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Selective Inhibition of in vitro Synthesis of Cancer DNA by Alkaloids of β-Carboline Class

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Abstract. The high template in vitro activity of native DNA from cancerous mammalian and plant tissues, compared to DNA from healthy tissues, enabled us to select substances which selectively inhibit cancer DNA synthesis. Among them, alstonine, serpentine, sempervirine and flavopereirine, all alkaloids which belong to the β-carboline class, distinguish cancer DNA from healthy tissue DNA and inhibit DNA in vitro synthesis when native DNA from different cancerous tissues or cells is used as template. They have practically no effect on DNA from healthy tissues. The inhibitory effect of alkaloids is due to their capacity to form an 'alkaloid-cancer DNA' complex which has been characterized by use of the Sephadex column. Evidence is presented showing that these alkaloids inhibit the initiation of DNA synthesis but not chain elongation. The stimulating action caused by carcinogens during cancer DNA in vitro synthesis may be prevented and reversed by alkaloids. Furthermore, the stimulating action of steroids during in vitro synthesis of hormone target tissue DNA might be neutralized by alkaloids. However, at relatively high doses, steroids reversibly compete with alkaloids for binding sites on breast cancer DNA. This is not observed with DNA from nonhormone target tissues.

Introduction

The search for naturally occurring or chemically synthesized substances which have a high affinity for DNAs from eukaryotic and prokaryotic cells has been carried out on a large scale [12, 14]. Some of these substances bind to bases of DNA, others intercalate between two chains of DNA. Their interaction with DNA results in preferential inhibition either of the transcription of DNA into RNA or in the inhibition of DNA replication [16, 18]. Several drugs used in cancer therapy have such properties and yet act as mutagens (as do carcinogens) toward normal cells [8, 9], but are toxic toward cancerous and normal cells.

We have already shown that DNA from cancerous and healthy mammalian and plant cells exhibit potentially different template ac-

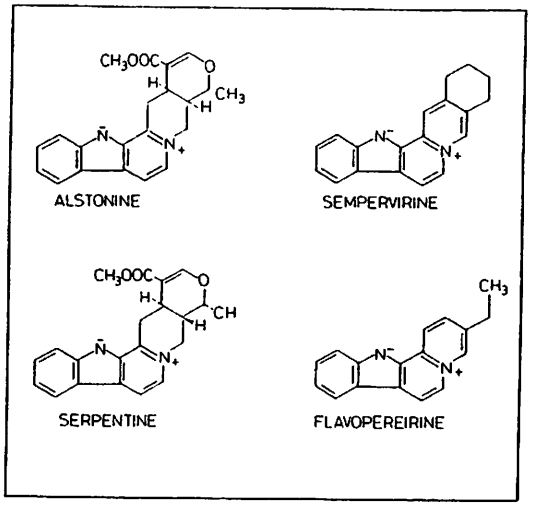

Fig. 1. Chemical structure of alstonine, serpentine, sempervirine and flavopereirine.

tivities in vitro. Thus, in a biochemical assay system (Oncotest) [1, 2, 4], it was possible to demonstrate that carcinogens strongly and preferentially stimulate the in vitro synthesis of DNA from cancerous cells. They stimulate synthesis of DNA from normal cells only slightly. Steroids also significantly stimulate cancer DNA in vitro synthesis provided that the DNA comes from cancer steroid target tissue (breast cancer for example). The differential behavior of cancer and normal DNA as template is due to relatively destabilized areas of cancer DNA which lead to the appearance of single-stranded chains and offer more binding sites to those substances. Some of them selectively bind to this type of DNA and consequently prevent DNA replication.

By submitting different commercially available drugs and plant extracts to the Oncotest, we became particularly interested in extracts of Rauwolfia plants *(Apocynaceae)*. While certain alkaloids of this plant (reserpine, ajmaline) behave like carcinogens, i.e. strongly and selectively stimulate the synthesis of DNA from cancerous tissues; others, such as alstonine and serpentine, selectively inhibit the in vitro synthesis of DNAs isolated from various cancerous cells.

We describe here several properties of alstonine and serpentine as well as of sempervirine and flavopereirine. These alkaloids belong to the β-carboline class containing a quaternary nitrogen (fig. 1). Their specific inbibiting effect in cancer DNA synthesis forms the core of this research.

