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Inhibitory Effects of the Tyrosine Kinase Inhibitor Genistein on Mammalian DNA Topoisomerase II[1]

Judith Markovits,[2] Claude Linassier, Philippe Fossé, Jeanine Couprie, Josiane Pierre, Alain Jacquemin-Sablon, Jean-Marie Saucier, Jean-Bernard Le Pecq, and Annette K. Larsen

Laboratoire de Pharmacologie Moléculaire, URA 158 du CNRS, U 140 de l'INSERM, Institut Gustave Roussy, 94805 Villejuif Cédex, France

ABSTRACT

Tyrosine phosphorylation plays a crucial role in cell proliferation and cell transformation which suggests that tyrosine kinase-specific inhibitors might be used as anticancer agents. When the cytotoxic effect of the potent tyrosine kinase inhibitor genistein on various cell lines was studied, we observed that 9-hydroxyellipticine-resistant Chinese hamster lung cells (DC-3F/9-OH-E) were markedly more resistant to genistein than the parental cell line (DC-3F). The DC-3F/9-OH-E cells have been shown to have an altered DNA topoisomerase II activity. We therefore examined the effects of genistein on DNA topoisomerase II-related activities of nuclear extracts from DC-3F cells as well as on purified DNA topoisomerase II from calf thymus. Our results show that genistein (*a*) inhibits the decatenation activity of DNA topoisomerase II and (*b*) stimulates DNA topoisomerase II-mediated double strand breaks in pBR322 DNA on sites different from those of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide, etoposide, and 2-methyl-9-hydroxyellipticinium. Structure-activity studies with six chemically related compounds show that only genistein has an effect on the cleavage activity of DNA topoisomerase II in the concentration range studied. Finally, genistein treatment of DC-3F cells results in the occurrence of protein-linked DNA strand breaks as shown by DNA filter elution. Viscometric (lengthening) studies demonstrate that genistein is not a DNA intercalator. Genistein is therefore an interesting compound because it induces cleavable complexes without intercalation.

Taken together, our results show that genistein is an inhibitor of both protein tyrosine kinases and mammalian DNA topoisomerase II. This could be accounted for by the sharing of a common structure sequence between the two proteins at the ATP binding site.

INTRODUCTION

Several retroviral oncogenes such as *src*, *yes*, *fgr*, *abl*, *fps*, *fes*, and *ros* code for tyrosine-specific protein kinases (1). Similar kinase activity is associated with the cellular receptors for EGF,[3] platelet-derived growth factor, insulin, and insulin-like growth factors (2). This suggests that tyrosine phosphorylation plays an important role in cell proliferation and cell transformation and that tyrosine kinase-specific inhibitors might be used as anticancer agents (3, 4). It has recently been shown that the tyrosine kinase inhibitor, erbstatin, has antitumor activity towards the L1210 mouse leukemia (5, 6). Another tyrosine kinase inhibitor, genistein, has been isolated from the fermentation broth of *Pseudomonas* sp. This compound, which is an isoflavone derivative, has been shown to be a specific inhibitor of the tyrosine kinase activity of the EGF receptor, $pp60^{v\text{-}src}$ and $p110^{gag\text{-}fes}$ *in vitro* (7).

Therefore we studied the cytotoxic effects of genistein on various cultured cell lines, and we observed that 9-hydroxyellipticine-resistant Chinese hamster lung cells (DC-3F/9-OH-E) were markedly more resistant to genistein than the parental line (DC-3F). The DC-3F/9-OH-E cells are cross-resistant to DNA topoisomerase inhibitors, such as NMHE, m-AMSA, and etoposide (8, 9). There is no difference with respect to uptake of these drugs through the plasma membrane (8–10), and subsequent studies have shown an alteration of the DNA topoisomerase II activity (10–14).

As a result of finding cross-resistance to genistein in the DC-3F/9-OH-E cells, we decided to examine the effect of genistein on mammalian DNA topoisomerase II. We now show that genistein inhibits the catalytic activity of DNA topoisomerase II and leads to the formation of cleavable complexes *in vitro*. We also show that genistein treatment *in vivo* results in the formation of protein-linked DNA strand breaks in DC-3F cells.

MATERIALS AND METHODS

Drugs and Chemicals

Genistein, apigenin, biochanin A, genistin, prunetin, and quercetin (Fig. 1) were purchased from Extrasynthese Laboratories (France). Flavone acetic acid (NSC 347512) was from Lyonnaise Industrielle Pharmaceutique (LIPHA, Lyon, France). Etoposide (VP-16-213, NSC 141540) and m-AMSA (NSC 249992) were a generous gift of Dr. W. T. Bradner (Bristol-Myers Co., Syracuse, NY) and Dr. Y. Pommier (National Cancer Institute), respectively. Twenty mM stock solutions in DMSO were always prepared just prior to use followed by dilution with medium or 20 mM Tris-HCl buffer, pH 8, to the desired concentration. The final concentration of DMSO in the culture medium never exceeded 1% (v/v). 2-Methyl-9-hydroxyellipticinium was a gift from SANOFI (France). Stock solution of the ellipticine derivative was prepared as a 10 mM stock in distilled water.

