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F 11782, a Novel Epipodophylloid Non-intercalating Dual Catalytic Inhibitor of Topoisomerases I and II with an Original Mechanism of Action

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ABSTRACT. F 11782, a novel epipodophylloid, proved a potent inhibitor of the catalytic activities of both topoisomerases I and II. Unlike classical inhibitors such as camptothecin or etoposide, F 11782 did not stabilise cleavable complexes induced by either topoisomerases I or II nor did it preferentially inhibit the religation step of the catalytic cycle of either enzyme. F 11782 neither intercalated DNA nor bound in its minor groove, and showed only weak inhibition of the ATPase activity associated with topoisomerase II. F 11782 appeared to act by inhibiting the binding of topoisomerases I and II to DNA in a manner dependent both on drug and enzyme concentrations, via a mechanism not previously described or shared by other known topoisomerase 'poisons' or inhibitors. In contrast, F 11782 had only a weak effect or none at all on various other DNA-interacting enzymes. In conclusion, F 11782, as a non-intercalating, specific catalytic inhibitor of both topoisomerases I and II with an original mechanism of action, may be considered to represent the first of a new class of topoisomerase-interacting agents. BIOCHEM PHARMACOL **59**;7:807–819, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. topoisomerase I; topoisomerase II; dual catalytic inhibitor; F 11782; antitumour agent; non-intercalating agent

Topoisomerase I is an enzyme that catalyses an ATP-independent relaxation of DNA supercoils by transiently breaking and resealing (religating) single-stranded DNA, allowing the passage of the other strand through the nick and changing the linking number by one unit [1, 2]. Topoisomerase I is considered to play a major role in DNA replication and in transcription [see Ref. 3 for review]. The antitumour drug camptothecin specifically targets topoisomerase I [4], stabilising cleavable complexes through inhibition of the religation step [5]. Much research effort has been devoted to discovering new drugs targeting this important enzyme, although many of the newly identified inhibitors are DNA binders and their specificity for topoisomerase I has often not been documented, as reviewed recently [6]. Although a number of compounds have been reported to possess dual inhibitory activities of both topoisomerases I and II [7], no major new chemical family with clinical potential specifically acting on topoisomerase I has yet been identified.

Topoisomerase II is an important nuclear enzyme controlling DNA topology through catalysis of a transient breakage of double-stranded DNA in an ATP-dependent fashion, allowing for the passage of double-standed DNA followed by a resealing of the DNA [3]. Relaxation of DNA supercoils by topoisomerase II is considered crucial to its role in DNA replication and in transcription and, furthermore, topoisomerase II plays a critical role in chromosome condensation and separation during mitosis and in the attachment of DNA loops to the nuclear matrix and chromosomal scaffold [see Refs. 1 and 3 for reviews]. Two isoforms of topoisomerase II, namely α and β, have been characterised [8, 9] and the corresponding human genes cloned [10, 11] and expressed [12, 13] in yeast. Both human topoisomerases IIα and IIβ have been shown to complement defective topoisomerase II mutants in yeast [14, 15]. However, expression of the α isoform is strictly dependent on cellular proliferation status, whilst the β isoform is expressed throughout the cell cycle [16]. During mitosis, topoisomerase IIα remains bound to the mitotic chromatin, whilst the IIβ isoform was found to diffuse into the cytosol [17]. Moreover, in human cells [18], topoisomerase IIβ could not complement a defect in the IIα isoform. Both isoforms have been shown to be overexpressed in human tumours [19], with the proportion of cells in individual tumours showing an elevation in topoisomerase IIβ generally being higher than that showing an elevation in topoisomerase IIα. These data, together with numerous *in vitro* studies using tumour cell lines [20–22] and yeast strains [12, 15, 23] expressing either topoisomerases IIα or

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IIβ, which have linked sensitivity to various topoisomerase II inhibitors with the presence or the absence of either isoform, suggest that the β as well as the α isoform could be a target for topoisomerase-interacting drugs.

