Sb-choline could also be assayed in lysed tissue extracts containing choline, by first separating these analogs on the cation exchange column described Fig. 2. The eluate fractions containing Sb-choline or choline were brought to pH 8.6 with a small volume of 1 N NaOH, and aliquots thereof were assayed radioenzymatically with the choline kinase method. Neither choline nor any other tissue constituents interfered with the assay of Sb-choline following this column separation (not shown). Further, this column step obviated the tetraphenylboron/heptanone extraction which McCaman and Stetzler [4] found necessary to reduce excessively high tissue blanks (yielding apparently high choline and ACh levels).

The synthesis described here for Sb-choline is similar to those described for analogs of choline in which the nitrogen is replaced by phosphorus [5] or arsenic [6]. The use of a sealed vessel and "diglyme" as solvent, however, hastened our synthesis by allowing us to use a higher reaction temperature than previously reported. Other chloride and iodide forms of Sb-choline are hygroscopic white powders that are apparently stable indefinitely at −20°.

Sb-choline and Sb-ACh should be useful not only as readily assayable cholinergic analogs for biochemical studies, but as excellent substrates for the X-ray microanalytical localization of Sb-choline and Sb-ACh in cell structures. X-ray microanalysis coupled to electron microscopy is a technique with tremendous potential for localizing and quantifying specific elements in small biological samples, e.g. lead [7] or calcium [8] containing compounds in synaptosomes [see Ref. 9 for a review]. Cholinergic nerve terminals are unique in that they have a high affinity uptake system for choline as well as storage vesicles for ACh. Since we have found that Sb-choline is taken up into synaptosomes in a Na-dependent manner in low concentrations, acetylated, and then released as Sb-ACh by depolarization with 60 mM potassium (E. M. Meyer and J. R. Cooper, unpulished observations), it should be possible to specifically identify structural components in cholinergic terminals and localize Sb-ACh in them. We are currently conducting experiments along these lines.

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Induction of deoxyribonucleic acid damage in HeLa S_3 cells by cytotoxic and antitumor sesquiterpene lactones

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Sesquiterpene lactones constitute one of the largest classes of antitumor and cytotoxic substances of plant origin [for review, see Refs. 1–3]. The biological activities of these compounds seem to be related to their alkylating properties [4–6], although their modes of action are still not established. Alkylation of sulfhydryl groups in enzymes has been postulated as the major mechanism by which sesquiterpene lactones exert their effects on cell growth [7, 8]. However, an alternative hypothesis that DNA is a target for these agents seems to be more relevant to biological and biochemical properties of sesquiterpene lactones [9 and references therein]. Recently we demonstrated that cytotoxic and antitumor sesquiterpene lactones preferentially inhibit DNA synthesis in HeLa cells, and this inhibition seems to play a role in the growth inhibitory action of the compounds studied [9]. This paper explores the possibility that inhibition of DNA synthesis and of cellular growth by sesquiterpene lactones may be a result of damage to DNA that alters DNA template properties.

Parthenolide was isolated from *Tanacetum vulgare* L., and 1,10-epoxyparthenolide was obtained by epoxidation of the former [9]. Alatolide and eupatoriopicin were provided by Prof. B. Drozdz from the Medical Academy, Poznan, Poland; vernolepin and elephantopin by Dr. A. T. Sneden from the University of Virginia, Charlottesville, VA, U.S.A.; and costunolide by Prof. R. W. Doskotch from the Ohio State University, Columbus, OH, U.S.A. HeLa S_3 cells were grown in suspension culture as described previously [10] and were at mid-log at the time of drug addition. DNA synthesis in subcellular systems from G_1/S

synchronized cells [11] was assayed by incorporation of [^{3}H]dTTP into lysates, purified nuclei, and reconstituted lysates [12]. Single-strand breaks in DNA from HeLa S_3 cells were analyzed by alkaline sucrose density gradients [13]. Ability of parthenolide to nick isolated DNA was assayed using the ethidium bromide fluorescence technique to measure conversion of superhelical PM2 DNA to the nicked circular duplex DNA form [14].

