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Three Alkaloids as Selective Destroyers of Cancer Cells in Mice

Synergy with Classic Anticancer Drugs

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Abstract. Alstonine, serpentine and sempervirine, when used at appropriate concentrations cure a relatively important proportion of BALB/C mice inoculated with transplantable YC8 lymphoma ascites cells, as well as Swiss mice bearing Ehrlich ascites carcinoma cells. The development of some solid tumors was only partially prevented. However, when one alkaloid was administered in association with either 5-FU, daunorubicin, 1-(2-chloroethyl) nitrosourea (CCNU) or cyclophospahmide (CP) to mice bearing either ascites carcinoma cells or solid tumors, a high rate of cure was obtained without toxicity. The role of the three alkaloids in the curing of mice and prevention of carcinogenesis is discussed.

Introduction

The ultimate aim of cancer chemotherapy should be the use of compounds which selectively destroy *in vitro* and *in vivo* the proliferative capacity of cancer cells without noticeably affecting the multiplication and survival of normal cells. This is an urgent task because the undesirable side effects of practically all anticancer drugs at present used in cancer chemotherapy are their tissue toxicity and the particularly severe damage they inflict on hematopoietic cells. The mutagenic and/or carcinogenic potential of these drugs observed in eukaryotic cells and animals [1–6], in the Salmonella/microsome test [7], in the Oncotest [8, 9], and in the Salmonella-Oncotest [10] point clearly to the need for a new generation of anticancer drugs possessing selective properties to the highest possible degree. As recently suggested, the 'destabilized' (relaxed) physicochemical structure of mammalian and plant cancer DNAs offers a great possibility for discovering new anticancer drugs. Using the Oncotest [8, 9], we selected substances, including some particular alkaloids with the capacity to distinguish between cancer and normal DNAs. These alkaloids bind to the initiation sites of destabilized cancer DNAs, prevent them from being replicated into a new DNA and also protect them from any further increase of destabilization due to carcinogens, classic anticancer drugs or steroid hormones [8, 9, 11]. Since they appear not to recognize stabilized DNA, i.e. normal cell DNA, they are expected not to exhibit toxicity on normal cells, hematopoietic cells in particular, at doses necessary to destroy cancer cells.

In this paper we report that alkaloids alstonine, serpentine and sempervirine, each when used either alone or in association with some classic anticancer drugs, successfully cure mice bearing transplantable carcinoma cells.

Materials and Methods

Mice

We used 6- to 8-week old mice (BALB/C, Swiss, DBA/2), purchased from IFFA Credo, L'Arbresle, France. Maintained under constant temperature (21 ± 0.3 C) with humidity, the mice were fed with common mouse food and had tap water ad libitum.

Transplanted Cell Lines

Transplanted YC8 lymphoma cells, Ehrlich ascites carcinoma and L1210 cells were maintained in the ascitic form by a 10-day passage of counted tumor cells into the appropriate strain of origin.

Drugs

Alstonine and serpentine were isolated and purified in our laboratory by a procedure described elsewhere [12]. Sempervirine was purchased from Roth-Sochiel Co, Lauterbourg, France. Daunorubicin, Rhône-Poulenc, Paris, France. 5-FU, Roche Co., Basle, Switzerland; 1-(2-chloroethyl-1)nitrosourea (CCNU), Laboratoires R. Bellon, Paris, France. Solution of drugs: alkaloids were dissolved in a small volume of 96 alcohol and diluted with Tris buffer 0.01 *M*. pH was ajusted to 7.5. The solution (5 mg/ml) was filtered on Millipore under sterile conditions.

Anticancer Effect of Alkaloids

Cancer cells inhibiting action of alstonine, serpentine and sempervirine, each administered alone or in combination with either 5-FU, CCNU or daunorubicin, was tested on mice bearing lymphoma YC8 cells (BALB/C mice inoculated i.p. with $5 \cdot 10^3$–1.10^4 cells/mouse), Ehrlich ascites carcinoma cells (Swiss mice inoculated i.p. with 10^4 cells) and L 1210 leukemia cells (DBA2 mice injected i.p. with 10^4–10^5 cells). In each experiment (see tables) 20 untreated mice served as control. A group of 20 mice was used for treatment with each alkaloid and for combination with one of the classical anticancer drugs tested here. Drugs were administered intraperitoneally 24–48 h after inoculation of cancer cells, twice/day for 8 consecutive days. When two drugs were applied the classic one was administered just before the alkaloid. Survival time of mice was recorded daily. Mice were weighed every 2nd day for 26 consecutive days. Only those mice which did not develop tumors and survived in excellent condition for 70 days after the death of untreated mice were considered as cured. DBA2 mice bearing L1210 leukemic cells were treated with each of the three alkaloids, using different concentrations ranging from 0.2 mg × 2 to 1 mg × 2. No success was obtained.

