Strychnopentamine, a Potential Anticancer Agent

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Abstract

We analysed the effects of strychnopentamine, an alkaloid isolated from *Strychnos usambarensis*, on an Ehrlich ascites tumor growing in the mouse after inoculation. Four subcutaneous injections of 1.5 mg strychnopentamine (1 per day) induce a significant decrease of the number of tumor cells and a significant increase of the survival of the treated mice. Observed side effects are partial haemolysis and some liver damage.

Key words

Strychnopentamine, indole alkaloid *Strychnos usambarensis*, anticancer activity, Ehrlich ascites tumor.

Introduction

Strychnopentamine (SP) (1) is a dimeric indole alkaloid isolated from the leaves and stem bark of an African Loganiaceae: *Strychnos usambarensis* Gilg (1, 2). Our group showed that, *in vitro*, it can exert a relatively strong cytotoxic activity on cancer and non-cancer cell lines, apart from a concentration as low as 0.5 μg/ml (3, 4). The present paper is devoted to preliminary studies performed *in vivo* on mice bearing an Ehrlich ascites tumor in order to analyse the activity of strychnopentamine on this tumor and its eventual toxicity in the animal.

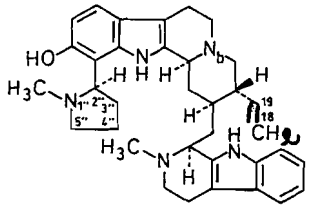
Materials and Methods

Strychnopentamine (SP) was extracted and purified following methods we previously described (1, 2). It is used in its diacetate form in solution in purified distilled water.

Animals used were 2 to 3 months old male C57BL/6J (Jackson) mice weighing approx. 25 grams.

Analysis of the effects of SP on the tumor in the mouse

$1 \cdot 10^6$ Ehrlich tumor cells (ELT line) are inoculated (*i.p.*) into 48 mice. Three mice are sacrificed on days 2, 4, 6,



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and 8 after the graft. On day 10, 1 ml of a solution containing a quantity of SP corresponding to 1.5 mg base is injected subcutaneously into 24 of these mice bearing a tumor. One ml of a normal saline solution is injected into the 12 control mice. Three treated mice are sacrificed 1 hour after the injection and 3 others 5 hours later on. Three control mice are also sacrificed 3 hours after the injection of the normal saline solution. The next day, the remaining treated mice received one *s.c.* injection of 1.5 mg SP and control mice, one *s.c.* injection of 1 ml of a normal saline solution. Three treated mice are sacrificed 1 hour after this second injection, and 3 others 5 hours later. Three control mice are also sacrificed 3 hours after their second injection. The same procedure is repeated during 2 other days on the remaining mice after a third and a fourth injection of 1.5 mg of SP or 1 ml of a normal saline solution.

Flow cytometry and DNA analysis

The ascites cancer cells are collected from the peritoneal cavity by successive washings with a solution of heparin in PBS (12 UI/ml). The DNA was stained with propidium iodide (Sigma) with or without previous lysis of the cells using the detergent-trypsin method of Vindelov et al. (5). This procedure allowed to distinguish between surviving and dead cells. For each sample, 20,000 nuclei were run on a flow cytometer FACS 440 (Becton Dickinson, Mountain View, CA, USA) equipped with a Consort 30 management system. See Melamed et al. (6) for a complete review of this technology. The excitation source was an argon-ion laser emitting a 488 nm beam at 200 mW of power. The red fluorescence of the DNA-propidium iodide complexes was collected through a 620/25 band-pass filter. The DNA content of the nuclei was displayed on a linear scale as monoparametric histograms of 255 channels of resolution. The G0, G1, S and G2M fractions were then mathematically extracted by the "sum of broadened rectangle" algorithm (7).

Detection of mitotic and pycnotic cells (Feulgen)

An aliquot of each cell suspension is centrifuged with a cytospin and the cells spotted on a slide and fixed for 24

hours in a mixture of absolute ethanol-acetone = 1 : 1 and then for 48 more hours in methanol. The cells are then stained with the Feulgen reaction. On these slides, we calculated the number (‰) of pycnotic and of mitotic cells and the percentages of the different phases of mitosis. For each condition, at least 1500 cells were counted. The results were compared to control values by the statistical Mann-Whitney test.

