Pergamon

Biochemical Pharmacology, Vol. 48, No. 3, pp. 549–560, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0161-D

INHIBITION OF DNA TOPOISOMERASES I AND II AND INDUCTION OF APOPTOSIS BY ERBSTATIN AND TYRPHOSTIN DERIVATIVES

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(*Received* 25 *August* 1993; *accepted* 25 *March* 1994)

Abstract—Inhibitors of protein tyrosine kinases (PTK) and DNA topoisomerases are potential antitumour agents. Drugs which bind to the ATP site of PTK, such as genistein, are common inhibitors to both types of enzymes. Eleven erbstatin and tyrphostin derivatives, which inhibit epidermal growth factor receptor PTK activity by competing with both the peptide substrate and ATP were tested for their capacity to inhibit DNA topoisomerases I and II. Erbstatin, two synthetic derivatives with a modified side chain and the tyrphostin AG 786 inhibited both topoisomerases in the same range of concentrations (20–50 μM). The tyrphostin AG 213 inhibited only topoisomerase II. In this series, absence of PTK inhibitory effect was correlated with the absence of DNA topoisomerase inhibition, while the detection of PTK inhibition may or may not be associated with DNA topoisomerase inhibition. In contrast to genistein, none of these molecules induced the stabilization of the topoisomerase–DNA cleavable complex, either *in vitro* or *in vivo*. Alcaline elution analysis revealed that erbstatin did not induce the formation of protein associated DNA strand breaks. However, an extensive degradation of the cellular DNA was observed which was shown to result from an internucleosomal fragmentation. Furthermore, typical morphological modifications associated with apoptosis were observed in the erbstatin treated cells by electron microscopy. These data indicate that erbstatin induces an apoptotic cell death.

Key words: topoisomerases; protein tyrosine kinases; inhibitors; erbstatin; tyrphostins; apoptosis

Many growth factor receptors and proto-oncogene products possess an intrinsic PTK§ activity which is essential for signal transduction in response to various stimuli [1]. PTK catalyse the transfer of the gamma-phosphate of ATP (or eventually GTP) to specific tyrosine residues in certain proteins. The catalytic function of all PTK requires the binding of a nucleoside triphosphate and an appropriate tyrosyl-containing substrate.

All protein kinases contain a common catalytic domain which usually extends over 240 amino acids, including the binding sites for ATP and the protein substrate. The ATP binding site, characterized by the GXGXXG motif, is located at the amino terminus of the catalytic domain while the protein binding site, less precisely defined, is likely to be located at the carboxy terminus [2, 3]. The local sequence around the phosphorylation site plays a vital role in defining the enzyme specificity.

Because of the importance of PTK in signal transmission and the association of an altered PTK expression with several proliferative diseases, an extensive effort has been made to develop PTK inhibitors. Bioflavonoids, such as genistein and quercetin, are competitive ATP binding inhibitors [4]. However, genistein also interfers with other enzymes downstream of the growth factor receptors, such as S6 kinase [5]. Furthermore, genistein is also a DNA topoisomerase II inhibitor which stabilizes the covalent DNA–enzyme intermediate, the so-called "cleavable complex" [6, 7]. It was observed that PTK and topoisomerase II, as well as other enzymes such as protein kinase C, or cyclic AMP-dependent protein kinase, all share a common sequence at the ATP site, characterized by the GXGXXG motif [7]. In addition, both PTK and DNA topoisomerase II catalyse a phosphoesterification reaction between a phosphate group of a nucleotide and a tyrosine residue in a protein. These similarities may account for the existence of inhibitors common to both types of enzyme.

Erbstatin was isolated from culture filtrates of a *Streptomyces* and characterized by Nakamura *et al.* [8]. *In situ*, this compound inhibits the PTK activity of both the EGF receptor and the $p60^{src}$ protein [9]. However, erbstatin was later reported to also inhibit the activity of protein kinase C and cAMP dependent protein kinase A [10, 11]. Erbstatin inhibits the growth of L1210 leukemia cells injected into mice [12], and has an antitumour activity on human

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§ Abbreviations: PTK, protein tyrosine kinases; EGF, epidermal growth factor; kDNA, kinetoplast DNA; VP-16, etoposide; 9-OH-E, 9-hydroxyellipticine; CPT, camptothecin; DPC, DNA-protein cross-links; MDR, multidrug resistance.