Topoisomerase II is the target of several clinically important anticancer drugs. One class, referred to in the literature as "poisons," stabilises cleavable complexes, including the non-intercalating agents etoposide and its derivatives [24, 25], as well as compounds such as genistein [26], azatoxin [24], and the DNA-intercalating anthracyclines [24]. Another class, referred to as catalytic inhibitors, which characteristically do not stabilise but can suppress cleavable complexes, comprises aclarubicin, the bis(dioxopiperazines), fostriecin, suramin, and merbarone [27]. These catalytic inhibitors express a variety of inhibitory mechanisms. Aclarubicin intercalates DNA and is thought to prevent its binding to the topoisomerase enzyme [28], while ICRF-193 has been shown to trap yeast topoisomerase II in a closed clamp conformation [29], thereby acting at the postreligation step of the catalytic cycle. More recently, merbarone has been shown to inhibit the cleavage step of the catalytic cycle of topoisomerase II [30]. Finally, an interesting and diverse family of molecules has been identified as dual inhibitors of both topoisomerases I and II, notably including saintopin [31], fagaronin [32], intoplicine [33], and TAS-103 [34], all of which share DNA-intercalating properties.

F 11782 is an original, lipophilic, fluorinated epipodophylloid, derived from the basic etoposide structure by esterification of both alcohol functions of the glucose moiety; its chemical synthesis is detailed elsewhere (PCT patent WO 96/12727). The goal of the present study was to characterise the pharmacological properties of this novel compound vis-à-vis topoisomerases I and II and to delineate the mechanistic basis for its inhibitory activities.

MATERIALS AND METHODS

Materials

Genistein, doxorubicin, distamycin A, coumermycin A1, and Cibacron Blue were purchased from Sigma. Suramin was obtained from RBI, camptothecin from Janssen, topotecan, GL-331 (4′-demethyl-4-*p*.nitro-anilino-4-deoxypodophyllotoxin), etoposide, and F 11782 (pentafluorophenoxy acetic acid 6β-[9α-(3,5-dimethoxy-4-phosphonooxyphenyl)-8-oxo-5α, 5aα,6,8,8aβ,9-hexahydro-furo[3′,4′:6,7]naphto[2,3-*d*][1,3]dioxol-5β-yl-oxy]-2-methyl-8-pentafluorophenyloxy acetoxy hexahydro pyrano[3,2-*d*][1,3]dioxin-7-yl ester, *N*-methyl-D-glucamine salt) were provided by Pierre Fabre Médicament. DMSO (Sigma) was used as the solvent. The plasmid YepWob6 encoding human topoisomerase IIα under the control of the GAL1 promoter was purchased from Professor J.C. Wang (Harvard University, Cambridge, MA) and YephTop2β expressed in the yeast JEL1 encoding human topoisomerase IIβ under the control of the GAL1 promoter was purchased from Dr. C. Austin (University of Newcastle, Newcastle upon Tyne, U.K.).

DNA Interactions

Inhibition of EtBr*–DNA interactions was evaluated according to published procedures [35, 36]. Test compounds were incubated for 10 min at room temperature in the presence of either 2 μM EtBr or 20 μM calf thymus DNA (Sigma) alone or a mixture of the two, in 2 mM acetate buffer pH 5.0 containing 9.3 mM NaCl, 0.1 mM NaEDTA. Inhibition of bisbenzimide–DNA interactions was monitored as detailed earlier [37, 38]. Test compounds were incubated for 10 min at room temperature in the presence of either 1 μM bisbenzimide (Hoechst 33258, Sigma) or 10 μM calf thymus DNA alone or a mixture of the two in 10 mM Tris–HCl buffer pH 7.5 containing 150 mM NaCl. The final solvent concentration used was 10% and experiments were performed in duplicate in 96-well fluorescence microwell plates (Dynatech). Fluorescence was read in a Perkin Elmer LS-50B spectrofluorimeter. With EtBr, excitation was at 546 nm and emission at 595 nm, while an excitation at 354 nm and emission at 450 nm was used with bisbenzimide. Background fluorescence due to interactions with either DNA or each of the individual chromophores alone was subtracted from the fluorescence detected in the presence of both DNA and the respective fluorochrome. Results are expressed as a percentage of the control fluorescence in the presence of the solvent alone.

Purification of Topoisomerase I

Topoisomerase I was purified from calf thymus obtained from the local slaughterhouse in Castres essentially according to the procedure described by Riou *et al.* [39]. Two 5-mL Hi-Trap (Pharmacia) SP columns were substituted for the original P11 column, and topoisomerase I was purified to homogeneity on hydroxyapatite column.