Effects of SP on the survival of the mice bearing a tumor

$1 \cdot 10^6$ Ehrlich ascites tumor cells are inoculated (*i.p.*) into 16 mice. Eight of them are treated with 4 successive injections of SP on days 10, 11, 12, and 13 (see above). The 8 other animals (controls) receive a saline solution. The number of surviving mice is noted every day. Survival curves are established in a Probit-Log grid of coordinates. We put the survival fraction of mice in ordinates and the number of days after inoculation of the tumors in abscissae. This graph allowed us to determine the mean survival time (50 % survival) for treated and control mice.

Possible toxicity of SP

4 healthy mice receive 4 subcutaneous injections of SP (as above) and are sacrificed 1 hour after the last injection. The blood is collected and aliquots are prepared for cell counts or quantitative measurements of creatinine, GOT (glutamic-oxaloacetic transaminase) and GPT (glutamic-pyruvic transaminase) using automatized methods as those used for human blood analysis on an ERIS® apparatus. The concentration of creatinine is measured following the Jaffé reaction (8). Enzymatic methods are used for GOT and GPT (9).

Mutagenic or antimutagenic effects

These tests are performed *in vitro* according to the Ames test (10) as modified by De Meo et al. (11) on *Salmonella typhimurium* with a solution of SP (final concentration: 500 µg/ml). The strains used for the analysis of the potential mutagenic effect of SP are TA97, TA98, TA100, and TA102 *S. typhimurium* with or without addition of a metabolic fraction (S9 MIX) extracted from a rat liver induced by Aroclor 1254. The antimutagenic effect is measured with benzo[*a*]pyrene and smoker urine as standard mutagens. The Ames test is used on TA98 *S. typhimurium* with the S9 MIX metabolic fraction.

Haemolytic properties

36,000 mouse red blood cells in 0.2 ml PBS (phosphate buffer solution) are inoculated into a NUNC 24 well plate. 0.2 ml of a solution of SP in a TGL solution (Tyrode-glycosol) containing 300, 150, 100, 50, 30, 15, 7.5, or 0 µg SP/ml are added. Erythrocytes are then observed under an inverted phase contrast microscope 1 hour and 24 hours later.

Statistical analysis

For comparing control values to values obtained for treated mice, the non-parametric Mann-Whitney test was applied. P values lower than 0.05 were considered as significant. All these analyses were performed on a PS2 (model 60) computer with Statgraphic software.

Results and Discussion

Figure 1 shows that 4 daily *s.c.* injections of 1.5 mg of strychnopentamine (SP) induce a significant decrease of the number of living Ehrlich ascites tumor cells. According to results obtained by FACS analysis, a decrease of the percentages of cells in S phase but an increase of the percentages of cells in G2M are observed in ELT cell populations treated with SP (Figs. 2 and 3). These results could be interpreted as a sign of a disturbance in the cell cycle, probably due to an increase of the duration of G2 and/or to some inhibition at the G2M transition. Furthermore, we did not observe any significant modification of the number of mitotic cells (*i.m.* treated: 34.9 ± 2.9, controls: 29 ± 7.3) and of the percentages of cells in each phase of the mitosis, thus indicating that the M phase is also longer. However, we did not find any significant modification of the number of pycnotic nuclei in the treated tumors even after 4 injections of SP (*i.p.* treated: 25 ± 0.93, controls: 19.6 ± 2.1). The discrepancy between the results of the pycnotic indexes and the increase of the number of dead cells by FACS analysis could be explained by the fact that most of these cells are lysed and undetectable on slides after staining.

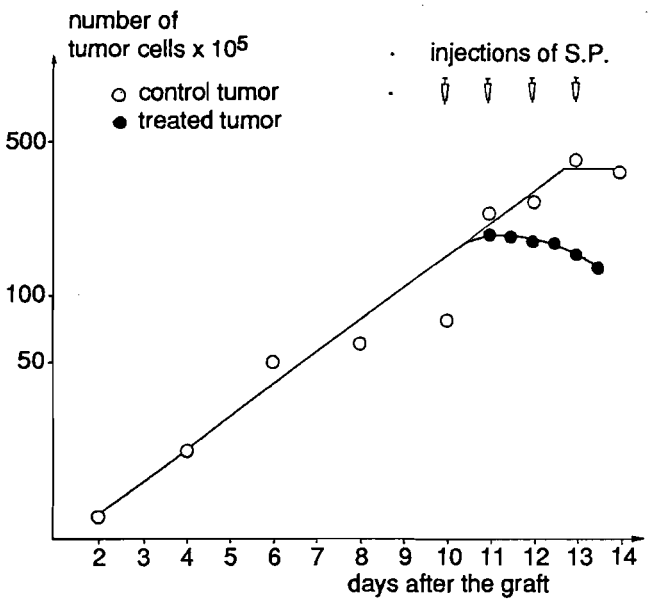

Fig. 1 Effects of 4 successive s.c. injections of 1.5 mg of SP (on days 10, 11, 12, and 13 after the graft) on the number of surviving Ehrich ascites tumor cells: treated –○–, controls –●–.

