Inhibition of DNA Topoisomerase I Activity by Diethylstilbestrol and Its Analogues

Taiko ODA,^a Yoshihiro SATO,^{*a} Masahiko KODAMA,^b and Motohisa KANEKO^b

Kyoritsu College of Pharmacy,^a Shibakoen 1-chome, Minato-ku, Tokyo 105, Japan and Biophysics Division, National Cancer Center Research Institute,^b Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan. Received December 10, 1992

Diethylstilbestrol and its derivatives were tested to determine their inhibitory effects on the relaxation activity of DNA topoisomerase I using cell lysates from Chinese hamster V79 cells. Among the derivatives investigated, (±)-indenestrol B, (±)-IB, showed the strongest inhibitory effect.

Keywords diethylstilbestrol; indenestrol; DNA topoisomerase; dienestrol; Chinese hamster V79 cell

trans-Diethylstilbestrol (DES) is a potent synthetic estrogen capable of eliciting biological responses similar to those induced by endogenous 17β -estradiol.¹⁾ Studies in humans and mice²⁾ have suggested that *in utero* exposure to DES induces cancerous lesions of the reproductive organs. In cultured cells from Syrian hamster embryo³⁾ or from C3H mouse,⁴⁾ DES has also been reported to induce or promote malignant cell transformation. Thus, participation of cellular targets other than steroid hormone receptors could be involved in the process of transformation,⁵⁾ and microtubule proteins have been identified as one of these cellular targets.⁶⁾

We reported previously⁷⁾ that various derivatives of DES inhibited microtubule assembly *in vitro*, and induced aneuploidy and disturbance of microtubule distribution in Chinese hamster V79 cells. There was a good correlation between the activity of DES and its monomethyl and dimethyl ethers (DES-monoMe and DES-diMe, respectively), as microtubule poisons and their toxic effect on V79 cells; however, for other related compounds, no such clear correlation was observed.⁸⁾ For this reason, we tried to look for other cellular targets of DES and its derivatives (Chart 1). Since some derivatives of podophyllotoxin, a microtubule poison, are inhibitors of DNA topoisomerase II,⁹⁾ we assumed that DES and its derivatives would also inhibit these topoisomerases.¹⁰⁾ We report here that DES and its derivatives are inhibitors of DNA topoisomerase I (Topo I) in V79 cell extracts, as assayed by *in vitro* relaxation of superhelical plasmid DNA.

Experimental

Materials The derivatives used were: DES, mono- and dimethyl ether (DES-monoMe and DES-diMe) of DES, *E,E*-dienestrol (*E*-DIEN), *Z,Z*-dienestrol (*Z*-DIEN), indenestrol A (IA), indenestrol B (IB), and estradiol (*E*₂), where *Z*-DIEN and IA are the products of *in vivo* and *in vitro* oxidative metabolism,¹¹⁾ and *E*₂ is a naturally occurring estrogen in mammals (Chart 1). DES, *E*-DIEN, *E*₂, and camptothecin¹²⁾ were purchased from Sigma Chemical Co. *Z*-DIEN, IA, and IB were prepared as described previously.¹³⁾ Dimethyl sulfoxide (DMSO) was purchased from Pierce Chemical Co. (Rockfield, IL). Fetal bovine serum (FBS) was obtained from Gibco after lot-checking.

Plasmid DNA pUC19 Plasmid DNA was isolated from *Escherichia coli* HB101 carrying pUC19 by alkaline lysis followed by cesium chloride/ethidium bromide ultracentrifugation.¹⁴⁾

Preparation of Cell Extracts for DNA Topo I Relaxation Chinese hamster V79 cells were grown in monolayer culture in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Ltd. (Tokyo)) with 10% (v/v) heat-inactivated FBS. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air. Cells from 100-mm dishes were trypsinized, pelleted (1000 × *g*, 3 min), and washed in phosphate-buffered saline (PBS) (2–3 ml). Then, 5 × 10⁶ cells from eight dishes were lysed by resuspending them in 250 μl of 10 mM Tris-HCl (pH 7.8), 0.5 mM EDTA, 0.005% Triton X-100, 1.0 M KCl, and kept frozen at –80°C until use. The resuspended lysate was centrifuged at 15000 rpm at 4°C [No. 20N 20000 rpm and RS-18GL refrigerated centrifuge; Tomy Seiko Co., Ltd., Tokyo, Japan] for 5 min, and the supernatant was used as the cell extracts of Chinese hamster V79 cells.

Assay of Enzyme Activity Topo I enzyme activity was assayed by monitoring the relaxation of supercoiled plasmid DNA (pUC19), according to the following modification of a procedure reported by Tricoli *et al.*¹⁵⁾ Enzyme reactions were performed in a total volume of 20 μl containing 10 mM Tris-HCl (pH 7.8), 200 μM EDTA, 500 μM dithiothreitol (DTT), 200 mM MgCl₂, pUC 19 (0.8 μg), the Chinese hamster V79 cell extract and various test compounds. The amount of compound/DMSO added was usually 1 μl. The reaction was started by addition of the Chinese hamster

Chart 1

V79 cell extract, and after incubation at 30 °C for 15 min, bromophenol blue (0.5% (w/v)), xylene cyanol (0.5% (w/v)) and glycerol (60%, v/v) were added. The extent of relaxation was followed by agarose gel (1.0% (w/v)) electrophoresis performed at 100 V for 2 h, and the bands were visualized under black light (302 nm) after staining with ethidium bromide (2 μ M) for 30 min. Photographs were recorded with an MP-3 Polaroid camera. The inhibition activities were determined by densitometric analysis of the photographs using a Shimadzu CS-9000 Dual-wavelength flying-spot scanner.

