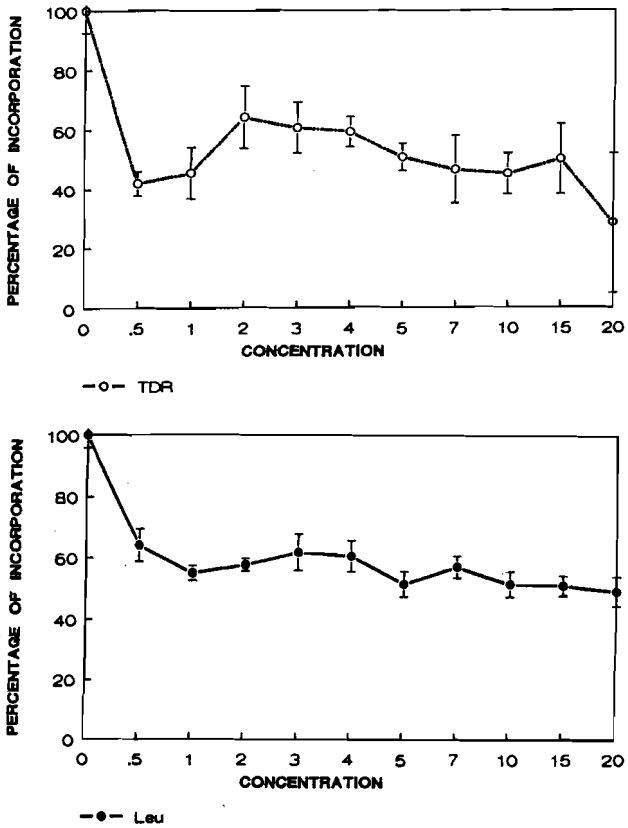


Fig. 3. [^{3}H]Thymidine (TDR) or [^{3}H]leucine (Leu) uptake by B16 melanoma cells as a function of the concentration of SP (μg/ml). Cells were incubated for 1 h with TDR or Leu and SP. Control values were considered as 100%. Vertical lines represent standard errors. All differences between treated and control groups were significant (Mann-Whitney test).

average amount of DNA, proteins or melanin per remaining cell is noted (Table II). No effects on the incorporation of [^{3}H]thymidine by the treated cells were observed when labelled thymidine was added during the last 24 h of such a 72 h treatment. Here the only measurable effect was a significant decrease of the number of living cells. The DNA content in individual cells was measured after Feulgen reaction by cytophotometry (Fig. 2). No differences between control and cells treated for 72 h were observed. However, in the treated populations,

Fig. 2. DNA content measured by cytophotometric absorption in individual cells after Feulgen reaction. B16 melanoma control cells: (T) or treated with strychnopentamine (SP) 0.5 or 0.4 μg/ml during 72 h. In ordinates, percentages of nuclei in each class. In abscissae, DNA contents in arbitrary units (a.u.). Nuclei (100) were measured in each case. The continuous line at the top of the control histogram indicates the mean diploid value (2DNA) measured on mouse leucocytes.