Production and Purification of Human Recombinant Topoisomerases IIα [adapted from 11, 40] and IIβ [adapted from 13] in Yeast

The procedures for the production and purification of human topoisomerases IIα and IIβ have been described in detail in Perrin *et al.* [41]. Briefly, the yeast strain JEL 1, expressing either human topoisomerase IIα or IIβ under the control of the GAL1 promoter, was grown in the presence of 2% galactose for 24 hr. Yeast membranes were disrupted by vortexing in the presence of glass beads. Topoisomerases were purified to homogeneity by successive chromatographic steps on celite, followed by a phosphocellulose column, and in the case of topoisomerase IIα by heparin

* *Abbreviations:* DTT, dithiothreitol; EtBr, ethidium bromide; kDNA, kinetoplast DNA; MAR, matrix-associated region; PCR, polymerase chain reaction; SV40, simian virus 40; and TBE buffer, Tris–borate–EDTA buffer.

column. Fractions of interest were aliquoted and stored in 50% glycerol at −70°.

DNA Relaxation Activity of Topoisomerase I

The total reaction volume was 20 μL, containing 50 mM Tris pH 7.5, 60 mM KCl, 0.5 mM DTT, 0.5 mM NaEDTA, 200 ng of pBR322 (Boehringer Mannheim), the amount of topoisomerase I which resulted after a 30-min incubation in 100% relaxation, and either DMSO solvent alone or the drug to be tested. After 30-min incubation, the reaction mixture was analysed on a 1% agarose gel and run at 35 mA overnight in TBE buffer (89 mM Tris, 89 mM borate, 2 mM NaEDTA, pH 8.3) [32]. Gels were stained with EtBr and scanned under UV illumination using a Bio-Rad Molecular Imager, the supercoiled DNA was quantitated, and the IC_{50} for the inhibition of relaxation was determined on at least 3 separate occasions.

kDNA Decatenation Activity of Topoisomerase II

Eighteen microlitres of buffer A (50 mM Tris pH 8.0, 120 mM KCl, 0.5 mM DTT, 0.5 mM ATP, 10 mM $MgCl_2$) containing 200 ng of kDNA (TOPOgen) and one unit of the human recombinant topoisomerase II (the amount of enzyme which resulted in the complete decatenation of 200 ng of kDNA) after a 30-min incubation were added to 2 μL of either solvent (DMSO) alone or a solution of the test drug [32]. After 30 minutes of incubation, the reaction mixture was analysed on a 1% agarose gel and run at 35 mA for 2 hr in TBE buffer. Gels were analysed as in the relaxation assay described above. Assays to determine the inhibition of decatenation by quantitating the amount of decatenated DNA were carried out on at least 3 separate occasions, and results are expressed as IC_{50} values.

Preparation of the DNA Probe

The nuclear MAR of the SV40-DNA, i.e. between positions 4100 and 4380 of its genome, was used as a DNA probe for cleavage assays as described previously [42, 43]. This MAR fragment was subcloned, using standard methods [44], into a commercially available pBS-SK+ plasmid (Stratagene). It was then cut with the restriction enzymes *Eco*RI and *Xba*I. The resultant 305 base-pair fragment was isolated by agarose gel electrophoresis and dephosphorylated with calf intestinal alkaline phosphatase, 5′ end-labelled with [γ-^{32}P] ATP (Amersham) with T4 polynucleotide kinase. One of the 5′ end-labelled regions was removed by a further digestion with the *Bam*HI restriction enzyme to obtain a single end-labelled fragment of 293 nucleotides. All the enzymes were purchased from Boehringer Mannheim. Alternatively, a DNA probe was synthesised by PCR in the presence of ^{32}P-radiolabelled nucleotide and the two sequence-specific oligonucleotides PR-118 (5′-CCC CCT CGA GGT CGA CGG-3′) and PR-119 (5′-TAG TGG ATC CCC CGG GCT-3′), according to standard methods [45]. PCR amplification classically yielded PCR products with a specific activity of 10–20 nCi/ng DNA probe.