The anticancer effect of alstonine administered either alone or in combination with cyclophosphamide (CP) was also tested on Swiss mice bearing solid Ehrlich carcinoma cells. To obtain solid tumors 10^6–2.10^6 Ehrlich carcinoma cells were injected subcutaneously to thigh of mouse (40 mice). 10 mice were not treated (control) while the treatment of others started when tumors became apparent. Drugs were administered in the region of tumor cell inoculation. 10 mice were treated with alstonine (0.4 mg × 2/day) for 10 consecutive days, 10 mice with CP (0.25 mg × 2/day) and 10 mice with CP (0.25 mg × 2/day) plus alstonine (0.2 mg × 2/day). In this later case CP was injected just before alstonine. On the 26th day after cancer cell inoculation, mice were sacrificed. Tumors were excised and weighed. Average weight was determined using the Student's t test ($p < 0.01$). In other experiments the mice were treated with drugs as indicated above but not sacrificed.

Results and Discussion

Our previously described observations [11] have shown that the alkaloids alstonine, serpentine and sempervirine selectively bind to initiation sites of cancer DNAs and prevent in vitro cancer DNA replication. These alkaloids poorly attach to stabilized DNAs isolated from healthy tissues. In in vitro conditions, each alkaloid destroys the proliferative capacity of cancer cells and exhibits practically no effect on normal cells [13].

Data described here show that each of these alkaloids acts as a selective destroyer of some carcinoma cells inoculated in mice. In fact several mice bearing YC8 lymphoma cells (table I, II) or Ehrlich ascitic cells (table III) and treated with either alstonine, serpentine or sempervirine were cured providing appropriate concentrations were used. The cured mice survived in excellent health. Sempervirine was active at lower concentrations compared to alstonine or serpentine. This is probably due to its excellent binding capacity to cancer cells DNAs [11]. Attempts to give unrepeated doses (one dose every 3–4 days) were not successful, both in the case of small and large doses. Two daily injections for 8 consecutive days were necessary since each of the alkaloids was eliminated from the mice within about 7 h. The best results were obtained when the alkaloids were administered twice daily by the intraperitoneal route and applied 24–48 h after carcinoma cell transplantation. Their anticancer effect appears to be direct as shown with different cancer cells cultured in vitro [13].

Since alkaloids bind to initiation sites of cancer DNAs thus blocking DNA synthesis [11, 13] we thought of associating them with some known anticancer drugs which induce the appearance of single-stranded DNA [14–17] thus increasing the number of initation sites for DNA replication. This approach has been sustained by our own results [9, 11, 18] as well as by the fact that drugs such as cis-platinium [14], adriamycin [19], proflavin and quinacrine [15] as well as X-rays induce unwinding and/or DNA strand separation without covalent attachement. Thus, they increase the capacity of cancer DNA for alkaloid binding. As illustrated in figure 1b, for example, CP efficiently induces cancer DNA strand separation while its effect on DNA from corresponding healthy tissues is negligible under our experimental conditions. Consequently this drug stimulates in vitro synthesis of DNA from cancer tissues thus facilitating the binding of alkaloids to such DNAs which results in prevention of DNA synthesis (fig. 1a). Regression of the solid Ehrlich ascites tumor in Swiss mice was reduced by 33% when alstonine alone was administered as shown in table IV. The weight of tumors reduced by 63% with CP was drastically reduced (99%) when alstonine was administered in association with CP. When mice were not sacrificed 50% of treated mice survived in good conditions without ap-

Table I. Survival of mice bearing lymphoma YC8 cells after treatment with alstonine

Days of survival	Untreated mice	Survival %	Mice treated with alstonine					
			0.2 mg × 2	survival, %	0.6 mg × 2	survival, %	1.2 mg × 2	survival, %
Start	20	100	20	100	20	100	20	100
20	18	90	20	100	20	100	20	100
25	4	20	16	80	20	100	18	90
30	0	–	8	40	17	85	16	80
40	0	–	8	40	12	60	16	80
50	0	–	8	40	12	60	16	80
60	0	–	8	40	12	60	16	80
90	0	–	8	40	12	60	16	80

All mice that did not survive had developed tumors (average tumor weight 8.6 ± 2.1 g). Cured mice survived in excellent condition.

Table II. Survival of mice bearing lymphoma YC8 after treatment with serpentine or sempervirine

Days of survival	Untreated mice	Survival %	Mice treated with serpentine (0.2 mg × 2)	Survival %	Mice treated with sempervirine (0.15 mg × 2)	Survival %
Start	20	100	20	100	20	100
20	17	85	20	100	20	100
25	14	70	15	75	20	100
30	2	10	8	40	17	85
40	0	–	8	40	16	80
50	0	–	8	40	16	80
60	0	–	8	40	16	80
90	0	–	8	40	16	80

All mice that did not survive had developed tumors (average tumor weight 8.6 ± 2.1 g). Cured mice survived in excellent condition.