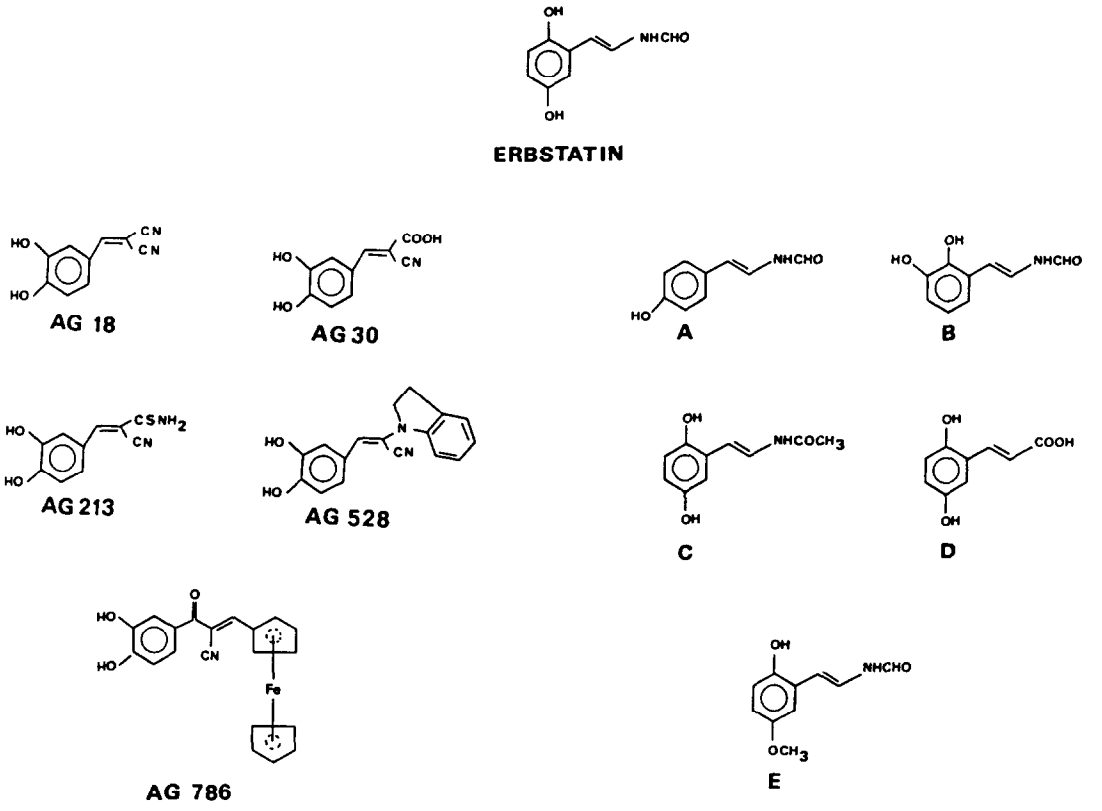

Fig. 1. Structure of erbstatin and tyrphostin derivatives used in the present study.

mammary and esophageal tumours in nude mice [13]. Initially described as binding competitively with the substrate and non-competitively with ATP [14], erbstatin was recently shown to exhibit a mode of partial competitive inhibition with respect to both the peptide substrate and ATP [11]. Among the multiple erbstatin derivatives which have been synthesized, the tyrphostins are some of the most interesting [15]. Some tyrphostins behave as peptidomimetic agents competing with the tyrosyl containing substrates at the catalytic site. Anti-EGF-receptor kinase tyrphostins block EGF-dependent autophosphorylation, EGF-dependent phosphorylation of exogenous substrates as well as EGF-dependent cell proliferation [16].

Starting from this group's observation that genistein, which binds to the ATP site, is a dual inhibitor of PTK and topoisomerase II, it was wondered whether other agents, with a different mode of kinase inhibition, would also inhibit topoisomerases I and II. In the present study, it is reported that indeed several erbstatin and tyrphostin derivatives inhibit topoisomerases I and II, but do not stabilize the cleavable complex.

MATERIALS AND METHODS

Drugs and chemicals. Erbstatin and its derivatives (Fig. 1) were synthesized as previously described [17]. Tyrphostins AG 18, 30, 213, and 528 (Fig. 1) were synthesized as described by Gazit *et al.* [18]. Synthesis of AG 786 is part of a manuscript in preparation. Etoposide (VP-16) was a kind gift from Bristol-Myers, Co. (Syracuse, NY, U.S.A.). Camptothecin was purchased from Sigma Chemical Co. Stock solutions (20 mM) of these drugs were prepared in DMSO and stored frozen at −20° for less than one week. Just before use, the solutions were diluted in distilled water, or in culture medium, to the desired concentration. The final DMSO concentration in the cell growth medium never exceeded 1%. All chemicals were of reagent or analytical grade. Foetal calf serum and all cell culture media were purchased from Gibco BRL (France).

DNA and enzymes. Supercoiled pBR322 DNA was purchased from Boehringer (Mannheim, Germany). Highly catenated kDNA was prepared from *Trypanosoma cruzi* pellets provided by Dr G. Riou (Institut Gustave Roussy, Villejuif, France). Calf thymus DNA topoisomerase I was from Gibco BRL, France and proteinase K was from New England Biolabs. Restriction endonucleases, the Klenow fragment of DNA polymerase I and dTTP were purchased from Boehringer (Mannheim, Germany).

Calf thymus DNA topoisomerase II was purified following previously published procedures with slight modifications [19, 20].

DNA topoisomerase assays. The standard reaction mixture for 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 and 30 μg/mL BSA. For type II DNA topoisomerase 1 mM ATP was added.

Relaxation assay. The reaction was initiated by the addition of DNA topoisomerases I or II to 150 ng of supercoiled pBR322 DNA and allowed to proceed at 37° for 10 min. Reactions were terminated by addition of SDS, Bromophenol blue and sucrose (1, 0.05 and 10% final concentrations, respectively). The samples were electrophoresed in 1% agarose gels at 2 V/cm for 18 hr in Tris–borate, EDTA buffer, pH 8. Ethidium bromide stained gels were photographed under UV light with Polaroid films.

Decatenation assay. Decatenation of kDNA was carried out by incubation of 0.5 μg of kDNA with purified DNA topoisomerase II (4 ng) in a final volume of 15 μL at 30° for 12 min. Reaction was terminated by the addition of SDS at a final concentration of 0.25%. The reaction products were analysed 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 hr. Ethidium bromide stained gels were photographed under UV light with Polaroid films.

Cleavable complex formation. *Eco*RI linearized pBR322 DNA was labelled at its 3'-ends by filling in restriction enzyme generated sticky ends with [α^{32}P]dATP using the large fragment of DNA polymerase I as previously described [21]. After purification, the DNA labelled on both ends was further cut with *Hind*III restriction endonuclease, thus removing a 3'-end labelled 29 base pairs fragment.

End labelled DNA (10^4–10^5cpm) and drug (1–100 μM) in a final volume of 15 μL were incubated with purified calf thymus topoisomerase II (50 ng) for 15 min at 37°. 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°, 5 μL of loading buffer containing 0.25% bromophenol and 40% sucrose were added. Whole samples were then analysed by agarose gel electrophoresis and autoradiography as previously described.

Cell lines and culture techniques. The Chinese hamster lung cell line DC-3F and the 9-OH-ellipticine resistant subline DC-3F/9-OH-E have been previously described [22], as have the media and growth conditions [22].

The mouse L1210 leukemia cells were maintained in exponential phase of growth in RPMI 1640 medium as described by Esnault *et al.* [23].

Cytotoxicity. For the determination of the cytotoxic effect of the tested compounds, $2–5 \times 10^5$ DC-3F cells were plated in 24-well dishes (Nuclon, Roskilde, Denmark) in the presence of increasing drug concentrations, the cells were then counted after 72 hr incubation. The ED_{50} value for each drug was determined.