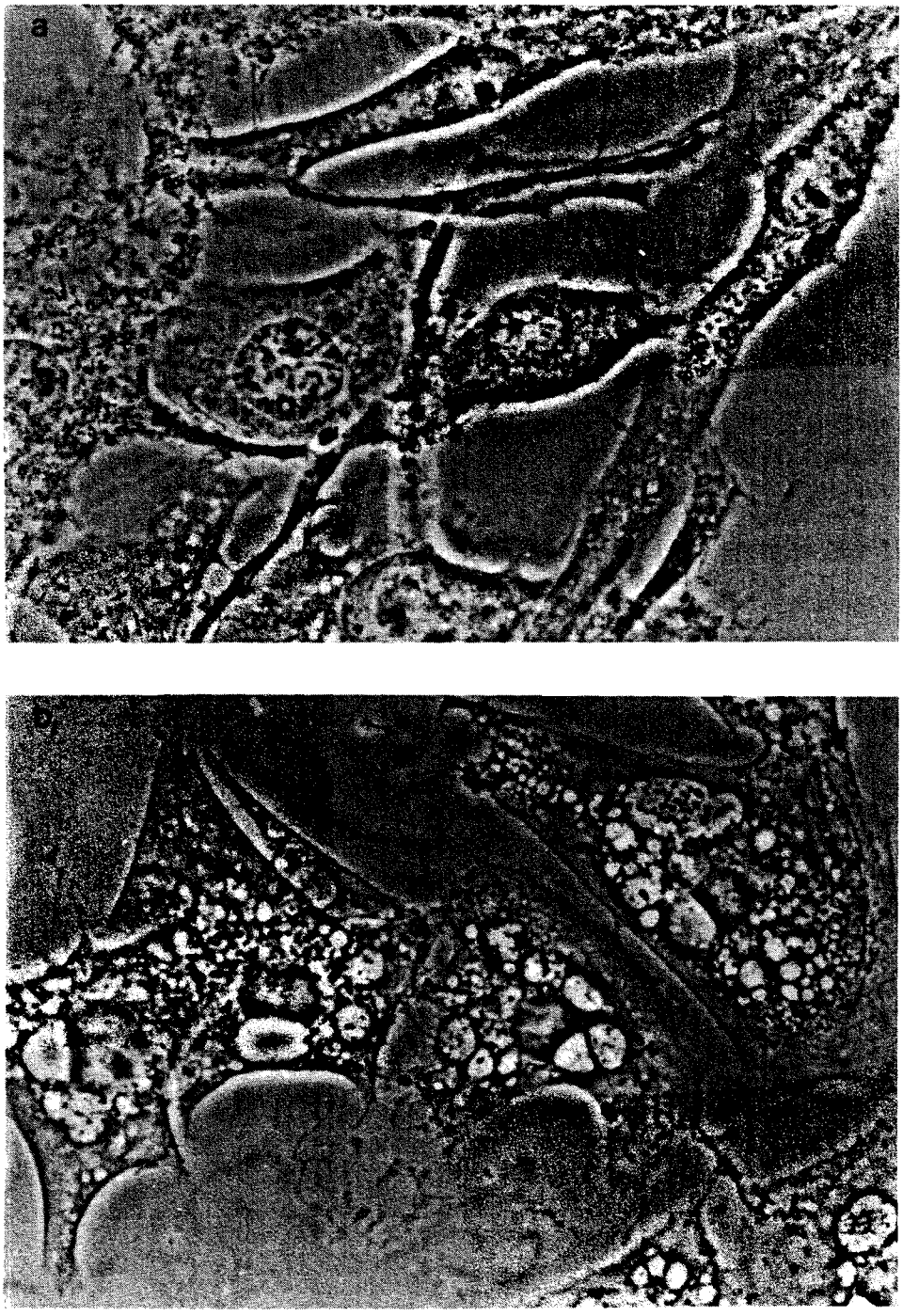


Fig. 4. Cells observed under an inverted phase contrast microscope (× 1170); (a) control cells, (b) living cells after 1 h of treatment with SP 5 μg/ml.