Nicking and Joining of Circular DNA The experimental procedure was according to that by Hayashi *et al.*¹⁶⁾ with a minor modification. Briefly, pUC19 form I DNA was treated with DNase I (Takara) to produce an average of one nick per molecule. This open circular DNA was treated with T4 ligase (Takara) at 16 °C for 2 h in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 10% DMSO and ethidium bromide or DES-derivatives. After ligation, the mixture was analyzed by agarose gel electrophoresis as described above.

Results and Discussion

Chart 1 shows various DES-related derivatives which were examined for their effects on the relaxation activity of Topo I. Topo I activity was assayed using lysates of V79

Fig. 1. Inhibition by DES of DNA Topoisomerase I Relaxation

Arrows F1 and F2 indicate intact supercoiled DNA and nicked DNA, respectively. Arrows F3, F4 and F5 indicate intact supercoiled DNA dimer, trimer, and tetramer, respectively. Lane 1, control pUC19 supercoiled DNA. Lane 2, no drug. Lanes 3–6, 1, 10, 100, and 1000 μ M of DES, respectively.

cells and supercoiled pUC19 DNA. A typical example of the apparent inhibition of relaxation activity of Topo I by DES is shown in Fig. 1. Since the apparent inhibition of relaxation by a drug could be due to its intercalation to DNA,¹⁷⁾ the possibility of intercalation of DES-related derivatives to DNA was checked by agarose gel electrophoresis of circular DNA which was closed in the presence or absence of these derivatives (Fig. 2).

Limited treatment of closed circular pUC19 (lane 1) DNA with DNase I yielded the open circular form (lane 2). Ligase treatment of this DNA produced several bands of closed circular DNA with low superhelical turns and the open circular DNA (lane 3). In the presence of a typical intercalator, ethidium bromide, more negative superhelical DNA was produced (lanes 5–7), while the DNA ligated in the presence of DES or IB (lanes 8–11) was indistinguishable from the control DNA (lane 4). Thus, we concluded that DES-related derivatives did not intercalate into DNA at the concentration where the inhibition of relaxation was observed. The inhibitory effects of various compounds were determined on the basis of densitometric analysis of photographs, similar to that in Fig. 1. As a measure of the inhibitory effect, the concentration required to inhibit 10% of the activity (IC₁₀) and the percentage inhibition at 1 mM drug concentration were used, as shown in Table I. In most cases except for ($\pm$)-IB, lower-IC₁₀ compounds showed higher inhibition at 1 mM. Further,

TABLE I. Inhibition of DNA Topoisomerase I Relaxation Activity by DES-Related Compounds

Compound	IC ₁₀ (μ M)	IC ₅₀ (μ M)	Inhibition at 1 mM (%)
($\pm$)-IB	200 $\pm$ 6	> 1000	43 $\pm$ 4
Z-DIEN	260 $\pm$ 8	590 $\pm$ 6	76 $\pm$ 4
($\pm$)-IA	400 $\pm$ 11	840 $\pm$ 6	61 $\pm$ 3
DES	470 $\pm$ 7	920 $\pm$ 8	67 $\pm$ 4
DES-monoMe	700 $\pm$ 15	> 1000	30 $\pm$ 4
E-DIEN	950 $\pm$ 7	> 1000	28 $\pm$ 3
DES-diMe	> 1000	> 1000	0
E ₂	> 1000	> 1000	0
CT	40 $\pm$ 4	— ^{a)}	— ^{a)}

CT, camptothecin. Each value represents the average $\pm$ S.D. of 3 experiments. The inhibition activities were determined by densitometric analysis. ^{a)} Not determined.

Fig. 2. Agarose Gel Electrophoresis of Circular DNA Closed in the Absence or Presence of Ethidium Bromide or DES-Related Derivatives

Lane 1, pUC19 supercoiled DNA. Lane 2, *Hind* III treated pUC19. Lane 3, open circular form produced by DNase I digestion. Lane 4, control (with ligase and DMSO). Lanes 5–11, with ligase and 3.2, 6.4, and 12.7 nM of ethidium bromide, 1 mM and 2 mM of DES, and ($\pm$)-IB, respectively.

though the inhibitory activities at IC_{50} were tested, the inhibition was found not to be dose-dependent. Although the inhibitory activity differed for each DES derivative, the structural factors essential for the inhibition were two hydroxyl groups on two aromatic rings in DES related compounds.

In the DES series inhibition of topoisomerase I relaxation activity decreased when the phenolic hydroxyl groups were methylated to monoMe and then to diMe derivatives. Compared with isomers of dienestrols, Z-DIEN showed about 3.5 times higher inhibitory activity than did E-DIEN. In indenestrols, IB showed higher inhibitory activity than IA, but only about 1/5 that of camptothecin.

In conclusion, a weak but consistent inhibitory effect on the relaxation activity of Topo I was observed for various DES derivatives.

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