Cell survival after a 3 hr exposure to the drug was also determined, as previously described [24].

The toxicity of erbstatin on exponentially growing L1210 cells was determined as described by Esnault *et al.* [23].

Preparation of crude nuclear extracts. Nuclear extracts were prepared from 0.5 to 1×10^8 exponentially growing cells as previously described [7]. Briefly, DC-3F cells were scraped into ice-cold nucleus buffer containing 1 mM KH_2PO_4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol and 10% (v/v) glycerol, pH 6.4, and centrifuged at 460 *g* for 10 min. All additional procedures were performed at 4°. Cells were rinsed once in nucleus buffer, spun again and resuspended in 1 mL of nucleus buffer. Nine millilitres of nucleus buffer containing 0.3% Triton X-100 was 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 *g* for 20 min. The supernatants were centrifuged again at 12,000 *g* for 15 min. The amount of protein was determined using the Biorad assay. The supernatant was stored in the presence of BSA (1 mg/mL) in a mixture of glycerol : nucleus buffer (1:1) at −20° and used within two weeks.

Measurements of DNA damage by alcaline elution. The determination of protein associated DNA strand breaks was carried out as previously described [7]. Cellular DNA in exponentially growing L1210 cells was radioactively labelled for 20 hr at 37° by adding to the growth medium either [2-^{14}C]thymidine (0.02 μCi/mL, 56 mCi/mmol; Amersham, France) or [*methyl*-^{3}H]thymidine (0.1 μCi/mL, 20 Ci/mmol; Amersham, France) diluted in 10^{-6}M unlabelled thymidine. After removal of the radioactive medium by centrifugation the cells were grown for 2 hr in label-free medium prior to all experiments. [^{14}C] thymidine labelled cells were treated with erbstatin at increasing concentrations for 24 hr or with 20 μM VP-16 for 1 hr. Drug treatments were terminated by centrifugation and resuspension of the cells in ice-cold Hanks balanced salt solution containing 0.02% EDTA.

Cells were lysed with LS-10 (2 M NaCl, 0.2% sarkosyl and 0.04 M EDTA, pH 10). The lysis fraction was washed from the filters with 5 mL of 0.02 M EDTA, pH 10, and the DNA was eluted with tetrapropylammonium hydroxide–EDTA, pH 12.1. Fractions were collected at 3 hr intervals. Fractions and filters were processed and radioactivity was counted by liquid scintillation spectrometry.

The fraction of DNA in the lysis was calculated as follows:

$$\frac{(\textit{DNA in lysis fraction}) + (\textit{DNA in EDTA fraction})}{\textit{total DNA}}.$$

DPC were then determined in DNA denaturing and non-deproteinizing conditions using a protein adsorbing filter (polyvinyl chloride; Gelman Sciences, Ann Arbor, MI, U.S.A.).

DPC frequencies were calculated according to the bound-to-one terminus model [25]:

$$P_x = [(1 - r)^{-1} - (1 - r_0)^{-1}]\ P_B,$$

where r_0 and r are the retentions on the filter of DNA from radioactively labelled control and treated L1210 cells, respectively, and P_B is the radiation dose administered (3000 rad).

DNA degradation. Exponentially growing L1210 cells seeded at an initial cell concentration of 5×10^4/mL were treated with various concentrations of erbstatin for 24 hr. The corresponding cytotoxicities were determined by counting the cell numbers in duplicated samples using an electronic cell counter (Coulter Counter ZM). After treatment, the cells were centrifuged, rinsed twice and resuspended in the same volume of drug-free medium. Untreated cells were submitted to the same procedure and diluted at 5×10^4/mL. After incubation in drug-free medium for 2, 24 and 48 hr, 10^6 cells were centrifuged at 400 *g* for 10 min, resuspended in 1 mL of PBS and centrifuged at 13,000 *g* for 30 sec. The pellet was stored at −20°. The cells were then lysed and the cellular DNA was analysed as previously described [26, 27].

Electron microscopy. Cells fixed in 2% glutaraldehyde in PBS were pelleted at low speed. The pellet was washed in Sorensen buffer (67 mM phosphate buffer, pH 7.4), post-fixed in 2% osmium tetroxyde, dehydrated with ethanol and included in "Epon" resin by usual techniques. Sections of cells were stained with uranyl acetate and lead citrate, and observed with a Zeiss EM 902 microscope. Enhanced contrast was obtained by selecting elastic electrons using the slit of the spectrophotometer.

RESULTS

Effect of erbstatin on DNA topoisomerase activities in 0.35 M NaCl nuclear extracts

Supercoiled DNA relaxation activity in 0.35 M NaCl nuclear extracts is shared by both DNA topoisomerases I and II. Therefore, analysis of erbstatin effects on these extracts allows comparison of its activity on both enzymes.

Figure 2 shows that supercoiled (form I) pBR322 DNA is completely relaxed by nuclear extract proteins (4 μg) after 10 min incubation in the presence of ATP (1 mM) (lane 5 compared to lane 2). The following lanes show the effect of erbstatin added at concentrations ranging from 1 to 50 μM: the DNA relaxation is completely inhibited at concentrations starting at 10 μM to be complete at 20 μM. Under the same experimental conditions the specific DNA topoisomerase II inhibitor VP-16 had no apparent effect on the relaxation reaction at concentrations of 50 and 100 μM (lanes 3 and 4). Therefore, this experiment shows that erbstatin has the capacity to inhibit the catalytic activity of both DNA topoisomerases I and II.

Inhibition of purified DNA topoisomerases by erbstatin and tyrphostin derivatives

Figure 3A shows that the relaxation of pBR322 DNA by the purified calf thymus DNA topoisomerase I was gradually inhibited by erbstatin at concentrations starting at 25 μM and was complete at 100 μM. In the same concentration range the ability of five erbstatin derivatives to interfere with this reaction were also examined. Compounds C and D completely inhibited the topoisomerase I activity at 50 μM, whereas compounds A, B and E at concentrations up to 100 μM had no effect.