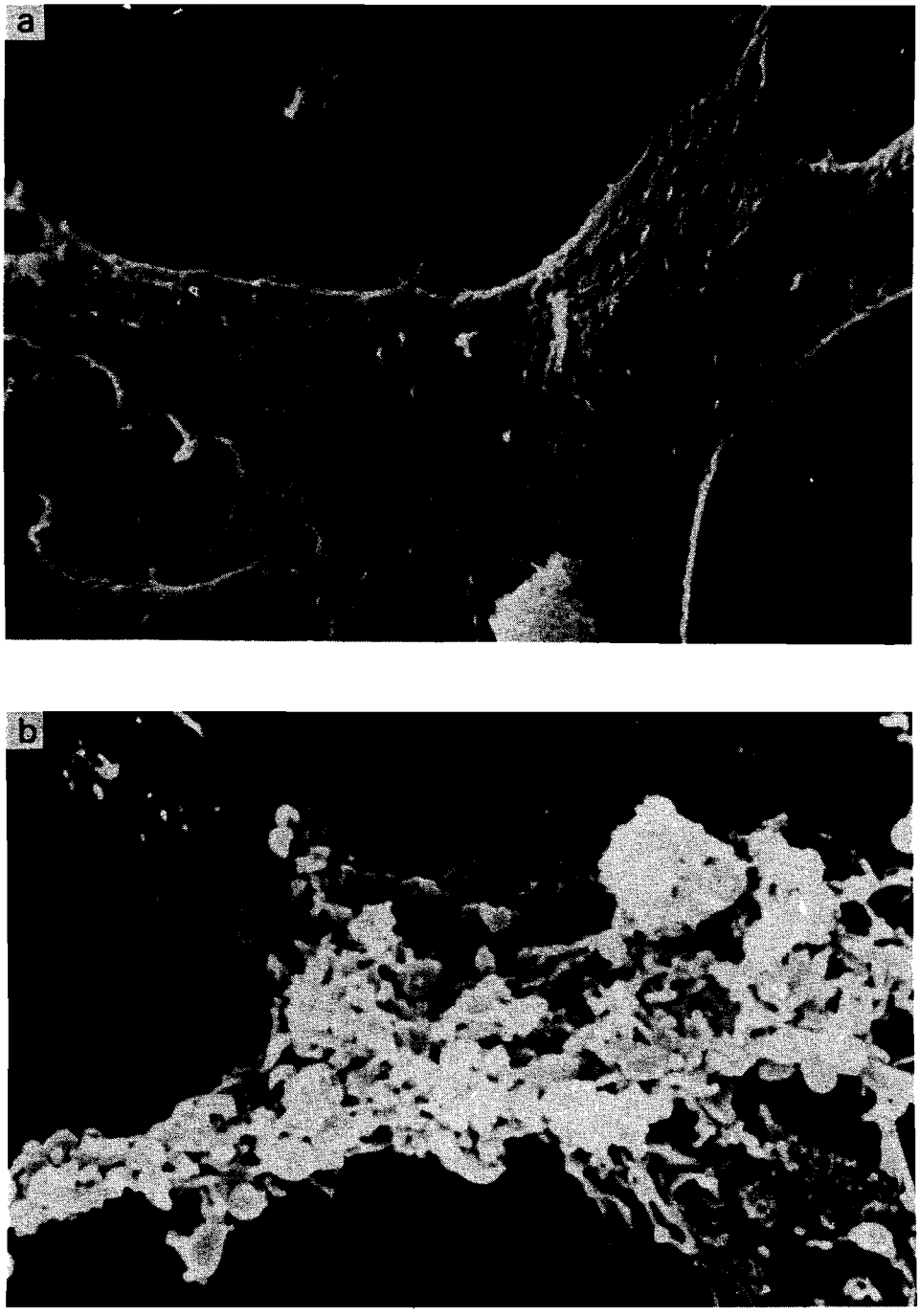
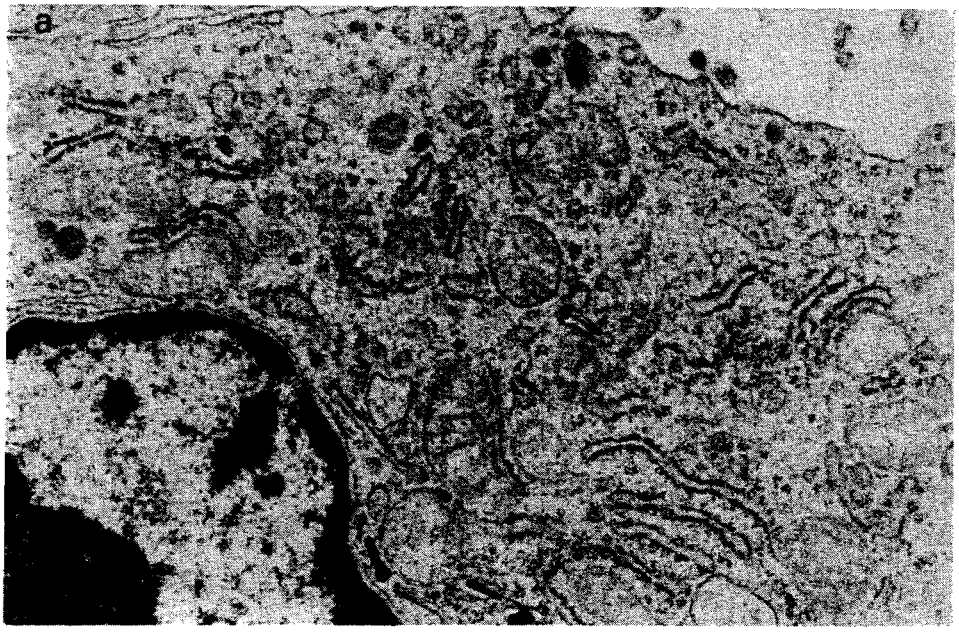