[2-^{14}C]Thymidine and [*methyl*-^{3}H]thymidine (specific activity, 0.055 and 20 Ci/mmol, respectively) and [α-^{32}P]dATP (3000 Ci/mmol) were from Amersham. Tetrapropylammonium hydroxide was purchased from Fluka. ATP was obtained from Boehringer. All other chemicals were of reagent or analytical grade, provided from Sigma Chemical Co. or Merck Laboratories. Fetal calf serum and all cell culture media were purchased from Gibco Laboratories.

DNAs and Enzymes

Supercoiled pBR322 DNA was either prepared from *Escherichia coli* (*Hsd*R, *Hsd*M, *rec*AF) as described (15) or purchased from Boehringer. Highly catenated kinetoplast DNA was prepared from *Trypanosoma cruzi* pellets (16, 17) provided by Dr. G. Riou, Institut Gustave-Roussy, Villejuif, France. Calf thymus DNA (Boehringer) was sonicated as described (18). DNA topoisomerase II was isolated from calf thymus nuclei and purified using the previously published procedures (19, 20). Restriction endonucleases, DNA polymerase I, the Klenow enzyme, and α-dTTP were purchased from either New England Biolabs or Boehringer Mannheim. Proteinase K was from Bethesda Research Laboratories.

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: EGF, epidermal growth factor; NMHE, 2-methyl-9-hydroxyellipticinium; m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; VP-16-213, etoposide; DMSO, dimethyl sulfoxide; kDNA, kinetoplast DNA; SDS, sodium dodecyl sulfate; MEM, Eagle's minimal essential medium; ED_{50}, drug concentration required to reduce growth by 50% compared to untreated controls; DPC, DNA-protein cross-links; MDR, multidrug-resistant.

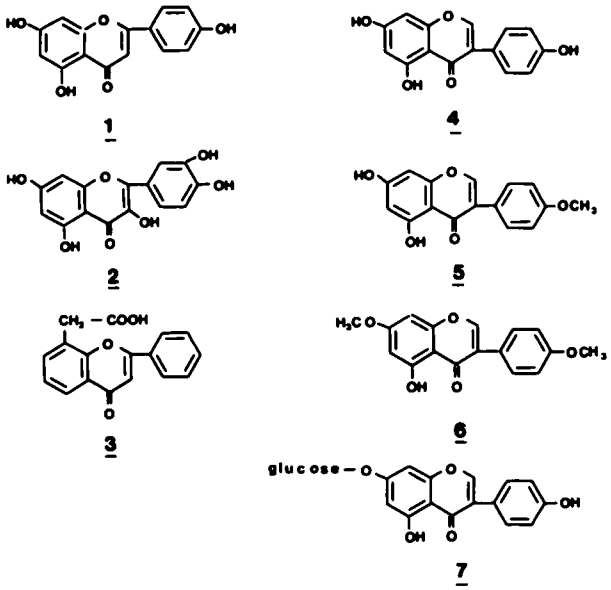

Fig. 1. Chemical structures of the compounds investigated. Flavonoids: apigenin (*1*); quercetin (*2*); flavone acetic acid (*3*). Isoflavonoids: genistein (*4*); biochanin A (*5*); prunetin (*6*); genistin (*7*).

Preparation of ^{32}P end-labeled pBR322 DNA

Linear pBR322 DNA obtained by *Eco*RI digestion was labeled at its 3'-ends as described (15, 21) by filling in restriction enzyme-generated sticky ends with [α-^{32}P]dATP using Klenow enzyme. The DNA labeled on both ends was cleaved with restriction endonuclease *Hin*dIII releasing a 29-base pair fragment.

DNA Interactions

Absorption spectra of genistein (10^{-4} M) in the absence and in the presence of sonicated calf thymus DNA (3.2×10^{-4} M base pair) were recorded on a Uvikon 860 (Kontron) spectrophotometer at 25°C.

The eventual binding of genistein to DNA was also tested by competition experiments with ethidium bromide as described (22).

Fluorescence was measured with a SLM 800 spectrofluorometer (Urbana, IL) equipped with a thermostated cell holder and a 9315 Ortec photon counter. Excitation and emission wavelengths were λ = 540 nm and λ = 590 nm, respectively.

Viscometric measurements were performed in a semimicrodilution capillary viscometer (Schott Geräte AVS 310) at 25°C as previously described (18). The length increase of short calf thymus DNA segments (100 μg/ml) was measured by the increase of intrinsic viscosity of the DNA in the presence of increasing concentrations of genistein as reported by Saucier *et al.* (18). These experiments were performed in 0.1 M Tris-HCl buffer, pH 7.4.

DNA Topoisomerase II Assays

The standard reaction mixture for all type II DNA topoisomerase assays contained 40 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml of bovine serum albumin, and 1 mM ATP.