Five tyrphostin derivatives were also examined. AG 18, AG 30, AG 213 and AG 528 are hydroxylated benzylidene malonitrile compounds. In these molecules, the nitrile group is in position *cis* to the aromatic ring, this configuration being necessary to achieve the maximal PTK inhibitory potency [15]. AG 786 has a ferrocene ring. Figure 3B shows that AG 30 had no effect on topoisomerase I activity at concentrations up to 100 μM. Similarly, AG 18, AG 213 and AG 528 had no effect on this enzyme (Table 1). In contrast, in the presence of AG 786 at 10 μM the reaction was partially inhibited, the inhibition being complete at 20 μM (Fig. 3B). Thus, AG 786 completely inhibits topoisomerase I activity at a concentration about five times lower than erbstatin. In addition, in the presence of AG 786 alone the relative amount of nicked DNA was increased (compare lanes 4 and 1, from the left in Fig. 3B), thus indicating that AG 786 induces some DNA single strand breaks. The mechanism of this strand breakage reaction is not yet precisely understood. However, this derivative essentially differs from all the other tested compounds by the presence of an iron atom which might be involved in this process.

The effect of these drugs on the topoisomerase II activity was examined by measuring the release of minicircles and small catenanes when the kDNA from *Trypanozoma cruzi* was incubated with purified calf thymus topoisomerase II in the presence of increasing drug concentrations. Figure 4 shows that the decatenation of kDNA by this enzyme is completely inhibited by erbstatin at concentrations higher than 20 μM. Table 1 shows that again erbstatin derivatives C and D inhibited topoisomerase II at 20 μM, while compounds A, B and E had no effect at concentrations up to 100 μM.

In the tyrphostin series, compounds AG 213 and AG 786 inhibited the topoisomerase II activity at a concentration of about 50 μM (Table 1).

Stabilization of the DNA–DNA topoisomerase II cleavable complex

DNA topoisomerase II inhibitors act either by trapping cleavable complexes formed between the enzyme and the DNA or by inhibiting the catalytic activity whithout stabilizing the cleavable complex. Stabilization of the cleavable complex results in single or double stranded DNA cleavage which can easily be detected by measuring the conversion of supercoiled pBR322 DNA to circular or linear molecules after treatment with SDS and proteinase K [28]. Using this technique, none of the five compounds which inhibited the topoisomerase catalytic activities were found to stabilize the cleavable complex. Erbstatin and two tyrphostin derivatives, AG 213 and AG 786, were then tested again using a more sensitive technique based on the detection of drug induced DNA double strand breaks in linearized 3′-end ^{32}P-labelled pBR322 DNA [21]. Figure 5 shows that a pattern of cleavage sites can be observed on the labelled DNA incubated with DNA topoisomerase II alone (compare lanes 1 and 2) and this pattern was not significantly altered when AG 213 was added to the reaction mixture at concentrations ranging from 10 to 200 μM. Similarly, erbstatin at 50 and 200 μM had no effect. In the presence of AG 786 at 100 and 200 μM this pattern

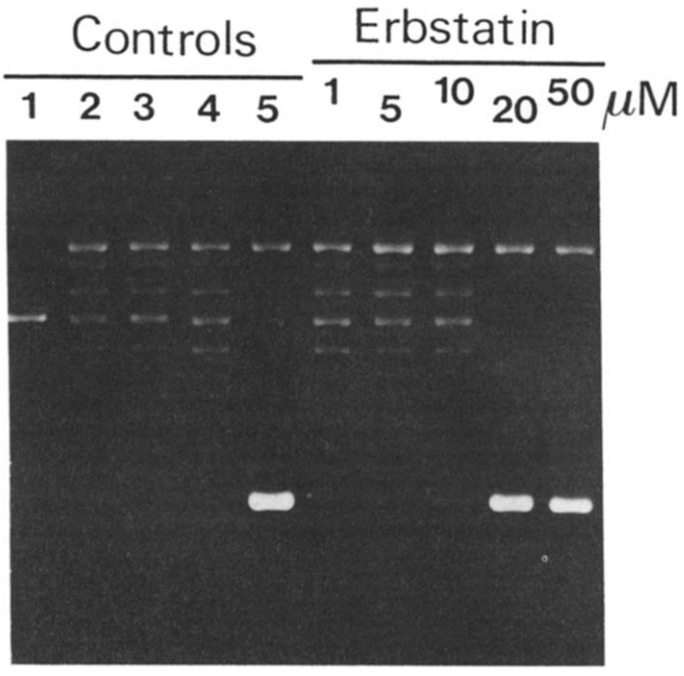

Fig. 2. Effects of erbstatin and VP-16 on the DNA relaxation activity in DC-3F 0.35 M NaCl nuclear extract. The reactions and electrophoresis were carried out as described in Materials and Methods. Supercoiled pBR322 DNA (lane 5) was incubated with 500 ng of nuclear extract proteins in the absence (lane 2) or presence of either VP-16 (50 and 100 μM in lanes 3 and 4) or 1, 5, 10, 20 and 50 μM erbstatin in the following lanes. Lane 1 shows the position of linear pBR322 DNA.

was suppressed, thus suggesting that this derivative prevents the interaction of the enzyme with the DNA.

These experiments show that, at least in the range of concentrations used, the compounds which inhibit the topoisomerase activities do not induce *in vitro* the stabilisation of the topoisomerase II mediated cleavable complex.

Determination of protein-linked DNA strand breaks in erbstatin treated cells

In vivo stabilization of the cleavable complex can be monitored by the determination of DPC. Production of DPC has been correlated to the cytotoxicity of DNA topoisomerase II inhibitors [29].