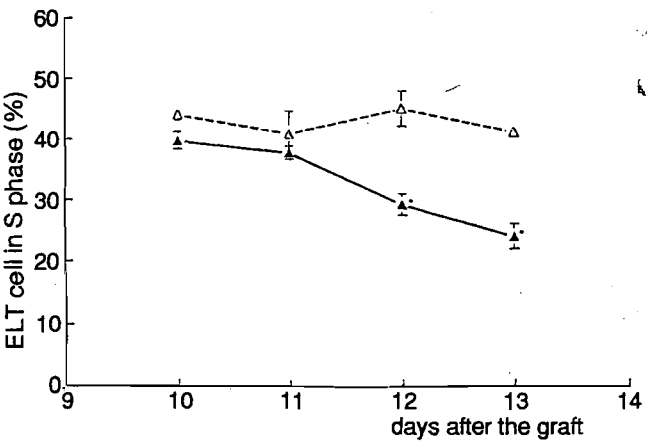

Fig. 2 FACS analysis of the effects of 4 successive s.c. injections of 1.5 mg of SP (on days 10, 11, 12, and 13 after the graft) on the percentages (means ± standard error) of ELT cells in S phase of the cycle: treated: –▲–, controls: –△–; *$p < 0.05$.

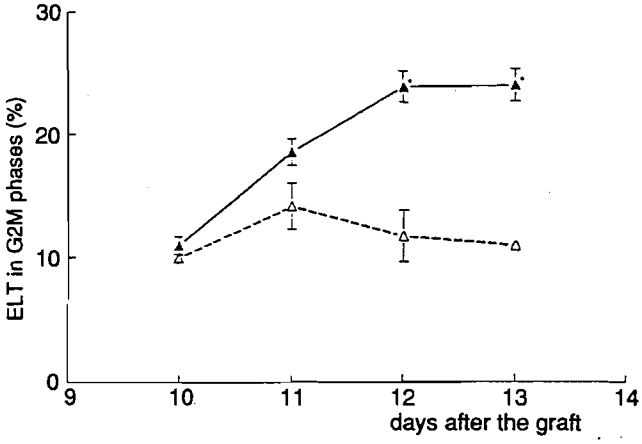

Fig. 3 FACS analysis of the effects of 4 successive s.c. injections of 1.5 mg of SP (on days 10, 11, 12, and 13 after the graft) on the percentages (means ± standard error) of ELT cells in G2M phases of the cycle: treated: –▲–, controls: –△–; *$p < 0.05$.

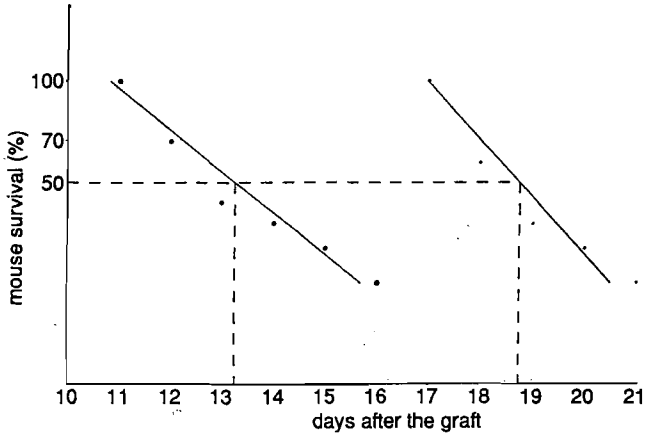

Fig. 4 Effects of 4 daily s.c. injections of 1.5 mg of SP (on days 10, 11, 12, and 13 after the graft) on the survival of mice bearing an ELT ascites: treated: –●–, controls –○–.

All these results agree with those we obtained for cells cultures *in vitro* and treated with SP (3) according to which the major cytotoxic effect of SP is a strong decrease of the number of cells.

Figure 4 shows that SP induces a significant increase ($P < 0.001$) of the mean survival time of the animals bearing an Ehrlich ascites (treated: 18.75 days ± 0.45/controls: 13.25 days ± 0.59).