Table III. Survival of mice bearing Ehrlich carcinoma cells after treatment with alkaloids

Days of survival	Untreated mice	Survival %	Mice treated with alstonine (0.2 mg × 2)	Survival %	Mice treated with sempervirine			
					0.15 mg × 2	survival, %	0.4 mg × 2	survival, %
Start	20	100	20	100	20	100	20	100
20	20	100	20	100	20	100	20	100
25	8	40	18	90	20	100	20	100
30	0	–	8	40	16	80	18	90
40	0	–	6	30	10	50	18	90
50	0	–	6	30	10	50	18	90
60	0	–	6	30	10	50	18	90
90	0	–	6	30	10	50	18	90

All mice that did not survive developed tumors (average tumor weight 9.5 ± 3.5 g). Cured mice survived in excellent condition without tumor development.

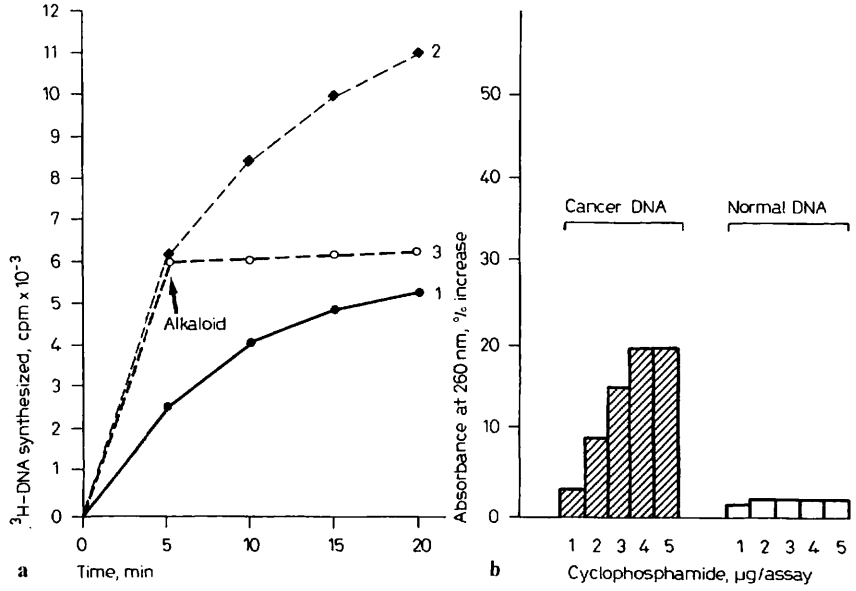

Fig. 1. a, b Effect of cyclophosphamide (CP) on cancer DNA in vitro synthesis and DNA strand separation. Ehrlich carcinoma DNA (0.25 µg) was used as template. 1: complete medium for DNA synthesis (^{3}H thymidine-5'-triphosphate was used as marker (11); 2: complete medium + CP (10 µg/assay) at time 0; 3: complete medium + CP (10 µg/assay) at time 0 and sempervirine (25 µg) at the 5th min. Conditions for DNA in vitro synthesis have been described elsewhere [11]. For DNA strand separation, see ref. [9].

parent tumor development. Similar results were obtained with the mammary carcinoma grafted cells of mice (RHO III) (results not shown here).

Another drug, 5-FU, commonly used to treat solid human tumors such as breast and colonic carcinoma, gave a response rate of about 20% [20]. The therapeutic synergism of 5-FU and alstonine studied here is illustrated in table V. It shows the absence of survivors when Swiss mice bearing Ehrlich ascites carcinoma cells were injected intraperitoneally and treated once per day with 5 µg of 5-FU. Few mice survived when 200 µg of this drug were injected twice per day. A combination of 5-FU with alstonine gave a large number of definite survivors. The effects of combinations of alstonine and CCNU (table VI) or alstonine and daunorubicin (table VII) gave superior results than when each drug was applied separately. These effects are synergistic and not additive, suggesting that alstonine and CCNU or daunorubicin acts by different pathways as previously shown using purified cancer DNAs [11]. Cured mice survive in perfect condition. Besides their anticancer potential, alkaloids used here possess a quaternary nitrogen which may interact with an excess of some classic anticancer drugs or their metabolites, thus explaining the decrease in the toxic effect of those drugs.

Lack of Curative Effect of Alkaloids on Mice Bearing L1210 Leukemic Cells

When a suspension of L1210 cells in Hanks' medium (2.10^4 cells/ml) was incubated with one of three alkaloids (100 µg/ml) for 30 min at room temperature and then injected into mice, there was no development of the leukemic cells. However, when alstonine, serpentine or sempervirine were used at different concentrations and administered every day intraperitoneally to preinoculated mice (DBA2) (2.10^4 cells/mouse 24 h before the treatment), the treated mice died at the same time as the untreated control mice (data not shown here). These results seem to indicate the inability of alkaloids to reach L1210 cells in sufficient concentrations in vivo for some unelucidated reason.