Fig. 5. Cells observed by scanning electron microscopy (× 4800); (a) control cell; (b) cell fixed after 2 h of treatment with SP 5 μg/ml.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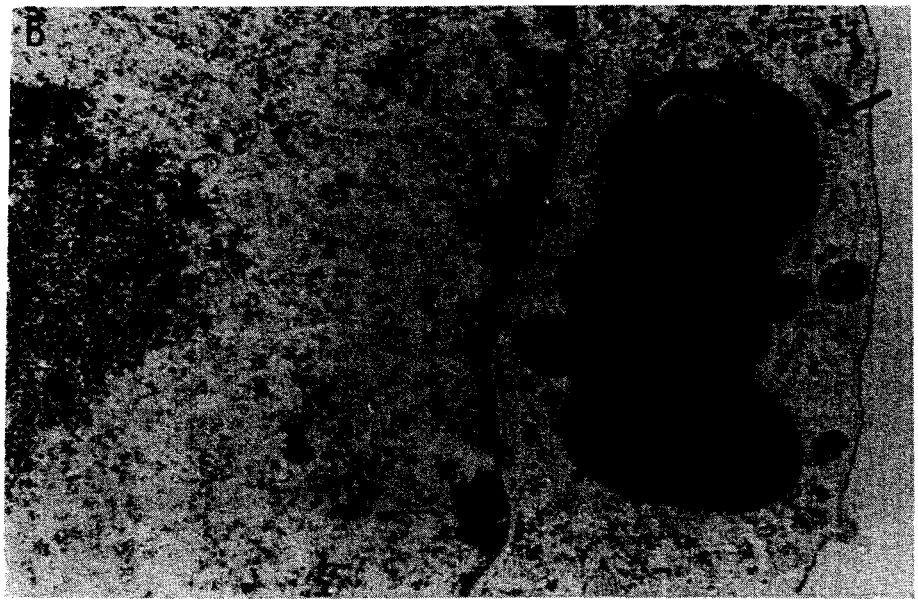
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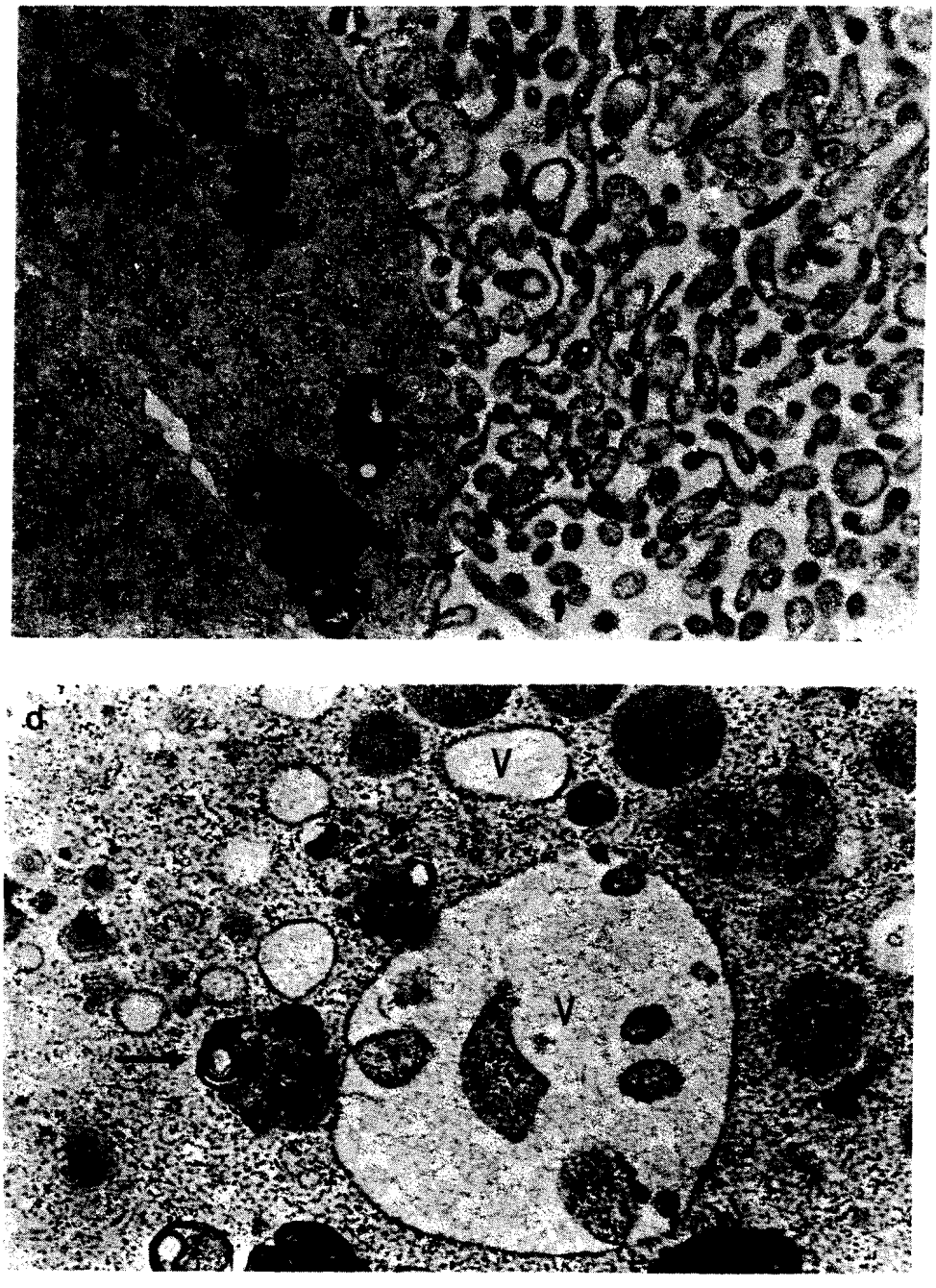