Altogether, these results indicate that SP could well have an interesting antitumor activity in mice bearing an Ehrlich tumor.

We also performed some preliminary tests in order to detect possible signs of toxicity due to a treatment with SP. Previous studies had showed that one *i.p.* injection of 2 mg SP is quite toxic for the mouse (4) while subcutaneous injections of 1.75 mg SP given during 3 consecutive days were apparently much less toxic (12). After receiving SP, mice were less active immediately after the injections but this transient apathy disappeared after a few hours and the mice were still alive thereafter.

We measured the amounts of creatinine, GOT, and GPT in the plasma of mice injected with SP in order to detect possible renal and hepatic lesions. Blood cell counts were also realised. These measurements were performed 1 hour after the fourth injection of 1.5 mg SP on day 13. The results given in Figure 5 indicate that a treatment consisting of 4 injections of 1.5 mg of SP induces a significant increase of GOT and GPT, related to some liver damage. No effect on glomerular filtration (level of creatinine not modified) could be suspected. We also noted a slight modification (not significant) of the percentages of leucocytes and platelets. The major effect is a considerable decrease of the haematocrit and of the number of erythrocytes, but without any decrease of total haemoglobin, suggesting a probable haemolysis. So we realized a test *in vitro* on mouse blood cells. The results (Table 1) show that SP effectively has haemolytic properties but at higher concentrations than 30 µg/ml. Such a high concentration could nevertheless be obtained for a short time in the blood of the treated mice, just after the injection of 1.5 mg of SP, which can explain the observed haemolysis.

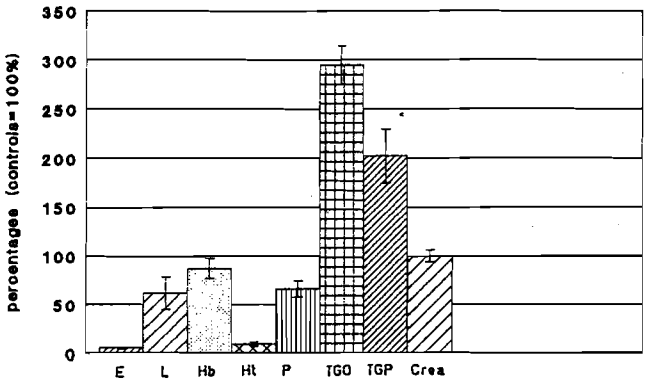

Fig. 5 Effects of 4 daily s.c. injections of 1.5 mg of SP (on days 10, 11, 12, and 13 after the graft) on the number of erythrocytes (E), leucocytes (L) and platelets (P), on the quantity of haemoglobin (Hb), on the haematocrit (Ht), and on the amount of GOT (TGO), GPT (TGP), or creatinin (Crea) in plasma. Values (means ± standard error) are expressed as percentages of the corresponding control values considered as 100 %; *$p < 0.05$.

Table 1 Haemolytic activity exerted by SP on mouse erythrocytes *in vitro*.

Concentration (µg/ml)	300	150	100	75	50	25	15	7.5
after 1 hour	+	+	+	+	–	–	–	–
after 24 hours	+	+	+	+	±	–	–	–

+ = total haemolysis;
± = partial haemolysis;
– = no haemolysis.

The *in vitro* experiments (3) also indicated that a cytotoxic activity was already detected at 0.5 µg/ml, that is the reason why experiments are in progress in order to lower the concentration of SP in the blood of the treated mice so reducing or avoiding any haemolysis. This could perhaps be achieved by using repeated injections of smaller

quantities of SP or another mode of administration. It is also interesting to note that experiments performed on *S. typhimurium* indicate that SP is devoid of any mutagenic or antimutagenic effects.

Conclusions

These preliminary experiments indicate that strychnopentamine which has been previously shown by our group to be able to inhibit cell proliferation *in vitro* can also exert an anticancer activity in mice bearing an Ehrlich ascites tumor (ELT). Strychnopentamine can decrease the number of these tumor cells and increase the duration of survival of animals inoculated with ELT cells.

However, strychnopentamine, like many other substances used in cancer chemotherapy, is not devoid of toxicity. The major side effect detected in our study are liver damage and haemolysis occurring at much higher concentrations than those necessary to obtain a cytotoxic effect in the tumor. Experiments now in progress are planned in order to define better therapeutic schemes. This probably could be achieved by using either lower doses given several times a day or another route of administration of the substance.

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