Assessment of DNA Topoisomerase II Catalytic Activity. Decatenation of kinetoplast DNA was carried out by incubation of 0.5 μg of kDNA with purified topoisomerase II (4 ng) in a final volume of 15 μl at 30°C for 12 min. Reaction was terminated by the addition of SDS (0.25% final concentration).

The reaction products were analyzed on a 1.2% agarose gel in Tris-acetate running buffer (40 mM Tris-acetate/1 mM EDTA, pH 7.8) at 4 V/cm for 3.15 h. Gels were stained with ethidium bromide, destained during 4 to 6 h, and photographed under UV light with Polaroid films. Negative films were scanned by a Microdensitometer MKIIIC double-beam recording (Joyce, Loebl, and Co., Ltd., United Kingdom) to determine the peak area of liberated decatenated kDNA minicircles.

Cleavable Complex Formation. Drug-stimulated DNA-topoisomerase II-mediated DNA cleavage activity was assayed by following the generation of linear DNA (Form III) from supercoiled (Form I) pBR322 DNA after separation of the different forms by agarose gel electrophoresis.

DNA (0.3 μg) and drug (1 to 100 μM) in a final volume of 15 μl were incubated with purified calf thymus topoisomerase II (50 ng) for 30 min at 37°C. The reaction was terminated by addition of a mixture of SDS and proteinase K (final concentrations, 0.4% and 0.4 mg/ml, respectively). After an additional incubation for 30 min at 50°C, 5 μl of loading buffer containing 0.25% bromophenol and 40% sucrose were added. Whole samples were then run overnight on a 0.8% agarose gel at 2 V/cm in 89 mM Tris-borate buffer, pH 8, with 2 mM EDTA, containing 0.5 μg/ml of ethidium bromide and processed as described above. The peak areas of linearized DNA (Form III) were calculated.

The cleavage patterns of ^{32}P end-labeled linear pBR322 DNA by DNA topoisomerase II in the absence and in the presence of drugs were analyzed by electrophoresis using 1% agarose gel. The product of each cleavage reaction was loaded and run overnight at 11°C, at 2.5 V/cm in Tris-borate-EDTA buffer. The gels were dried, and autoradiography was performed.

Cell Lines and Culture Techniques

The parental hamster lung cell line DC-3F and the resistant sublines DC-3F/9-OH-E and DC-3F/AD X (23) were grown in MEM supplemented with 10% fetal calf serum and penicillin-streptomycin. Cells were routinely maintained in exponentially growing monolayer culture by seeding 10^5 cells/25-cm^2 flasks and splitting every 3 days.

Cytotoxicity

For determination of the cytotoxic effect of genistein, 2 to 5×10^5 cells were plated in 24-well dishes (Nuclon, Roskilde, Denmark) with various concentrations of drug for 72 h. The concentration of DMSO was adjusted to be less than 1%. The ED_{50} of each cell line was determined from the exponential part of the dose-response curve.

The *in vitro* colony formation was also used to determine survival fractions after 3-h drug exposure.

Preparation of Crude Nuclear Extracts

Between 5×10^7 and 10^8 DC-3F cells from an exponentially growing population were used to prepare nuclear extracts as described in detail elsewhere (24, 25). Briefly, control and genistein-treated cells (20 μM or 60 μM of genistein for 3 h) were scraped into ice-cold nucleus buffer containing 1 mM KH_2PO_4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM disodium EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, and 10% (v/v) glycerol, pH 6.4, and centrifuged at 460 $\times$ *g* for 10 min. All additional procedures were performed at 4°C. Cells were rinsed once in nucleus buffer, spun again, and resuspended in 1 ml of nucleus buffer. Nine ml of nucleus buffer containing 0.3% Triton X-100 were added, and the suspension was gently rotated for 10 min. Nuclei were centrifuged again and resuspended in 1 ml of nucleus buffer containing 0.35 M NaCl (final concentration). The salt extraction of the isolated nuclei was performed by gentle rotation for 30 min. The nuclei were centrifuged at 670 $\times$ *g* for 20 min. The supernatants were centrifuged again at 12,000 $\times$ *g* for 15 min. The amount of protein in the supernatant was determined using the Biorad assay (26). The supernatant was stored in the presence of bovine serum albumin (1 mg/ml) in a mixture of glycerol:nuclei buffer (1:1) at −20°C and used within 3 wk.

Measurements of DNA Damage by Alkaline Elution

The methodology of DNA alkaline elution has previously been described (27–29). Asynchronously proliferating DC-3F cells were labeled for 20 h with 0.02 μCi/ml of [2-^{14}C]thymidine or with a mixture of [*methyl*-^3H]thymidine (0.1 μCi/ml) and 10^{-6} M unlabeled thymidine. Cells were then washed with medium and grown in label-free medium (for 3 h) prior to drug treatments. [^{14}C]Thymidine-labeled DC-3F cells were treated with genistein for 24 h at 37°C. Reactions were terminated by washing and scraping the cells in drug-free medium (at 0°C).

DNA single-strand breaks were determined by DNA-denaturing (pH 12.1) alkaline elution carried out under deproteinizing conditions.