Materials and Methods

Deoxyribonucleoside-5′-triphosphates (d-XTP), Poly A, Poly G, Poly C and Poly U (Miles Laboratoires, USA); ^{3}H-thymidine-5′-triphosphate (TTP), specific activity 17.5 Ci/mol (Amersham, England); pancreatic DNase and RNase A and T_1 (Worthington Inc., USA); testosterone and estradiol (gift of Dr. *Ray*, Institut Pasteur); *dl*-ethionine (Calbiochemicals, USA); 7,12-dimethylbenz(a)anthracene (DMBA); Nutritional Biochemicals, USA). Alstonine and serpentine were respectively isolated from *Rauwolfia vomitoria* and *serpentina* [5].

Sempervirine was purchased from Roth-Sochiel, France. Flavopereirine, synthesized compound was a gift from Prof. *J.* Poisson, Faculté de Pharmacie, Châtenay-Malabry, France.

Origins of DNAs. Freshly removed healthy and cancerous human tissues from the mammary gland, lung, ovary and neurocarcinoma were kindly supplied by competent surgeons. Rat spleen and hepatoma were also used. KB cells cultured in vitro were supplied by Dr. *C. Bonissol*, Hôpital Necker, Paris. Gently broken tissues were treated several times, first with phenol and then with chloroform in the presence of lauryl sulfate and buffer containing hydroxychinolin (2%). The RNA contaminant was eliminated by incubating with RNase A and/or T_1 (RNase A: 20 μg/ml; RNase T_1: 10 units/ml) for 30 min at 36 °C in a 1 × SSC solution (0.15 *M* NaCl and 0.015 *M* sodium citrate). RNase was then removed by several chloroform treatments, each followed by centrifuga-

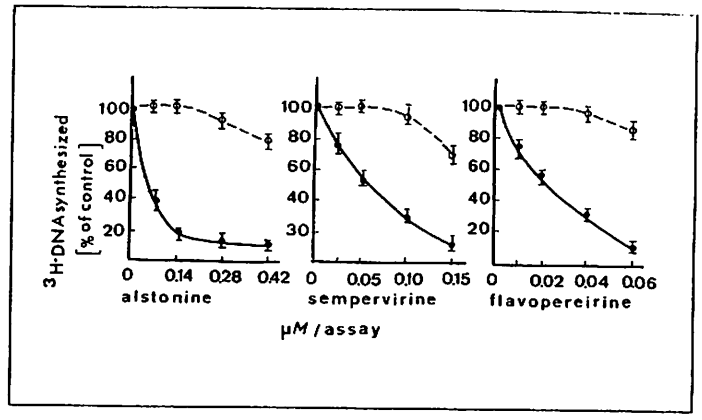

Fig. 2. Selective inhibition of breast cancer DNA in in vitro synthesis by alstonine, sempervirine and flavopereirine. For conditions, see text. Breast DNA (o) and breast cancer DNA (•; 0.5 μg) were used as templates. Each alkaloid was added at time 0. Incubation 10 min at 36 °C.

tion (5,000 *g* for 10 min). DNA was precipitated with 2 vol of 96% alcohol, dissolved in 2 × SSC solution and then dialyzed against 2 × SSC for 24 h at 4 °C. The amount of DNA was determined by UV absorbance at 260 nm (260/280 = 2.1). Purified DNAs were stored at −20 °C. Before use, they were dialyzed against distilled water for 2 h at 4 °C in order to eliminate salts which might interfere with DNA synthesis in the presence of the compounds due to be screened. The hyperchromic effect on incubation with NaOH is 35–52% for DNAs used. In alkaline sucrose gradient the material forms a homogeneous peak ranging between 36 and 50 S and a small one of 2.5–4.2 S. Denatured DNA was prepared by heat (water bath) at 100 °C for 5 min, followed by rapid cooling in an acetone dry-ice bath.

Isolation of DNA-Dependent DNA Polymerase. DNA polymerase was purified from *E. coli* T3000 extracts [3]. The purified enzyme 280/260 ratio should range between 1.5 and 1.7. Conditions for DNA synthesis have been described elsewhere [1, 2]. The synthesis of DNA was determined by measuring the amount of acid-precipitable radioactive product. It was performed in the absence and presence of each alkaloid, using DNA as template, DNA isolated from healthy and cancerous tissues or cells.