To test the ability of erbstatin to trap the topoisomerase II–DNA complex in mammalian cells, exponentially growing L1210 cells were exposed to increasing concentrations of erbstatin for 24 hr. In these conditions the IC_{50} value determined either by inhibition of cell growth or colony forming efficiency was 11 μM. The occurence of DPC was determined by the alcaline elution technique under DNA denaturing (pH 12.1) and non-deproteinizing conditions. Table 2 shows that, even at the highest tested dose (40 μM), erbstatin only induces very few protein-associated DNA strand breaks. Cells and isolated nuclei were also treated with erbstatin at 10, 20 and 30 μM for 1 hr and 30 min, respectively. In these conditions the DPC were undetectable (data not shown). VP-16, when used as a control at 20 μM for 1 hr, produced about 10 times more DPC than erbstatin at 20 μM for 24 hr (3000 rad-equivalent as compared to 245).

Table 2 also shows that the 24 hr erbstatin treatment resulted in a dose-dependent increase in the amount of DNA eluted in the lysis buffer. This observation reflects the degradation of the cellular DNA induced by the drug which remained moderate up to 10 μM and rapidly increased beyond that concentration. The accumulation of DNA in the lysis fraction was most likely the consequence of drug-induced DNA double strand breaks since the pH of the lysis buffer (pH 10) was too low to denature the DNA.

This experiment showed that erbstatin did not induce the fragmentation of the L1210 cellular DNA in alcaline elution detectable fragments which would result from the stabilization of the cleavable complex. It rather suggests that, at cytotoxic doses, the DNA is degraded in much smaller fragments through a mechanism reminiscent of the apoptosis phenomenon.

DNA degradation in erbstatin treated cells

L1210 cells were treated for 24 hr with erbstatin at 10, 15 and 20 μM. Cell survival at these concentrations was 52, 41 and 37%, respectively. DNA degradation was then examined by agarose gel electrophoresis 2, 24 and 48 hr after erbstatin treatment. A nucleosomal DNA ladder was observed 2 hr after drug treatment and the extent of DNA degradation was dose-dependent (Fig. 6). The cells

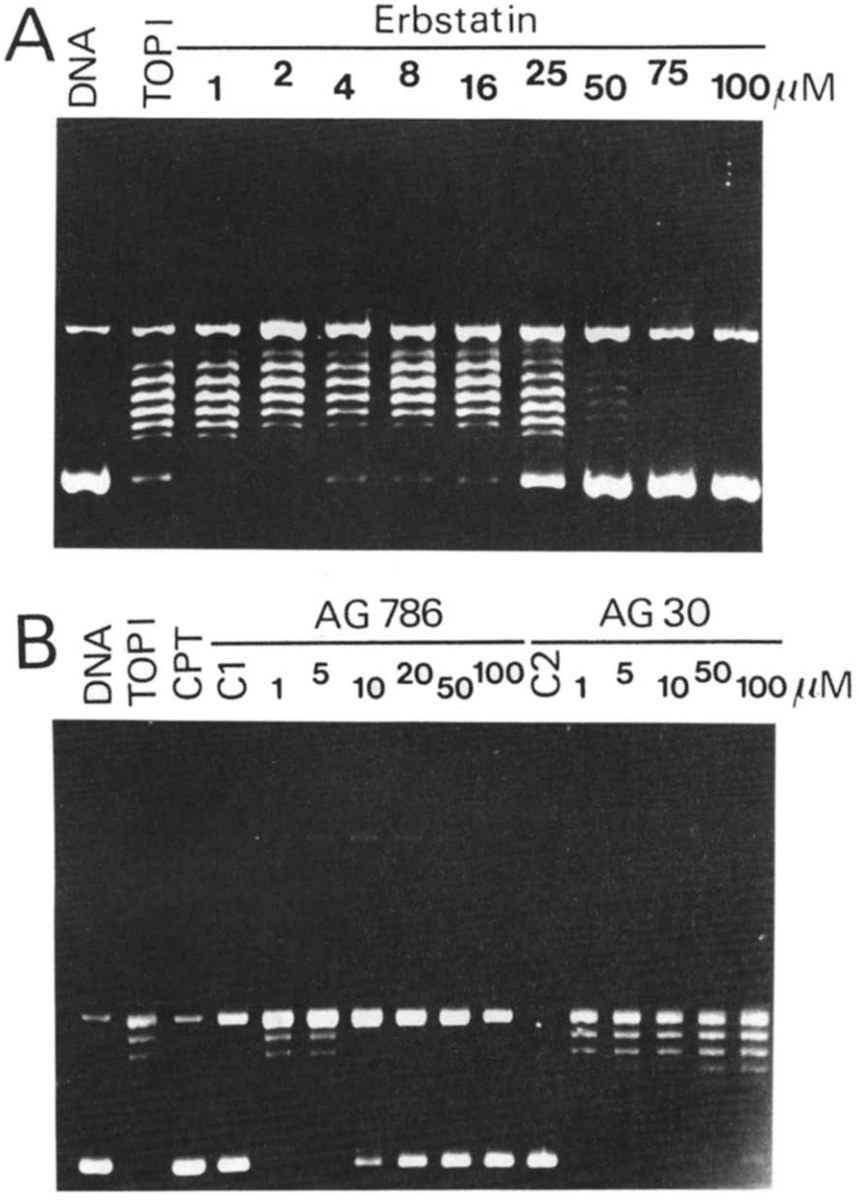

Fig. 3. Effects of erbstatin and tyrphostin derivatives on DNA topoisomerase I. The reactions and electrophoresis were carried out as described in Materials and Methods. (A) Supercoiled pBR322 DNA (lane 1) was incubated with 0.3 U DNA topoisomerase I alone (lane 2) or in the presence of 1, 2, 4, 8, 16, 25, 50, 75 and 100 μM erbstatin. (B) pBR322 DNA (lane 1) was incubated with 0.3 U DNA topisomerase I in the absence (lane 2) or presence of either 50 μM camptothecin (CPT) or 1, 5, 10, 20, 50 and 100 μM AG 786 or 1, 5, 10, 50 and 100 μM AG 30. The DNA was also incubated with either AG 786 or AG 30 alone at 100 μM (lanes C1 and C2).

treated for 24 hr with erbstatin at 20 μM were also examined by electron microscopy 24 hr after drug removal. Figure 7 shows that the DNA degradation observed in these cells is associated with typical morphological changes of apoptosis: margination and peripheral aggregation of nuclear chromatin into dense area along the nuclear membrane (B). In some cases a fragmentation of the nucleus was also observed (C).