Briefly, 3×10^5 ^{14}C-labeled, genistein-treated DC-3F cells were mixed in 4 ml of cold MEM with $\simeq 5 \times 10^5$ ^{3}H-labeled untreated DC-3F cells that had been irradiated with 300 rads on ice. The elution rate of the DNA of [^{3}H]thymidine-labeled cells (internal standard cells) served to normalize the elution rate of DNA from cells labeled with [^{14}C]thymidine.

DPC were assayed for a mixture of genistein-treated [^{14}C]thymidine-labeled cells and ^{3}H-labeled internal standard cells. Cell mixtures were X-ray irradiated with 3000 rads on ice just prior to elution. Cells were layered on protein adsorbing filter (polyvinyl chloride; Gelman Sciences, Ann Arbor, MI), then lysed with 5 ml of a solution containing 2% SDS, 0.02 M disodium EDTA, and 0.1 M glycine (pH 10). The detergent was then washed away with 5 ml of 0.02 M disodium EDTA solution. Elution of DNA was carried out under DNA-denaturing (pH 12.1) and nondeproteinizing conditions. DPC frequencies were calculated using the bound to one terminus model of Ross *et al.* (30).

RESULTS

Cytotoxicity of Genistein. The cytotoxicity of genistein on Chinese hamster lung cells (DC-3F) and on two variants (DC-3F/9-OH-E and DC-3F/AD X) is shown in Fig. 2. DC-3F/9-OH-E cells have been shown to be resistant to the compound which inhibits DNA topoisomerase II due to an altered DNA topoisomerase II activity (10–14), whereas DC-3F/AD X, a variant resistant to actinomycin D, has a MDR phenotype and expresses a large quantity of the M_r 170,000 to 180,000 glycoprotein which is coded by the *mdr* gene (31). Fig. 2 shows that genistein is almost equally toxic to the DC-3F parental line and to its MDR variant DC-3F/AD X. In contrast, DC-3F/9-OH-E cells are about 10-fold more resistant to genistein than the parental line. Differences of the same order of magnitude were found by the colony-forming assay, where the ED_{50} for DC-3F was about 50 μM. The limited hydrosolubility of genistein ($\simeq$400 μM) did not permit a precise determination of the ED_{50} of the drug on the DC-3F/9-OH-E; however, these results show clearly that the resistant ratio is at least 8 in agreement with the data derived from growth inhibition assay. This suggests that genistein might interact with mammalian DNA topoisomerase II, for which reason, the effects of genistein on mammalian DNA topoisomerase II were studied.

Genistein Inhibits the Catalytic Activity of Purified Mammalian DNA Topoisomerase II *in Vitro*. The effect of genistein on the catalytic activity of purified calf thymus DNA topoisomerase II was assayed by following the decatenation of mitochondrial kinetoplast DNA to minicircles and small catenanes. Under the experimental conditions the reaction progressed linearly with time and reached a plateau after 12 min, at which time more than 90% of the original kinetoplast DNA was converted into decatenated products (results not shown). Fig. 3 shows that the decatenation activity of purified calf thymus

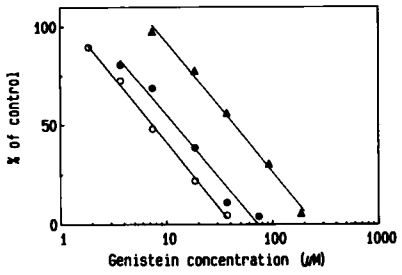

Fig. 2. Effect of genistein on the growth of DC-3F (○), DC-3F/AD X (●), and DC-3F/9-OH-E (▲) cells. Cells were exposed to various concentrations of genistein for 72 h. Following drug removal cells were trypsinized and counted. Each *point* represents an average of two independent experiments performed in duplicate.

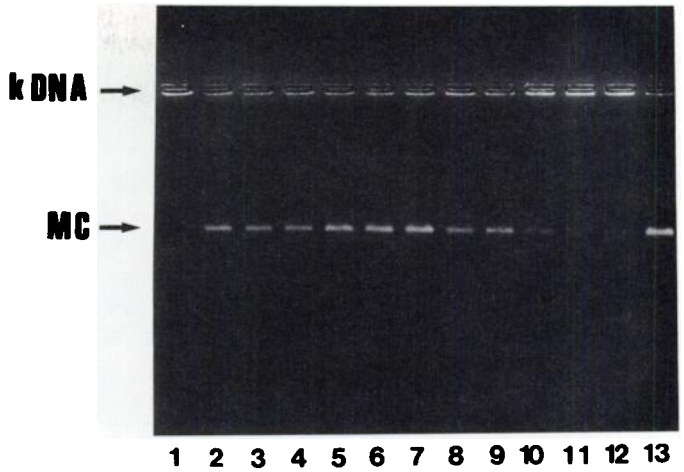

Fig. 3. Inhibition of purified calf thymus DNA topoisomerase II-mediated decatenation by genistein. The decatenation reaction and electrophoresis were performed as described in "Materials and Methods." *Lane 1*, substrate kDNA alone; *Lane 2*, kDNA reacted with DNA topoisomerase II. The enzymatic activity was assayed by incubation for 9 min in the presence of 0.5, 1, 2, 4, 8, 20, 40, 80, 150, and 370 μM genistein (*Lanes 3* to *12*) or 1% DMSO (*Lane 13*).