Formation and Isolation of the 'Alcaloid-DNA' Complex. 100 μg of each purified breast cancer or healthy DNA dissolved in the Tris-HCl buffer solution (100 μmol, pH 7.50) were incubated with 40 μg of serpentine previously dissolved in an aqueous solution (pH adjusted to 7.5; final volume 0.5 ml). After 15 min of incubation at room temperature, the mixture was filtered on G-25 Sephadex fine column (30 × 1 cm) previously equilibrated with Tris-HCl buffer (0.01 *M*, pH 7.5). The column was eluted with the same buffer. 2-ml fractions were collected and UV absorbance measured at 260 nm.

Results

The first observation, to the effect that the amount of radioactive (acid precipitable) DNA synthesized on DNA template isolated from cancer tissues is always higher than that synthesized in the presence of DNA from healthy cells (fig. 3A), indicated the existence of different receptive properties in the two DNA templates. The very considerable difference of template activity has already been demonstrated in the presence of carcinogens and steroids, which strongly enhance cancer DNA in vitro synthesis [1, 2]. Here we show that, in contrast to carcinogens, each of the four alkaloids strongly inhibits the in vitro synthesis of DNA from breast cancer and has a slight inhibiting effect on DNA isolated from corresponding healthy tissue (fig. 2, 3). DNAs from several cancer and healthy tissues or cells cultured in vitro behave as do

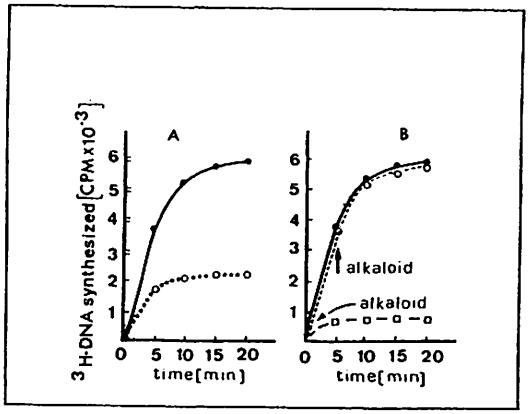

Fig. 3. Breast DNA, and breast cancer DNA in in vitro synthesis. Inhibition of cancer DNA synthesis. Conditions for DNA synthesis, see text. Breast DNA and breast cancer DNA (0.5 µg) were used as templates. A = Breast cancer DNA; o = breast DNA. B ● = Breast cancer DNA; □ = breast cancer DNA + alkaloid (serpentine 80 µg) added at time 0; o = breast cancer DNA + alkaloid (serpentine 80 µg) added at the 5th min of incubation.

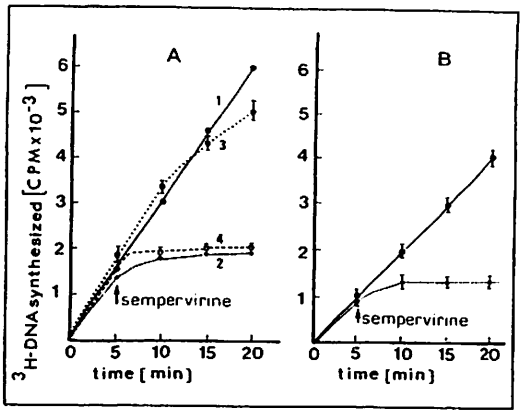

Fig. 4. Effect of sempervirine on cancer DNA in in vitro synthesis in the presence of testosterone, *dl*-ethionine or 7,12 DMBA. Incubation conditions, see text. A Lung cancer DNA (0.25 µg) was used as template under two conditions: 1 (●——●) complete medium + ethionine (60 µg) at time 0 (control); 2 (●····●) complete medium + ethionine (60 µg) at time 0 and sempervirine (25 µg) at the 5th min of incubation; 3 and 4, breast cancer DNA (0.25 µg) was used as template; 3 (o····o), complete medium + testosterone (40 µg) at time 0 ; 4 (o– – –o) complete medium + testosterone (40 µg) at time 0 and sempervirine (25 µg) at the 5th min of incubation ($p < 0.001$, value significance in comparison to control, for all points except for 10 min, $p < 0.01$). B Breast cancer DNA (0.25 µg) was used as template. ●——● = Complete medium + 7,12 DMBA (60 µg) at time 0 (control); ●····● = complete medium + 7,12 DMBA (60 µg) at time 0 and sempervirine (25 µg) at the 5th min of incubation ($p <$ 0.001, value significance in comparison to control).

DNAs from breast cancer and healthy tissues when incubated in the presence of each of the four alkaloids (table II, III; fig. 4). DNA synthesis requires the presence of all four deoxyribonucleoside-5′-triphosphates and does not take place in the presence of DNase (table I).