We found previously that parthenolide, used by us as a model sesquiterpene lactone, inhibits DNA synthesis in HeLa cells by acting after formation of deoxynucleoside triphosphates [9]. In this work we extended the previous studies in an attempt to determine whether the observed inhibition may result from impaired template properties of DNA. DNA synthesis in subcellular systems derived from inhibitor-treated cells could be measured by incorporation of radioactive deoxynucleoside triphosphate. This assay can be done using unfractionated cellular homogenates ("lysates") or purified nuclei, although in the latter the rate of DNA synthesis is slower [11]. DNA synthesis in nuclei, however, can be stimulated by the cytoplasmic fraction which contains most of the DNA-polymerase activity and other factors involved in replication [11]. Table 1 shows that incubation of HeLa S_3 cells with 202 μM parthenolide resulted in an almost complete inhibition of DNA synthesis in subcellular systems derived from these cells. The effect in unfractionated lysates was similar to that in purified nuclei (Table 1). Addition of the cytoplasmic fraction from control cells did not reverse the inhibition in nuclei from lactone-treated cells (Table 1), suggesting that the template, itself, was damaged. Thus, the data suggest that interference with DNA template plays a major role in the inhibition of DNA synthesis by parthenolide, although some effects on DNA-polymerases and/or other replication factors cannot be excluded.

One possible explanation of the effect of parthenolide on DNA replication is that the lactone induced damage to DNA as do other alkylating compounds (for review see Ref. 15). Alkaline sucrose density gradient centrifugation revealed that DNA from cells incubated with parthenolide was markedly reduced in size, when compared with DNA from control cells (Fig. 1). Thus, parthenolide induced single-strand breaks in DNA. The observed effect was clearly dose dependent (Fig. 1a) and time dependent (Fig. 1b). The DNA damage could be seen after incubation of cells with the lactone at 202 μM for 30 min. The size of DNA, however, was reduced considerably more after longer incubation of cells with parthenolide (Fig. 1b). When the cells were incubated with parthenolide for 16 hr a significant decrease of DNA size was observed at lactone concentrations as low as 3 and 15 μM (data not shown). The former value corresponded to the parthenolide concentration that inhibits the growth of HeLa cells by 50 per cent (EC_{50}) as determined previously [9]. To elucidate further the relevance of DNA damage to biological activity, we selected six other cytotoxic and antitumor sesquiterpene lactones. The EC_{50} values of these compounds against HeLa cells range from 0.5 up to 6.15 μM [9]. To determine the abilities of the selected lactones to induce DNA single-strand breaks, each or these compounds was given to HeLa cells at concentrations corresponding to 35× and 70× its EC_{50} value. All of the lactones studied were found to induce DNA damage, as in the case of parthenolide (Fig. 2). Moreover, the extent of the effect (reduction of DNA size) was similar for all of these agents when the results for biologically equieffective concentrations were compared (Fig. 2). Thus, the data suggest that there is a correlation between DNA damage and ability of sesquiterpene lactones to inhibit cellular growth.

We questioned whether the lactones could act directly on isolated DNA and also cause DNA breaks. Using an assay based on conversion of PM2 DNA from the superhelical form to the relaxed one [14], we found that this is

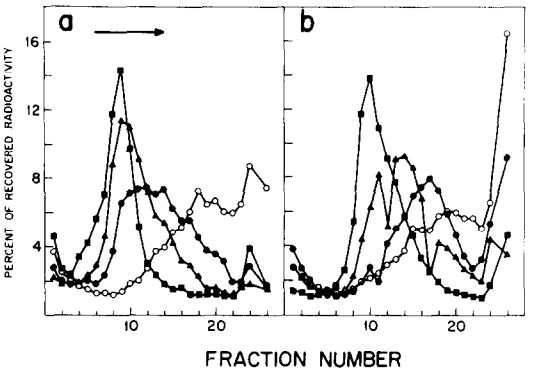

Fig. 1. Induction of DNA single-strand breaks by parthenolide in HeLa S_3 cells. The cells (0.5 × 10^6/ml), prelabeled with [^{14}C]thymidine, were incubated at 37° with parthenolide as indicated. Panel a: incubation for 4 hr with parthenolide at 0 μM (control) (—○—); 45 μM (—●—); 105 μM (—▲—); and 210 μM (—■—). Panel b: incubation with 202 μM of parthenolide for 0 min (control) (—○—); 30 min (—●—); 1 hr (—▲—); and 2 hr (—■—). Cells were lysed on the top of a 5–30% alkaline sucrose gradient and were centrifuged in an SW 50.1 rotor for 60 min at 25,000 rpm as described elsewhere [13].