DNA topoisomerase II was inhibited gradually starting at 20 μM with complete inhibition at high (80, 150, and 370 μM) concentrations of genistein. It is interesting that genistein totally inhibited the decatenation at a significantly lower concentration (20 μM) when crude nuclear extracts were used instead of purified enzyme (results not shown). This supports the notion that a catenation factor or cofactor might be present in crude nuclear extracts (32–34) and that genistein may interact with this factor.

Genistein Stimulates the Generation of a Cleavable Complex between DNA and DNA Topoisomerase II. DNA topoisomerase II inhibitors might interfere with the breakage-rejoining step of the DNA strand-passing reaction by forming a stable drug-topoisomerase II-DNA ternary complex called cleavable complex. Treatment of this cleavable complex with SDS and proteinase K results in the formation of both single and double DNA strand breaks (35–41). To test whether genistein stabilizes the cleavable complex, supercoiled pBR322 DNA was used as a substrate with purified calf thymus DNA topoisomerase II or crude nuclear extracts from DC-3F cells. Because linear DNA (Form III) cannot be easily distinguished between DNA topoisomers, the reaction mixtures were analyzed on ethidium bromide-containing agarose gel. Due to ethidium bromide intercalation, the covalent circular DNA relaxed by DNA topoisomerase II became much more positively supercoiled (more compact) than the original negatively supercoiled DNA, and the relaxed DNA migrates faster than the original DNA. In addition, in such gel DNA relaxed by DNA topoisomerase II and negatively supercoiled DNA can be distinguished. Fig. 4*A*, *Lanes 4* to *11*, shows that genistein (0.5 to 100 μM) stimulates the generation of linear full length DNA from supercoiled pBR322 in a concentration-dependent manner. In addition, the difference of DNA migration in *Lanes 1*, *3*, and *13* (samples without topoisomerase II) *versus Lanes 2*, *4* to *12*, and *14* to *20* (samples with topoisomerase II) gives evidence that DNA relaxation has occurred in the presence of ATP. The formation of DNA double strand breaks was further stimulated 3- to 4-fold in the presence of 1 mM ATP (Fig. 5).

Analysis of the Genistein-induced Cleavage Sites. The cleavable complex formation depends on the nature of the drug, the drug concentration, and the DNA sequences involved in the complex (21, 42–44). The location of the genistein-induced