Results in table II illustrate that alstonine inhibits both the in vitro synthesis of native double-stranded cancer DNA and that of denatured DNA (see Methods). In slightly sensitive healthy cells, DNA becomes more sensitive once denatured by heat. This indicates the very important role of single strands in DNA regarding its binding capacity toward these alkaloids. This corroborates observations made using carcinogens [1], that is to say that native cancer DNA is less stabilized compared to DNA of healthy cells. This confirms that DNA is the target for alkaloids and that the two types of DNA respond differently. The activity of DNA-dependent DNA polymerase is not modified by alkaloids.

Alkaloids Inhibit the Initiation of Cancer DNA Synthesis

We have investigated the effects of each of the four alkaloids on the synthesis in vitro of DNA using as template cancer or healthy tis-

Table I. Effect of sempervirine on breast cancer DNA in vitro synthesis under various conditions

Compounds used	^{3}H-TTP incorporated cpm in 20 min at 36 °C	% inhibition
Complete medium	3,015 ± 48	–
– d-ATP, d-CTP, d-GTP	373 ± 24	87
+ DNase, 1 μg	243 ± 16	91
+ sempervirine, 0.24 μmol	268 ± 23	91
+ ethionine, 0.12 μmol	638 ± 15	78
+ ethionine, 0.24 μmol	635 ± 21	78
+ ethionine, 0.48 μmol	589 ± 22	80
+ DNA, 1 μg	989 ± 29	64
+ enzyme, 180 μg	663 ± 30	78

For incubation conditions, see text. Breast cancer DNA (0.25 μg) was used as template. $p < 0.001$, value significance of comparison to control (complete medium) of four independent experiments. Background (DNA omitted) varying between 145 and 175 cpm was not subtracted.

sue DNAs. Alkaloids were added either at time 0 of the reaction or once the reaction had been started. When serpentine for instance is introduced before DNA synthesis has started, it strongly inhibits the reaction; but if introduced during DNA synthesis (arrow on fig. 3B), the alkaloid at the concentration used has practically no inhibitory effect. The same results were obtained with each of the four alkaloids. It should be emphasized that approximately four times less sempervirine and flavopereirine is needed to observe about the same level of inhibition as that observed in the presence of serpentine or alstonine. The inhibition of DNA synthesis was not overcome by a dose of the enzyme three times higher than that initially used. DNA added in excess (four times the initial amount) overcomes the inhibition to a certain extent (table I). This suggests that the excess alkaloid present binds to newly added

Table II. Effect of sempervirine on native and denatured DNA in vitro synthesis (cancerous and normal DNA)

Compounds, μmol	^{3}H-TTP incorporated, cpm, in 10 min at 36 °C							
	rat hepatoma DNA				rat spleen DNA			
	native DNA	% inhibition	denatured DNA	% inhibition	native DNA	% inhibition	denatured DNA	% inhibition
Complete medium	2,519 ± 57	–	2,433 ± 21	–	2,102 ± 34	–	2,064	–
+ sempervirine 0.04	1,354 ± 22	46	1,241 ± 23	48	1,954 ± 20	7	1,716	16
+ sempervirine 0.08	933 ± 25	62	1,029 ± 29	60	1,797 ± 31	14	1,612	21
+ sempervirine 0.16	410 ± 14	83	446 ± 22	81	1,769 ± 23	16	1,426	38

For incubation conditions see text. Rat hepatoma DNA (0.25 μg) and spleen DNA (0.5 μg) were used as templates. Significance of comparison to control (complete medium): $p < 0.001$ for rat hepatoma native and denatured DNA; $p < 0.001$ for spleen native and denatured DNA (data from three independent experiments).

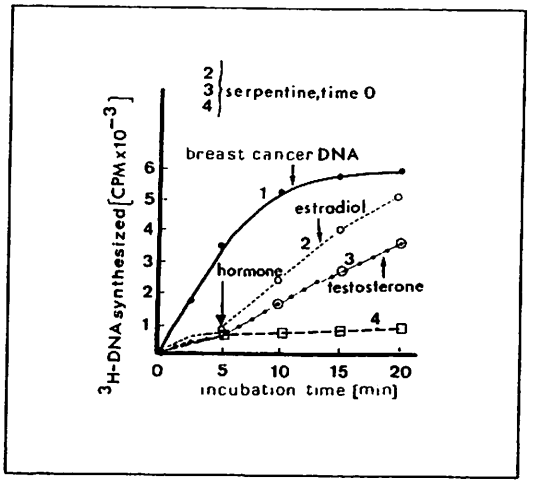

Fig. 5. Steroids may overcome breast cancer DNA synthesis inhibited by serpentine. Incubation conditions, see text. Breast cancer DNA (0.5 µg) was used as template. 1 (●) = complete medium; 4 (□) = complete medium + serpentine (80 µg) at time 0; 2 (○) = complete medium + serpentine (80 µg) at time 0 and estradiol (50 µg) at the 5th min of incubation; 3 (⊙) = complete medium + serpentine (80 µg) at time 0 + testosterone (50 µg) at the 5th min of incubation.