Fig. 6. Analysis by transmission electron microscopy of B16 melanoma cells. Control cell (a, ×27 000), cell treated with SP 0.4 μg/ml during 72 h (b, ×27 000), with SP 3 μg/ml during 1 h (c, ×10 800), or SP 2 μg/ml during 1 h (d, ×27 000). Lamellar bodies probably inside phagolysosomes (→), blebs (◂), vacuoles (V).

some cells with a relatively low DNA content were present (< 2.5 arbitrary units).

The analysis of cells treated for 24 h but at higher concentrations revealed that strychnopentamine is cytotoxic from 2 μg/ml on, but a concentration of 4 μg/ml is necessary for inducing a decrease in the average amount of proteins per remaining cell (Table II). Furthermore, the incorporation of [^{3}H]thymidine and [^{3}H]leucine is reduced by SP (0.5 μg/ml and more) after only 1 h of treatment (Fig. 3).

The observation of living treated cells under an inverted phase contrast microscope revealed that after only 2 or 3 h of treatment (3 μg/ml), many large vacuoles were present in the cytoplasm. We also noted that blebs on the surface of treated cells were much more numerous than on control cells (Figs. 4a,b); this was confirmed by scanning electron microscopy (Figs. 5a,b). Lipidic structures were shown to be present in the cytoplasm by fat red staining of the treated cells.

Cells treated for 72 h by 0.4 μg/ml SP were analysed by transmission electron microscopy. Structures analogous to phagolysosomes containing melanosomes, premelanosomes and lamellar bodies and limited by a single membrane were abundant in the cytoplasm (Fig. 6b). However, the other cell organelles seemed to be unaltered. At higher concentrations (2 or 3 μg/ml) and after only 1 h of treatment, we observed, besides lamellar bodies, swelling of the rough endoplasmic reticulum, formation of many blebs at the cell surface and, in some cases, clumping of chromatin leading to cell death (Figs. 6c,d).

DISCUSSION AND CONCLUSIONS

We show in this work that SP can be very cytotoxic for mammalian cells cultured in vitro. It induces inhibition of proliferation and cell death in cancer and non-cancer cells at relatively low concentrations (< 1 μg/ml) after 72 h of treatment. Higher concentrations (4 μg/ml) are necessary for inducing a decrease of the cell protein content, after 24 h of treatment. Furthermore, the incorporation of [^{3}H]thymidine and [^{3}H]leucine are significantly reduced already after only 1 h of treatment (0.5 μg/ml).

We also show here that SP can provoke the formation of lamellar bodies in the cytoplasm. The presence of bodies of this kind in cells treated with different drugs possessing, as SP does at physiological pH, a cationic amphiphilic structure, has been attributed to drug-induced impairment of lysosomal phospholipid metabolism [12]. But obviously, this type of molecule could have other multiple effects at the cell level. That is why it is not possible to explain the toxicity of these compounds only by the presence of such structures. In the case of SP, the metabolism of the remaining living cells, after 72 h of treatment (0.4 μg/ml) does not seem to be affected by the presence of these lamellar bodies because no alteration of the rate of incorporation of [^{3}H]thymidine or of protein, melanin or DNA contents was noted. The formation of a drug-phospholipid complex concentrated in cytoplasmic lamellar bodies was observed with amphiphilic drugs possessing, as SP does, an ionisable amino group and an aromatic structure [13]. A similar phenomenon could perhaps explain the relative absence of effects of

SP on the remaining living cells, after 72 h of treatment. During the treatment, lamellar structures could have progressively concentrated SP, consequently eliminating the alkaloid from the culture medium.

Blebs and vacuolization rapidly appear in cells treated with SP. They could be related to cell suffering, as observed under various experimental conditions [14]. But obviously, additional experiments are necessary for understanding more precisely the mode of action of SP at the cell level and for establishing structure-activity relationships with related compounds.

On the other hand, experiments actually in progress in our laboratories confirm that SP can induce in vivo a significant decrease of the number of cancer cells in mice bearing an Ehrlich ascites [15]. The possible toxicity of this molecule has yet to be explored in the animal.

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