These data, which are consistent with the previous experiment, indicate that erbstatin induces an apoptotic degradation of the cellular DNA at cytotoxic doses.

DISCUSSION

Because of their role in oncogenic transformation, a large effort has been made to identify inhibitors

Table 1. Comparative effects of erbstatin and tyrphostin derivatives on EGFR tyrosine kinase, DNA topoisomerases and DC-3F cell growth

Compounds	$IC_{50}(\mu M)$*	$IC_{100}(\mu M)$ Topo I†	Topo II‡	$IC_{50}(\mu M)$§
Erbstatin	3.4	100	20	18
A	>60	No effect	No effect	>120
B	1.7	No effect	No effect	47
C	15.5	50	20	31
D	4.1	50	20	31
E	>30	No effect	No effect	>100
AG 18	11	No effect	No effect	ND
AG 30	18	No effect	No effect	ND
AG 213	0.85	No effect	50	22
AG 528	ND	No effect	No effect	ND
AG 786	ND	20	50	ND

* EGF-receptor autophosphorylation.
† Relaxation of pBR322 DNA.
‡ Decatenation of kDNA.
§ DC-3F cell growth inhibition.
ND, not determined.

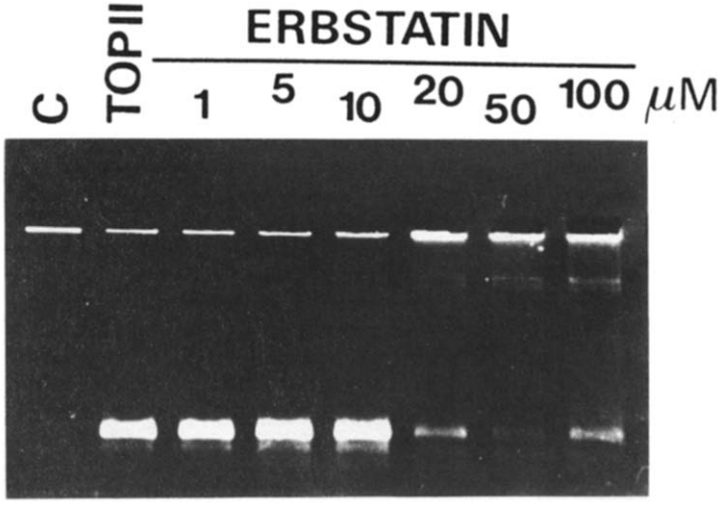

Fig. 4. Effect of erbstatin on the decatenation of kDNA by DNA topoisomerase II. The reaction and electrophoresis are described in Materials and Methods. kDNA (lane 1) was incubated with 4 ng DNA topoisomerase II either in the absence (lane 2) or presence of 1, 5, 10, 20, 50 and 100 μM erbstatin.

of PTK. Many of them were found to inhibit PTK by competing with ATP for binding to the enzyme. However, most likely because the ATP binding site is highly conserved in a variety of proteins, some of these molecules were also reported to inhibit other enzymes like serine/threonine kinases and DNA topoisomerase II. The cross-resistance of 9-OH-ellipticine resistant cells to genistein indicated that inhibition of DNA topoisomerase II plays a part in the mechanism of action of genistein [7]. It is reported here that other PTK inhibitors, which for most of them compete on PTK with both the peptide substrate and ATP, also inhibit DNA topoisomerase activities. However, in contrast to genistein, they do not stabilize the DNA–enzyme covalent "cleavable" complex. Furthermore, it is shown that erbstatin induces an internucleosomal fragmentation of the cellular DNA.

Four of the 11 tested molecules, including erbstatin, compounds C and D, and the tyrphostin AG 786, inhibit the catalytic activities of both DNA topoisomerases I and II in the same range of concentrations (20–50 μM). Changes in the position of the hydroxyl groups on the erbstatin aromatic ring or substitution of one of them by a methoxy group abolish the inhibitory effect on both enzymes. Therefore, the presence and the position of these hydroxyl groups seems to be important in determining the capacity of these molecules to inhibit the topoisomerase activities. In the tyrphostin series the

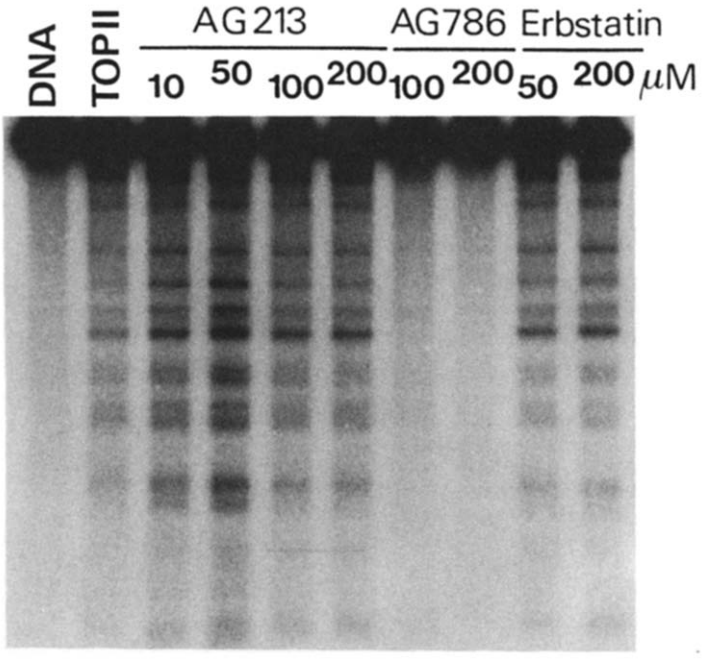

Fig. 5. Patterns of double strand breaks in pBR322 DNA incubated with purified DNA topoisomerase II in the presence of AG 213, AG 786 and erbstatin. ^{32}P end labelled linearized DNA (lane 1) was incubated with 50 ng DNA topoisomerase II either in the absence (lane 2) or in presence of 10, 50, 100 and 200 μM AG 213, or 100 and 200 μM AG 786 or 50 and 200 μM erbstatin. The reactions and electrophoresis are described in Materials and Methods.