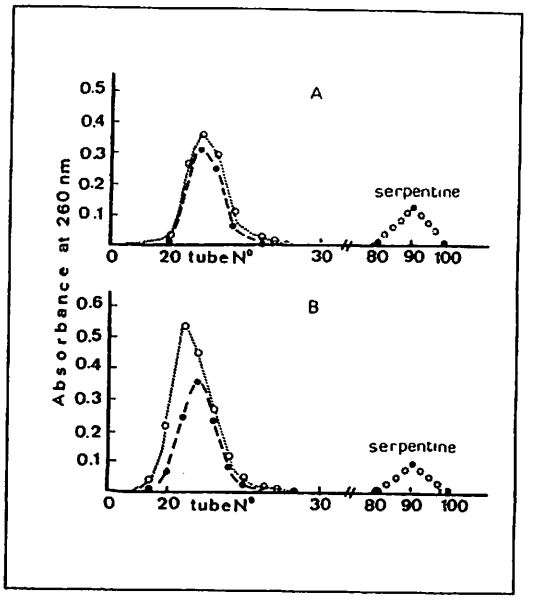

Fig. 6. 'Cancer DNA-serpentine' complex in in vitro formation. For conditions, see Materials and Methods. **A** ● = Breast DNA; ○ = breast DNA + serpentine. **B** ● = Breast cancer DNA; ○ = breast cancer DNA + serpentine.

Table III. Effects of sempervirine and steroids on cancer DNA in vitro synthesis

Compounds, µmol	^{3}H-TTP incorporated, cpm, in 10 min at 36 °C				
	Breast cancer DNA			KB cells DNA	
		p[a]	% inhibition		% inhibition
Complete medium	2,310 ± 50	–	–	2,246	–
+ sempervirine, 0.15	413 ± 19	<0.001	83	476	80
+ estradiol, 0.16	1,901 ± 58	<0.01	17	436	81
+ estrone, 0.16	2,190 ± 70	$<0.5 <0.9$	6	412	82
+ progesterone, 0.16	1,970 ± 53	<0.01	14	367	84
+ testosterone, 0.16	2,345 ± 82	$<0.5 <0.9$	0	373	83

Breast cancer DNA (0.25 µg) and KB cells (0.25 µg) were used as template. Steroids were first dissolved in 0.2 ml alcohol, 96°, and then diluted with distilled water. Enzyme was added as last component. p[a], p, value significance of comparison to control (complete medium) of four experiments.

DNA. These data show that the activity of DNA template is affected by the alkaloid while that of DNA polymerase is not.

Carcinogens, known to increase the number of initiation sites in DNA of cancerous cells [19], also strongly enhance cancer DNA in vitro synthesis and induce local cancer DNA strand separation [1]. On this basis one might expect that alkaloids which also selectively recognize cancer DNA will inhibit the synthesis having been enhanced by carcinogens. In fact, sempervirine or serpentine (fig. 3), which in the absence of carcinogens (ethionine or DMBA) inhibits only the initiation of cancer DNA replication, prevents DNA synthesis which has already started in the presence of a carcinogen (fig. 4A, B). Thus alkaloids appear to bind faster to liberated cancer DNA initiation sites than does DNA polymerase, and consequently inhibit DNA synthesis. *dl*-Ethionine (carcinogen) present in the incubation mixture at time 0 *does not stimulate cancer DNA synthesis in the presence of sempervirine* (table I).

Steroids also induce the separation of the strands of cancer DNA isolated from steroid target tissue, breast cancer for instance, and liberate the initiation sites for DNA replication [1]. At given concentrations, steroids allow alkaloids to prevent cancer DNA synthesis which has already started (fig. 4A). However, at high concentrations, they compete with alkaloids for template when the DNA originates from steroid target tissues (fig. 5) and not, for instance, with DNA from KB cells (table III).