The mechanism through which alkaloids and classic anticancer drugs operate in the destruction of tumor cells may be of great importance in preventing the appearance of cancer cells, which may be generated by carcinogens, drugs, steroid hormones, their derivatives or even by radiation. In fact, as soon as these DNA-destabilizing agents induce DNA strand

Table IV. Regression of the solid Ehrlich ascites tumors in Swiss mice treated with anticancer drugs

Mice	Average weight of tumors, g	Mean value	Inhibition, %
Control	10.4–12.4	11.4	–
Treated with alstonine	5.5–10.4	7.6	33
Treated with CP	3.6– 5.0	4.3	63
Treated with alstonine + CP	0.04–0.11	0.07	99

Table V. Survival of mice bearing Ehrlich carcinoma cells after treatment with either 5-FU or 5-FU in combination with alstonine

Days of survival	Survival of mice treated with 5-FU (5 μg)		Survival of mice treated with 5-FU (200 μg × 2)		Survival of mice treated with 5-FU (5 μg) + alstonine (50 μg × 2)		Survival of mice treated with 5-FU (200 μg × 2) + alstonine (200 μg × 2)	
	n	%	n	%	n	%	n	%
Start	20	100	20	100	20	100	20	100
20	20	100	20	100	20	100	20	100
25	8	40	18	90	20	100	20	100
30	0	–	6	30	10	50	17	85
40	0	–	4	20	10	50	16	80
50	0	–	4	20	10	50	16	80
60	0	–	4	20	10	50	16	80
90	0	–	4	20	10	50	16	80

All mice that did not survive developed tumors (average tumor weight 10.7 ± 2.8 g). Cured mice did not develop tumors and survived in excellent condition.

Table VI. Survival of mice bearing lymphoma YC8 cells after treatment with alstonine or CCNU and in combination with both

Days of survival	Survival of untreated mice		Survival of mice treated with alstonine (0.2 mg × 2)		Survival of mice treated with CCNU (0.2 mg × 2)		Survival of mice treated with alstonine (0.2 mg × 2) + CCNU(0.2 mg × 2)	
	n	%	n	%	n	%	n	%
Start	20	100	20	100	20	100	20	100
20	18	90	20	100	20	100	20	100
25	7	35	18	90	20	100	20	100
30	0	–	10	50	9	45	20	100
40	0	–	6	30	9	45	20	100
50	0	–	6	30	9	45	20	100
60	0	–	6	30	9	45	20	100
90	0	–	6	30	9	45	20	100

All mice that did not survive developed tumors (average tumor weight 9.6 ± 3 g). Cured mice survived in excellent condition without tumor development.

Table VII. Survival of mice bearing lymphoma YC8 cells after treatment with alstonine or daunorubicin and in combination of both

Days of survival	Survival of untreated mice		Survival of mice treated with alstonine (0.2 mg × 2)		Survival of mice treated with daunorubicin (10 μg × 2)		Survival of mice treated with alstonine (0.2 mg × 2) + daunorubicin (10 μg × 2)	
	n	%	n	%	n	%	n	%
Start	20	100	20	100	20	100	20	100
20	20	100	20	100	20	100	20	100
25	17	85	20	100	16	80	19	95
30	0	–	10	50	9	45	19	95
40	0	–	6	30	8	40	19	95
50	0	–	6	30	8	40	19	95
60	0	–	6	30	8	40	19	95
90	0	–	6	30	8	40	19	95

All mice that did not survive developed tumors (average tumor weight 9.0 ± 1.8 g). Cured mice did not develop tumors and survived in excellent condition.

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separation, alkaloids which only recognize destabilized DNA prevent further DNA chain opening and stop the function of such DNA [10, 11, 16]. For this reason one may expect that the process of carcinogenesis in animals and humans will be prevented by these alkaloids. The low affinity of alstonine, serpentine or sempervirine (used at curative doses) for healthy cells [13] or their DNA [11] suggests the absence of toxicity, in particular towards blood cells. No change in the cell counts of the circulating blood of rabbits (3-kg rabbits that received i.v. 20 mg of alstonine or serpentine twice per week for 6 consecutive months showed no change in circulating blood cells; unpublished results), nor in the body weight of mice was observed; the animals survived in excellent health subsequent to the cessation of treatment. From data reported here and also described elsewhere [10, 11, 16], it appears reasonable to use the above alkaloids either alone or in therapeutic synergism with different classic anticancer drugs or radiation for the treatment and curing of cancers.

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