Table 2. DNA damages following 24 hr exposure of L1210 cells to erbstatin

Treatment	Percent of DNA in the lysate	DPC frequency (rad-equivalents)
None	5.3	35
Erbstatin (μM)		
2	7.5	60
5	9.9	48
10	12.7	210
15	32.0	182
20	41.4	245
40	75.0	366
VP-16, 20 μM (1 hr)	7.9	3210

benzenemalonitrile derivatives AG 18, 30, 213 and 528 had no effect on topoisomerase I, and only AG 213 inhibited topoisomerase II. AG 213 is also a more potent PTK inhibitor than the three other tested derivatives [16]. The ferrocene derivative AG 786 inhibited both enzymes in the same concentration range as the erbstatin derivatives.

In both groups there was no quantitive relationship between the effect on PTK and on topoisomerases. However, the absence of PTK inhibitory effect was always associated with an absence of effect on the topoisomerases, whereas a kinase inhibitory effect may or may not be associated with topoisomerase inhibition. This suggests that the structural requirements are similar but not identical for both classes of inhibitors and eventually some small structural changes may be enough to achieve the specificity for one or the other enzyme.

Comparison of the compounds used in this work with genistein suggest a possible relationship between the mode of PTK inhibition and the effects of these drugs on topoisomerases. Genistein was the first PTK inhibitor shown to also inhibit topoisomerase II [6, 7]. Because genistein is considered as a competitive inhibitor with respect to ATP on the EGF receptor tyrosine kinase activity [4], it was assumed that it would also bind to the ATP site on topoisomerase II [7]. This hypothesis also explained the absence of genistein effect on topoisomerase I, which is not an ATP requiring enzyme. All the

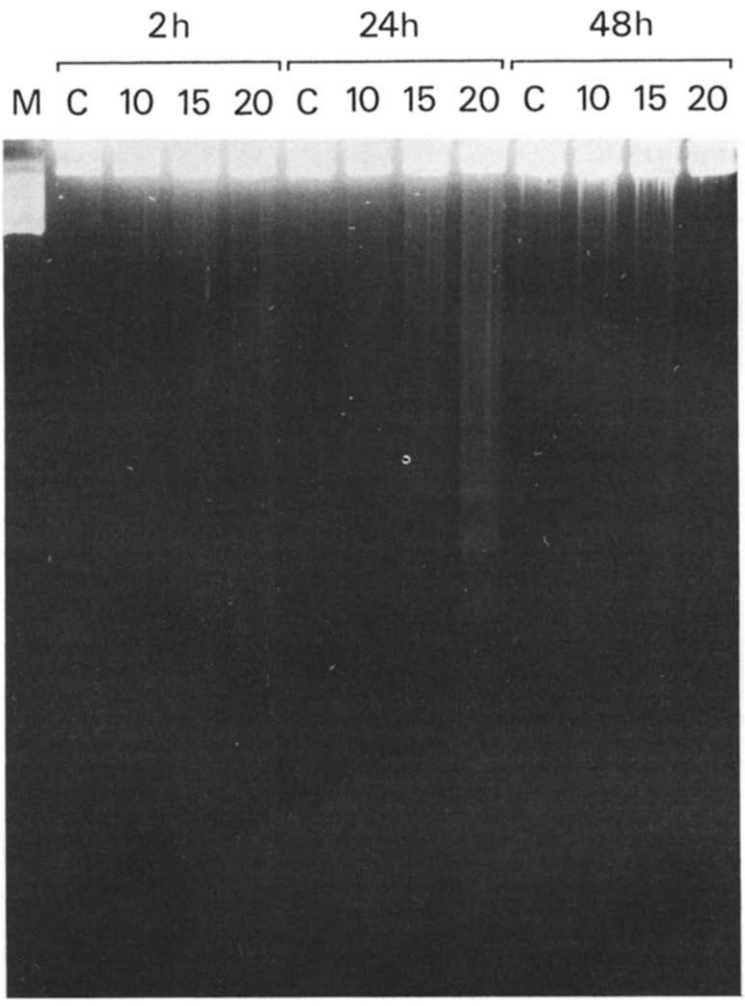

Fig. 6. Oligonucleosome-size DNA fragmentation in erbstatin treated L1210 cells. L1210 cells were exposed to erbstatin at 10, 15 and 20 μM for 24 hr as described in Materials and Methods. Two, 24 and 48 hr after treatment the cells were lysed and the DNA was analysed by agarose (2%) gel electrophoresis. M, molecular weight markers; C, untreated cells; the numbers above each lane indicate the drug concentrations.

compounds tested in this work were shown to compete with both the substrate and ATP on the EGF receptor PTK activity. Four of these molecules, erbstatin, compounds C and D, and AG 786, inhibited both topoisomerases I and II, while AG 213 was only active on topoisomerase II. However, in cell-free systems, none of these compounds were found to induce any significant stabilization of the cleavable complex between DNA and topoisomerase II at concentrations ranging from 1 to 200 μM. In the same conditions, stabilization of this complex by genistein was detected at ~20 μM [7]. The behaviour of AG 786 was markedly different: this drug inhibited the formation of topoisomerase II-mediated DNA strand breaks (Fig. 5), thus suggesting that it prevents the DNA–enzyme interaction. A similar behaviour has been observed previously with other molecules interacting either with the DNA, like ethidium bromide and ditercalinium [30, 31], or with the enzyme, like suramin [32].

Proteins carrying a PTK activity are involved in multiple metabolic pathways. Therefore, PTK inhibitors are expected to display a variety of pharmacological effects depending on their relative capacity to inhibit different enzymes [15]. The relative contribution of these different inhibitory effects to the antiproliferative activity of these derivatives is presently difficult to evaluate. The toxicity of these molecules is relatively low (>10 μM). Among the tested compounds, the most toxic are those which are the most efficient PTK inhibitors (erbstatin, compounds B and D, and AG 213). However, the toxicity may also be modulated by the effect on the topoisomerases: for example, compound B, a strong PTK inhibitor which has no effect on the topoisomerases, has a lower toxicity than expected from its effect on PTK; in contrast, compound C inhibits both topoisomerases and displays the same cytotoxicity as compound D which inhibits PTK at a concentration three-fold lower.