Table 1. DNA synthesis in subcellular systems derived from control and parthenolide-treated HeLa S_3 cells*

System	Control (dpm ± S.D.)	Parthenolide-treated cells (dpm ± S.D.)	(% control)
A Unfractionated lysates	11735 ± 1494	1939 ± 141	17
B Purified nuclei	1301 ± 79	243 ± 11	19
C Purified nuclei + cytoplasmic fraction from control cells	5677 ± 560	847 ± 41	15

* The cells (0.5 × 10^6/ml), synchronized at G_1/S, were incubated with parthenolide (202 μM) for 4 hr. Then lysates, purified nuclei, and cytoplasmic fraction were obtained and assayed for incorporation of [^{3}H]dTTP [11]. Results (dpm) show average values of triplicate samples (2.25 × 10^6 nuclei each) ± S.D. after a 20-min incubation with [^{3}H]dTTP (1.04 Ci/mole).

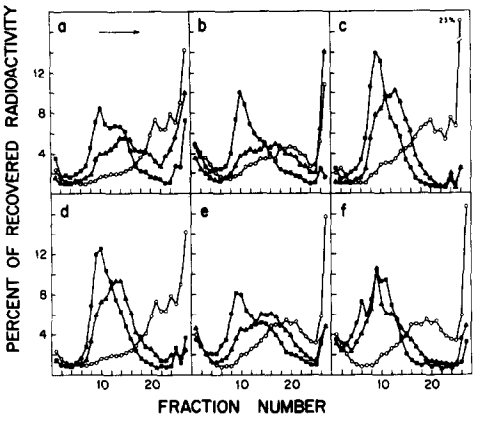

Fig. 2. Induction of single-strand breaks by sesquiterpene lactones in HeLa S_3 cells. The cells were incubated for 2 hr with lactones given at concentrations of 35 × EC_{50} (—▲—); 70 × EC_{50} (—■—); and control (—○—). (EC_{50} = concentration inhibiting by 50 per cent growth of HeLa cells.) The following lactones were used (EC_{50} values [9] given in parentheses): (a) constunolide (6.15 μM); (b) epoxyparthenolide (4.35 μM); (c) eupatoriopicrin (1.15 μM); (d) elephantopin (0.5 μM); and (f) vernolepin (2.4 μM). Other details are given in the legend to Fig. 1.

not the case. PM2 DNA was incubated with parthenolide (204 μM) at 37° for 4 and 24 hr in the absence and presence of 2 mM dithiothreitol, as some of the DNA-damaging agents are activated by sulfhydryl compounds [16]. Parthenolide was ineffective under any of these conditions (data not shown). A similar lack of effect on PM2 DNA was observed in the case of elephantopin, vernolepin and eupatoriopricin (data not shown).

The results suggest that sesquiterpene lactones may not damage DNA in the cells directly but rather after some kind of metabolic activation. On the other hand, DNA single-strand breaks found in lactone-treated cells may result from enzymatic excision of the sites in DNA that have been damaged by the lactone action (e.g. alkylation). Another possibility is that single-strand breaks observed on alkaline sucrose gradients correspond to alkali labile bonds created in DNA at the sites damaged by lactones. These explanations are consistent with the hypothesis that sesquiterpene lactones can alkylate cellular DNA.

In summary, parthenolide, a cytotoxic sesquiterpene lactone, inhibited DNA replication in HeLa cells, most likely by interfering with DNA-template. This lactone was also found to induce DNA single-strand breaks in cellular DNA of HeLa S_3 cells as shown by alkaline sucrose gradient centrifugation. The extent of DNA damage was dose and time dependent. Six other antitumor and cytotoxic sesquiterpene lactones were demonstrated to induce damage to DNA in HeLa S_3 cells. This effect seems to have been related to the abilities of these compounds to inhibit DNA synthesis and cellular growth. The results support the hypothesis that alkylation of DNA is a molecular basis of biological activity of sesquiterpene lactones.

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