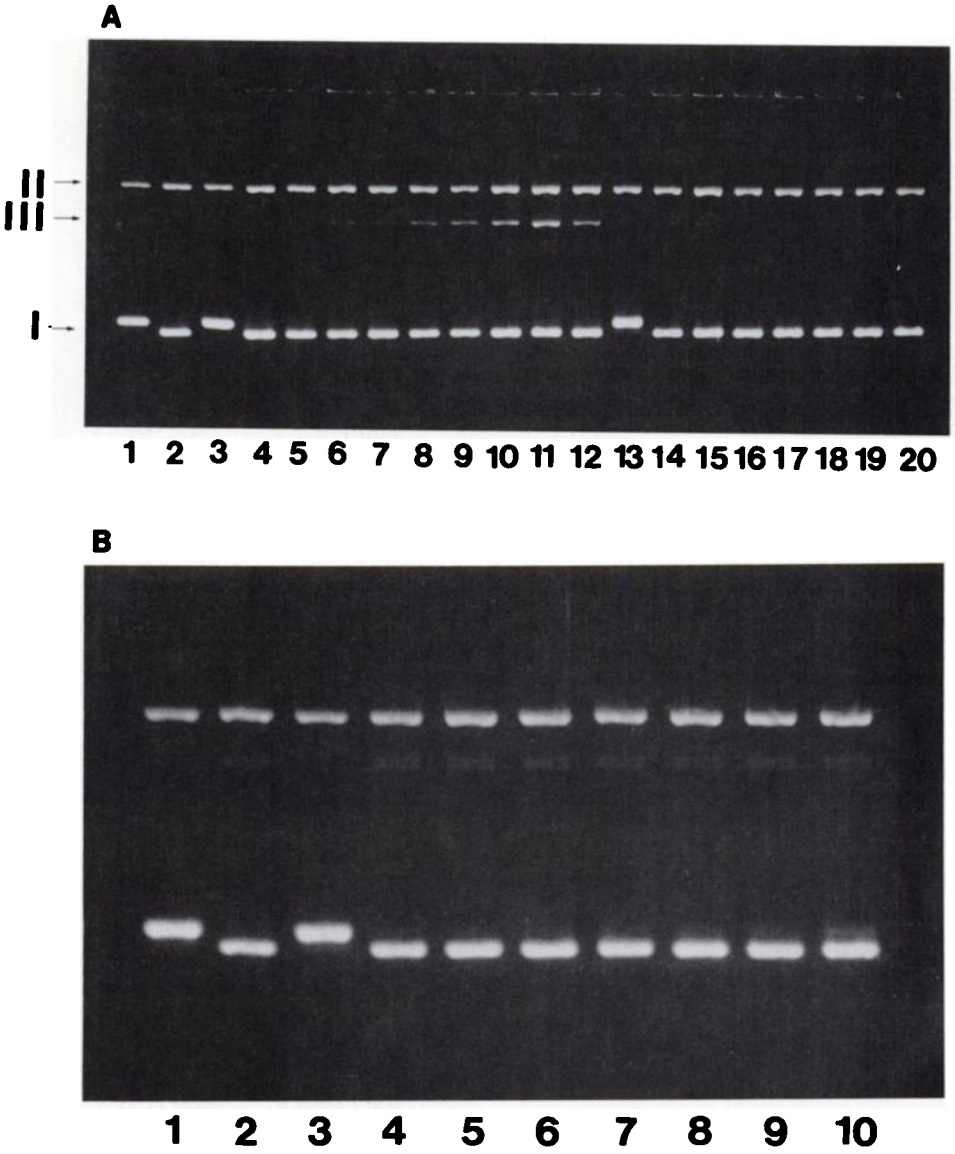

Fig. 4. *A*, comparative effects of an isoflavonoid (genistein) and a flavonoid (apigenin) on the DNA cleavage activity of calf thymus DNA topoisomerase II in the presence of 1 mM ATP. pBR322 DNA was incubated alone (*Lane 1*) or in the presence of either 50 μM genistein (*Lane 3*) or 50 μM apigenin (*Lane 13*). DNA topoisomerase II was present in all other samples. Samples 4 to 11 contained 0.5, 1, 2, 5, 10, 20, 50, and 100 μM genistein. Samples 14 to 20 contained 0.5, 1, 2, 5, 10, 20, and 50 μM apigenin. Sample 12 contained 50 μM VP-16. *I*, *II*, and *III* indicate the position of supercoiled, nicked, and linear pBR322 DNA, respectively. *B*, biochanin A does not affect the DNA cleavage activity of DNA topoisomerase II. Native pBR322 DNA (*Lane 1*) was reacted with 50 μM biochanin A (*Lane 3*) or with purified calf thymus DNA topoisomerase II in the absence (*Lane 2*) or presence of biochanin A (0.5, 1, 2, 5, 10, 20, and 50 μM in *Lanes 4* to *10*, respectively). Reaction mixtures contained ATP (1 mM).

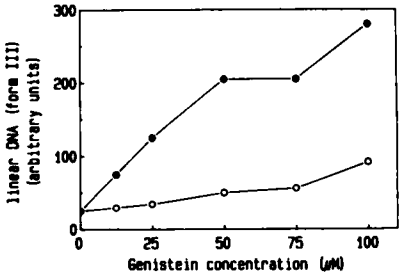

Fig. 5. Effect of ATP on genistein-induced DNA topoisomerase II-mediated DNA double strand cleavage activity in pBR322 DNA *in vitro*. Cleavable complex assays (see "Materials and Methods") were performed in either the presence of 1 mM ATP or the absence of ATP with 12.5, 25, 50, 75, and 100 μM genistein. Negative films of the gels were scanned, and the peak areas of linearized DNA (Form III) were calculated.

double strand breaks was studied using linear pBR322 which was ^{32}P labeled at the *Eco*RI end. Fig. 6 shows that little DNA topoisomerase II-mediated DNA cleavage occurred in the absence of drug. Genistein at 50 or 100 μM induced a characteristic cleavage pattern which was clearly distinct from the pattern obtained with other topoisomerase inhibitors. The cleavage sites were less numerous, and at least one was different from the VP-16, m-AMSA, and NMHE patterns (see *top arrow* on Fig. 6). A major cleavage site (*bottom arrow*) appeared at position 4250 ± 50 of the pBR322 map.

Comparative Effects of Genistein and Structurally Related Compounds on the Cleavable Complex Formation. Some isoflavonoids and flavonoids structurally related to genistein were evaluated for their ability to induce the cleavable complex. The structures of these compounds are depicted in Fig. 1. Fig. 4 shows the effects of genistein, apigenin, and biochanin A on the cleavable complex formation. Whereas 5 to 100 μM genistein stimulated the cleavable complex formation, no appreciable change, as compared to untreated controls, was observed with apigenin and biochanin A. Prunetin, flavone acetic acid (45, 46), and quercetin showed no effect in the concentration range studied, while genistin seemed to induce the formation of a limited amount of the cleavable complex (Form III). However, the amount of Form III did not vary as a function of the genistin concentration (data not shown).