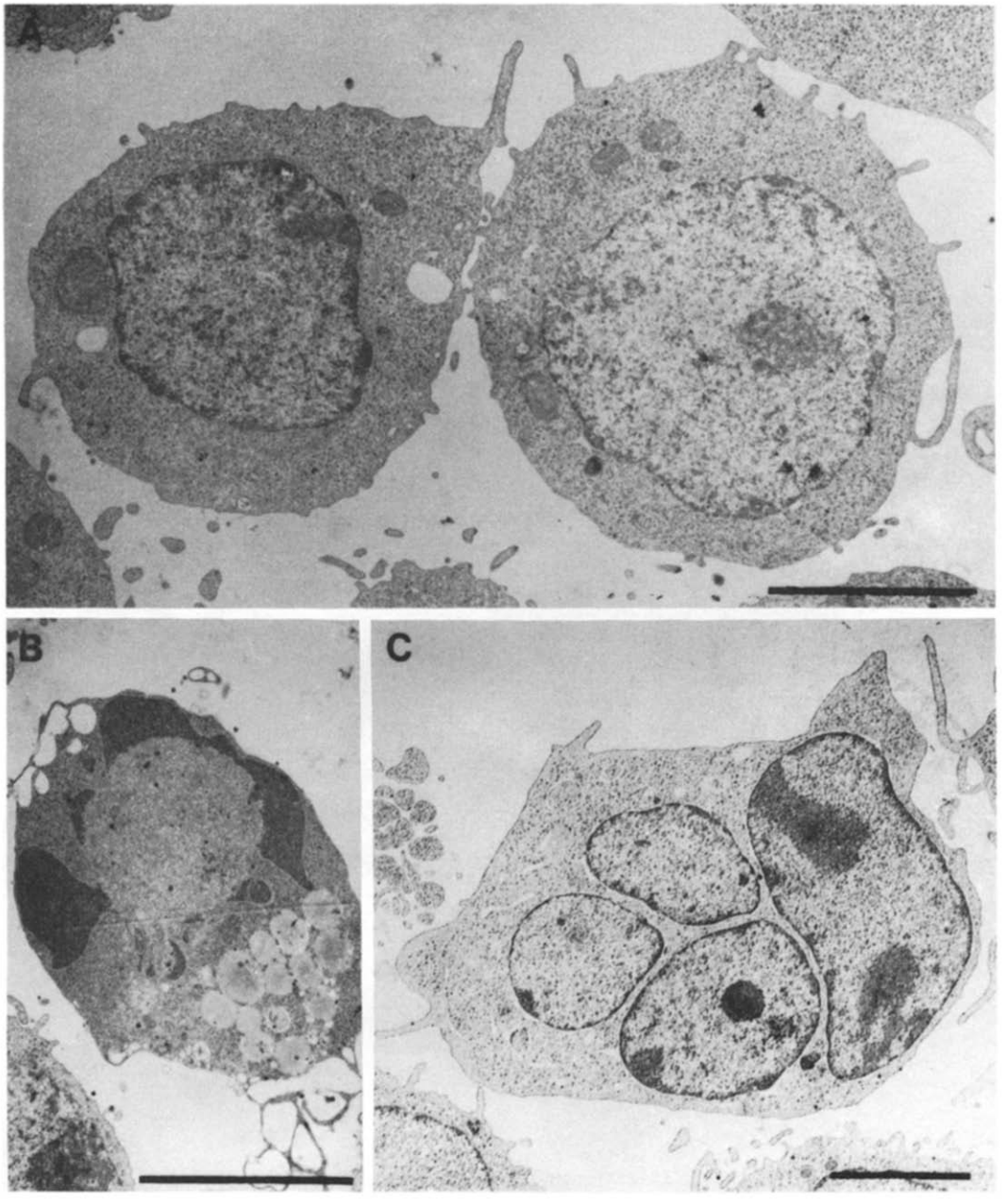

Fig. 7. Electron micrographs of erbstatin treated L1210 cells. Exponentially growing L1210 cells were treated with erbstatin at 20 μM for 24 hr and examined by electron microscopy 24 hr after drug removal. (A) Untreated cells. (B) Characteristic apoptotic cell with condensed chromatin along the nuclear membrane, dense cytoplasm and blebbed plasma membrane. (C) Cell with nuclear fragmentation and patches of dense chromatin. Scale bar, 0.5 μm.

Cells resistant to topoisomerase II inhibitors (DC-3F/9-OH-E) showed only a minor cross-resistance to these drugs (1.5- to 2-fold), thus confirming the marginal role of topoisomerase II inhibition in their mechanism of toxicity. DC-3F/AD X, a cell line resistant to actinomycin D through the expression of the MDR phenotype, did not exhibit any cross-resistance to any of these compounds.

As expected from the *in vitro* studies, analysis of *in vivo* erbstatin effects did not reveal any significant stabilization of the topoisomerase I or II mediated cleavable complexes. However, the presence of DNA fragments in the lysis fraction of the alcaline elution experiment suggested that this drug induced the degradation of the cellular DNA. Indeed, a dose-dependent internucleosomal fragmentation of

the DNA was observed at 2, 24 and 48 hr after treatment of the mouse L1210 cells with erbstatin. This DNA degradation was associated with nuclear morphological alterations typical of apoptosis. These observations indicate that erbstatin induces the apoptotic cell death. Previous reports demonstrating that the dephosphorylation of some specific proteins, through the activation of a phosphatase or the inhibition of a protein kinase, is of central importance in the apoptosis of lymphoid tumour lines [33], is consistent with these data.

Acknowledgements—This work was supported by grants from the Ligue Nationale Française contre le Cancer and the Association pour la Recherche contre le Cancer (Villejuif, France). We thank Monique Charra for expert technical assistance, Dominique Coulaud for expertise in electron microscopy and Jeanine Seité for her assistance in preparing the manuscript.

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