'Alkaloid Cancer DNA' Complex Formation

The strong inhibition that each alkaloid exerts on the in vitro synthesis of DNA from cancerous tissues and the only slight inhibition on that of DNA from healthy tissues prove that alkaloids have a preferential affinity for DNA from cancerous cells. In fact, breast cancer DNA binds a relatively large amount of serpentine, while DNA from the corresponding healthy tissues binds little, if any (fig. 6). This may explain why alkaloids preferentially inhibit the synthesis of DNA from cancer tissues but not that of DNA from healthy tissues. It should be noted that alkaloids also have a high affinity for RNA fragments rich in G and A nucleotides or for ribopolymers of the A, G or AG type. In contrast, they do not bind to poly C or poly U or free nucleotides (results not presented here). Thus, it seems quite possible that cancer cell DNA possess some 'accessible AG sites' in the destabilized DNA which are consequently capable of reacting with alkaloids.

Conclusion and Discussion

We have shown previously that DNAs from various cancer tissues are destabilized, i.e. contain a large amount of single-stranded areas compared to DNAs from healthy tissues. A correlation between in vitro DNA synthesis, DNA-strand separation and multiplication of cancer cells was demonstrated [1]. Molecules with an affinity for DNAs (for instance carcinogens, steroids, antimitotic drugs, etc.) induce further in vitro destabilization of cancer DNAs, which is a very receptive template for the binding of different molecules. The alkaloids used here exhibit strong and selective affinity for DNA from cancer tissues but little affinity for DNA from healthy tissues. Unlike carcinogens, antimitotic drugs and steroids (for hormone target tissues), none of the four alkaloids enhance cancer DNA in vitro synthesis. In fact

they specifically inhibit it and have practically no effect on DNA from healthy tissues. We have shown here that these alkaloids act by inhibiting the initiation of cancer DNA synthesis and not of chain elongation. If the same experiments are performed in the presence of a carcinogen which, by inducing DNA-strand separation in some areas, provides more initiation sites, the alkaloids, at appropriate concentrations, may then prevent cancer DNA replication at any moment. Steroids which are nonmutagenic substances induce the appearance of cancerous tissues in the mammary gland of mice [11, 13], stimulate the in vitro multiplication of breast cancer cells [10, 17], enhance breast cancer DNA in vitro synthesis and separate the chains of the DNAs isolated from breast cancer tissues [1]. During this last process, alkaloids bind to newly liberated initiation sites and cause inhibition of DNA synthesis even if it has already started. However, it should be stressed that steroids at given concentrations may overcome the inhibition of cancer DNA synthesis caused by alkaloids. This reversible competition between steroids and alkaloids for binding sites on breast cancer DNA is not observed when DNAs from non-hormone target tissues are used. This observation indicates that some small-size molecules present on the DNA of steroid target tissues interact with hormones.

Alkaloids inhibit DNA in vitro synthesis in a large variety of cancer tissues. On this basis, one is inclined to postulate that DNAs from different cancerous tissues, although purified, contain a certain amount of molecules, peptides, amino acids, small size RNA, etc., the nature and amount of which probably differ from those of healthy DNAs. This is quite possible, since analysis of DNA and m-RNA from cancer and healthy tissues did not reveal any difference at the level of nucleotide sequences [14, 19].

Cancer DNAs interact in vitro with alkaloids, producing a 'DNA-alkaloid complex', which has been isolated. The DNA-alkaloid complex is also produced when DNAs from different cancer tissues are incubated with each of the four alkaloids (results not shown here). The binding of alkaloids to DNAs from healthy tissues is hardly measurable under the same experimental conditions. These results explain the specific inhibition of cancer DNA synthesis by alkaloids. It seems very probable that alkaloids intercalate between the strands of destabilized cancer DNAs and have little affinity for the stabilized structure of healthy cell DNA. This is strengthened by the observation that there is competition between alkaloids and psoralen, an intercalating agent (unpublished results).

The fact that alkaloids prevent and neutralize the stimulating effect of carcinogens or steroids (DNA from breast cancer) suggests that they may play an important role in preventing the effects of carcinogens or steroids on cell DNA. The slight inhibitory action of alkaloids on DNAs from healthy tissues or cells suggests that DNA in these cells does not have a destabilized structure, i.e. contains far less single-stranded areas than cancer DNA.

Data on the effects of alkaloids on cancerous and normal cells cultured in vivo and on the evolution of experimental animal cancers will shortly be described.

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