DNA Cleavage and Religation

The total reaction volume of the topoisomerase I-mediated cleavage reaction was fixed at 20 μL. Eighteen microlitres of buffer (50 mM Tris pH 7.5, 60 mM KCl, 0.5 mM DTT, 0.5 mM NaEDTA) containing 10 ng (+/− 45,000 dpm) of labelled DNA probe and 1 unit of topoisomerase (the amount necessary to completely relax 200 ng of pBR322 after a 10-min incubation) were added to 2 μL of either solvent alone or the drug to be tested in the solvent. The solvent, DMSO, was used at a final concentration of 5%. After a 10-min incubation at 37°, the reaction was stopped by the addition of 5 μL of a mixture containing 5 mg/mL proteinase K (Boehringer Mannheim) and 5% SDS, and further incubated for 30 min. In order to evaluate any interference with complex stabilisation, after 10-min incubation at 37° (first incubation) with the first drug or solvent alone, camptothecin (or solvent alone) was added at a final concentration of 10 μM for another 10 min (second incubation), and the reaction was stopped as described above. Five microlitres of denaturing loading buffer (1 M NaOH, 60 mM EDTA, 0.2% bromophenol green, 30% sucrose) was then added. After 3 min at 96°, the tubes were stored on ice or frozen [46]. One negative control (DNA probe and topoisomerase I) and one positive control (DNA probe, topoisomerase I, and camptothecin 10 μM) were included in each experiment. Denatured samples were loaded onto a 7% acrylamide gel containing 7 M urea in TBE buffer. Electrophoresis was performed using a mini-gel apparatus (Polylabo) at 35 mA for 30 min. The gel was then dried and exposed against autoradiographic film (Amersham) between two intensifying screens for 16–18 hr at −70°.

The protocol for assessing topoisomerase II-mediated cleavage and religation is essentially derived from cleavage assays described previously [42, 43]. Briefly, the total reaction volume of 20 μL contained: i) 2 μL cleavage buffer 10x (200 mM Tris–HCl pH 7.5, 550 mM KCl, 65 mM $MgCl_2$, 20 mM ATP, 75 mM mercaptoethanol, 225 μg/mL BSA); ii) 4–8 ng 5′ end-radiolabelled DNA probe; iii) a final concentration of 2.5% DMSO with or without test compound; and iv) 1 μL (2 units) of purified human topoisomerase IIα (TOPOgen) or human recombinant topoisomerase IIβ. The reaction mixture was incubated for 15 min at 37°, and the reactions were stopped by the sequential addition of 2 μL SDS 10% (w/v) (GIBCO BRL) for 5 min at 37° and 2 μL proteinase K (GIBCO BRL) at 2 mg/mL in 30 mM NaEDTA (GIBCO BRL) for 60 min at 42°. To evaluate any interference with complex stabilisation, after 15-min incubation at 37° (first incubation) with the first drug or solvent, the second drug (or solvent) was added at a final concentration of 100 μM for another 15 min (second incubation), and the reactions were stopped as

described above. Alternatively, after the initial 15-min incubation, kinetics of religation were studied by the addition of 2 μL of 5 M NaCl and postincubation at 37°, and the sequential arrest of the reaction, from 15 to 1200 sec, by the addition of SDS and proteinase K as described above. Samples were precipitated with 65 μL ethanol for 120 min at −20°, and pellets were resuspended in 6 μL loading buffer (95% formamide, 20 mM NaEDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) (Amersham). Samples were heated for 3 min at 95°, then chilled for 2 min on ice before being loaded onto a 31 × 38.5 cm 6% polyacrylamide DNA sequencing gel containing 7 M urea in TBE buffer. Electrophoresis was performed at 55 W for 100 min. Gels were then vacuum-dried at +80° and exposed against an autoradiographic film (Amersham) between two intensifying screens for 16–18 hr at −70°. Signals on dried gels were also numerised and quantitated using a Molecular Imager apparatus and its Molecular Analyst software (Bio-Rad).