The inhibitory effects of these drugs on purified DNA topoisomerase II (as measured by cleavable complex formation) were compared with their effects on the tyrosine kinase activity (as measured by autophosphorylation of the EGF receptor) in Table 1. These data show that, among the flavonoids and isoflavonoids tested, only genistein had a clear ability to form the cleavable complex. It is interesting to point out that genistein is also the most potent inhibitor of tyrosine kinase and

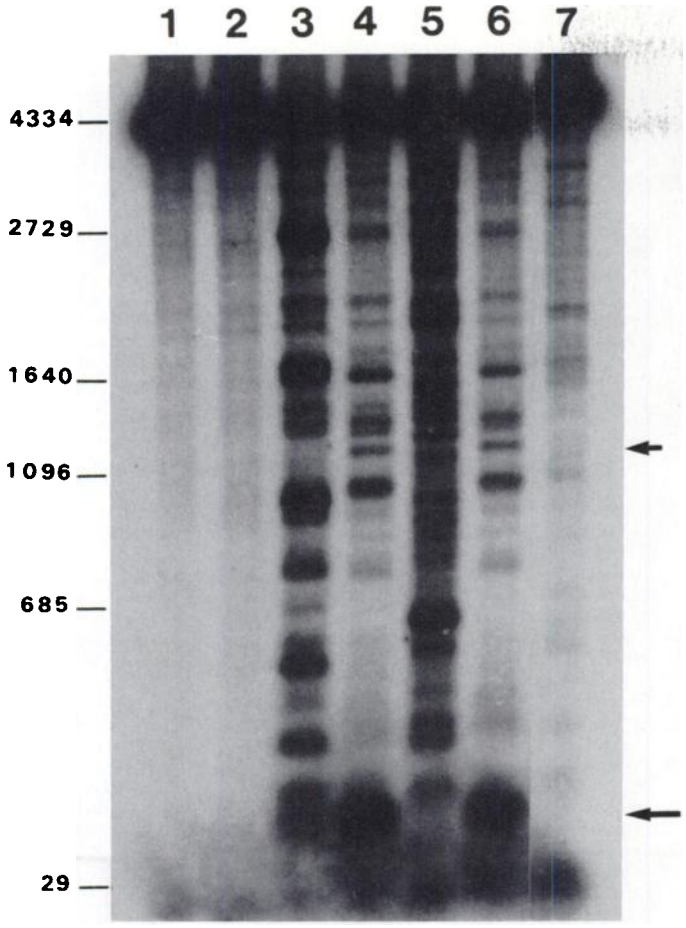

Fig. 6. DNA double strand break patterns of purified calf thymus DNA topoisomerase II in the absence or presence of various drugs in ^{32}P end-labeled pBR322 DNA. The reactions and electrophoresis were performed as described in "Materials and Methods." Linearized pBR322 DNA (*Lane 1*) was reacted with purified calf thymus DNA topoisomerase II in the absence (*Lane 2*) or presence of either m-AMSA (20 μM in *Lane 3*), genistein (50 and 100 μM in *Lanes 4* and *6*, respectively), VP-16 (100 μM in *Lane 5*), or 2-methyl-9-hydroxyellipticinium (2 μM in *Lane 7*). The size (in base pairs) of the fragments of pBR322 DNA is indicated at the *left* of the picture. *Arrows* indicate new cleavage sites.

Table 1 *In vitro effects of the studied compounds on the DNA topoisomerase II-mediated DNA cleavage activity and on the autophosphorylation of the EGF receptor*

	Formation of cleavable complex[a] (μM)	Inhibition (50% inhibitory dose, μM) of autophosphorylation of EGF receptor (taken from Ref. 7)
Genistein	5	2.6
Genistin	50	227
Prunetin	>100	14.1
Biochanin A	>100	91.8
Apigenin	>100	92.9
Quercetin	>100	16.6

[a] Formation of at least 2 times more linear pBR322 DNA (Form III) than with calf thymus DNA topoisomerase II alone.

that both reactions are inhibited at comparable concentrations of genistein.

Genistein Binding to DNA. The ability of genistein to compete with ethidium bromide for calf thymus DNA was determined in a 0.1 M Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl. The results show that the binding curves of ethidium bromide in the absence and presence of genistein were similar, indicating that ethidium bromide was not displaced by genistein. The binding of genistein to DNA was also tested by viscometric titration. One characteristic of DNA intercalative binding drugs is their ability to increase the DNA length. The helical extension of short calf thymus DNA fragments in the presence of increasing concentrations of genistein was measured by viscometric titration as described (18). The slope of the curve of log η/η_0 *versus* log $(1 + 2r)$, where η and η_0 are the intrinsic viscosity of DNA in the presence and absence of dye, respectively, and r, the number of dye bound per nucleotide, was first measured for the monointercalating agent ethidium bromide as a control. A slope of 2.1 was obtained for ethidium bromide under these experimental conditions in agreement with previous results (18). In contrast, no significant variation of DNA viscosity could be measured for genistein in the range of concentration at which genistein affects DNA topoisomerase II.

Genistein Induces Protein-linked DNA Strand Breaks in DC-3F Cells. The occurrence of DNA single strand breaks in DC-3F cells treated with genistein (0.5 to 20 μM) for 24 h was studied by the alkaline elution assay. The elution kinetic values of [^{14}C]DNA from control cells were the same as from cells treated with low levels of genistein (0.5 to 5 μM). However, the elution kinetic values of DNA from cells exposed to 10 and 20 μM genistein were significantly different from that of DNA from untreated cells (results not shown). Simultaneously performed "frank break" assays indicated that these DNA single strand breaks were protein associated. Additional alkaline elution assays were therefore performed in order to quantify the frequencies of genistein-induced DPC. Table 2 shows that the frequencies of DPC are significantly elevated only in cells treated with 20 to 100 μM genistein. This is consistent with the notion that at least part of the cytotoxic effects of genistein is related to its ability to induce protein-linked DNA strand breaks in mammalian cells.

DISCUSSION

Genistein is one of the first specific protein tyrosine kinase inhibitors which has been described (7). Because of the potential usefulness of such inhibitors in cancer treatment, a detailed study of their mechanism of action at the cellular level is required.

The comparison of the cytotoxic effects toward a series of variant cell lines is one of the most powerful tools to identify potential targets of drugs. We therefore compared the cytotoxicity of genistein toward three Chinese hamster cell lines: the sensitive DC-3F parental cells and two drug-resistant variants (DC-3F/9-OH-E and DC-3F/AD X). DC-3F/AD X has the multidrug resistance phenotype (23, 31), whereas DC-3F/9-OH-E has an altered DNA topoisomerase II activity (10–14). Until now this cell line appears to be selectively more resistant to drugs acting at the level of DNA topoisomerase II which includes m-AMSA, doxorubicin, ellipticines, and VP-16. Our observation, that DC-3F/9-OH-E cells were significantly more resistant toward the cytotoxic effects of genistein than the

Table 2 *Frequencies of DNA-protein cross-links produced by various concentrations of genistein in asynchronously growing DC-3F cells*

Values are the means of at least two independent experiments, each performed in duplicate.

Genistein treatment for 24 h (μM)	DPC (rad equivalents)
0.5	0
1	0
2.5	0
5	0
10	52
20	126
50	150
100	765

parental cell line, whereas only a slight difference was observed compared to the DC-3F/AD X cell line, strongly suggests that DNA topoisomerase II is involved in the action of genistein. This is further reinforced by our results concerning the interaction of genistein with purified DNA topoisomerase II. Previous studies performed with DNA topoisomerase II inhibitors have shown that the cytotoxic effect of these drugs is associated with their ability to induce cleavable complexes between DNA topoisomerase II and DNA. Such complexes are best evidenced *in vitro* by the generation of DNA double strand breaks after SDS and proteinase K treatment. As for several DNA intercalating agents and podophyllotoxin derivatives, genistein is also able to induce DNA topoisomerase II-mediated double strand DNA breaks *in vitro* and protein-associated DNA strand breaks in whole cells which can be demonstrated by alkaline elution. It is interesting that the protein-linked DNA strand breaks are observed within the same dose range as the cytotoxicity, as measured by colony formation.

Because many antitumor DNA intercalating agents are supposed to act at the level of DNA topoisomerase II by stabilizing the cleavable complex, it was of importance to determine whether genistein was able to intercalate into DNA.

Our results with genistein-ethidium bromide competition and viscometry clearly show that genistein is unable to intercalate into DNA in the concentration range of interest. Genistein therefore belongs to the class of nonintercalative inhibitors of DNA topoisomerase II. In this class, only podophyllotoxin derivatives (VP-16 and VM-26) have been described. However, it is of importance to underline that the chemical structure of genistein is not related to that of podophyllotoxins. In addition, the action of genistein appears to be specific; closely related flavonoids (apigenin, flavone acetic acid, quercetin) and isoflavonoids (biochanin A and prunetin) are inactive.

It has recently been suggested that genistein was a protein tyrosine kinase inhibitor acting at the level of the ATP site (7). The different protein tyrosine kinases share a common sequence characteristic of the ATP site which is also found in protein kinase C and in cyclic AMP-dependent protein kinase. The consensus sequence is G X G XX G . . . . K.

We therefore looked for such a sequence in DNA topoisomerase II. A related sequence is indeed present in human DNA topoisomerase II (H topo II) and presents a striking homology with that found in protein tyrosine kinase, as shown for c-*erb*-B2 (47) and DNA topoisomerase II (48), as an example.

H topo II 465-482	K	T	L	A	V	S	G	L	G	V	V	G	R	D	K	Y	G	V
			|		|			‖										
c-*erb*-B2	R	K	V	K	V	L	G	S	G	A	F	G	T	V	Y	K	G	I

It is possible that DNA topoisomerase II could have retained an ATP site similar to that of protein kinase during evolution. In addition the action of both protein tyrosine kinase and DNA topoisomerase II involves the formation of a phosphate ester between a phosphate group of a nucleotide and the hydroxy group of tyrosine. This would account for the fact that both enzymes have some inhibitors in common. However, if genistein was competing with ATP, one would expect to see the reversal of the genistein effect by ATP. In fact, the opposite effect is observed (see Fig. 5). Such a behavior could be due to the alteration of the mode of ATP binding to its site in a noncompetitive manner by genistein. Confirmation of this hypothesis must await further results.

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