DNA religation activity of topoisomerase I was evaluated as previously described [47, 48]. Eighteen microlitres of a mixture containing 16.2 μL of buffer A (50 mM Tris pH 7.5, 60 mM KCl, 0.5 mM DTT, 0.5 mM NaEDTA), 0.8 μL of pBR322 (Boehringer Mannheim), and 4 units of topoisomerase I were incubated for 15 min at 37° to enable the nicking of DNA to occur. Two microlitres of either solvent alone or a solution of the drug to be tested were next added. DMSO was used as the solvent at a final concentration of 1%. After another 5-min incubation at 37°, the tubes were transferred onto ice for 5 min for the religation step to proceed. The reaction was then stopped by the addition of 5 μL digestion solution (5 mg/mL proteinase K, 5% SDS) with heating at 37° for 20 min. Twenty microlitres of the reaction mixture was analysed on a 1.1% agarose gel, run at 50 V in TBE buffer. Gels were analysed as in the relaxation assay and the amount of nicked DNA was quantitated, with assays carried out on at least 3 separate occasions. Results are expressed as IC_{50} values.

DNA-binding Activity of Topoisomerases I and II

DNA-binding activity was evaluated using a gel shift assay technique, as described by Svejstrup *et al.* [49]. Topoisomerase I (20 ng) and 0.4 ng of a PCR-amplified [^{32}P]DNA probe (10–20 nCi/ng) or alternatively, 2 μg of human recombinant topoisomerase IIα or 1.5 μg of human recombinant topoisomerase IIβ and 4 ng of [^{32}P]DNA probe were incubated in a buffer containing 10 mM Tris–HCl pH 7.6, 7.5% glycerol, 10 mM $MgCl_2$, and 50 mM KCl. After a 30-min incubation, samples were separated on polyacrylamide 5% mini-gels (Bio-Rad) by electrophoresis for 90 min at 4°, 150 V, and 20 mA. During electrophoresis, the free DNA probe migrated through the gel, whilst the DNA/topoisomerase complex remained at the top of the gel. Each experiment was confirmed by at least a second under identical conditions. Gels were dried and scanned with a Molecular Imager. The patterns of [^{32}P]DNA/topoisomerase complex and free [^{32}P]DNA probe were visualised using Molecular Analyst software. For each experimental point, the free [^{32}P]DNA probe was quantitated as a percentage of the free [^{32}P]DNA probe incubated without topoisomerase. Then, the inhibition of DNA binding of topoisomerase by each test compound was evaluated as a percentage of the DNA binding measured in the presence of solvent only.

ATPase Activity

The ATPase activity of human topoisomerase IIα was determined essentially as described earlier [50, 51], except that here the ATPase activity was coupled with decatenation activity and not with relaxation activity. Thus, five units of topoisomerase IIα were incubated with 5 μg of kDNA (TOPOgen) and 50 nM of [γ-^{33}P]ATP (2000–4000 Ci/mmol; NEN) in 100 μL of a buffer containing 10 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, and 50 mM KCl. After a 30-min incubation, 20 μL of reaction mixture was mixed with 30 μL 20% acetic acid. Ten microlitres of this mixture were then submitted to TLC on polyethyleneimine–cellulose sheets in 1 M KH_2PO_4 for 1 hr. Membranes were dried and scanned using a Molecular Imager. For each experimental point, the amount of hydrolysed ATP, characterised by the free ^{33}P, was quantitated as a percentage of total radioactivity. Results are expressed as IC_{50} values from at least three independent experiments.

Nuclear Enzymes

These assays were essentially based on standard methods using the matrix association region (MAR) DNA probe prepared as described above: i) T4 DNA ligase (New England Biolabs), bovine pancreatic DNAse (Boehringher Mannheim), and T4 polynucleotide kinase (Boehringher Mannheim) were assayed using conditions described in Sambrook *et al.* [44]; ii) the activities of DNA polymerases were assayed through the Taq polymerase by standard PCR amplification [45], and sequenase activities were assayed using the T7 sequenase version II DNA sequencing kit commercialised by Amersham; and iii), the hybridisation of two complementary 40mer long oligonucleotides was evaluated [44].

RESULTS

Evaluations of Interactions between F 11782 and DNA

Since compounds that alter the gross structure of DNA either by intercalation or by minor groove binding can have dramatic effects on the activities of the topoisomerases [6], the potential DNA-binding properties of F 11782 (Fig. 1) have been assessed by measuring the displacement of either EtBr or bisbenzimide from DNA. F 11782 appeared totally inactive in the EtBr/DNA displacement assay at concentrations up to 100 μM (data not shown), contrasting with the effects of true intercalators such as doxorubicin or

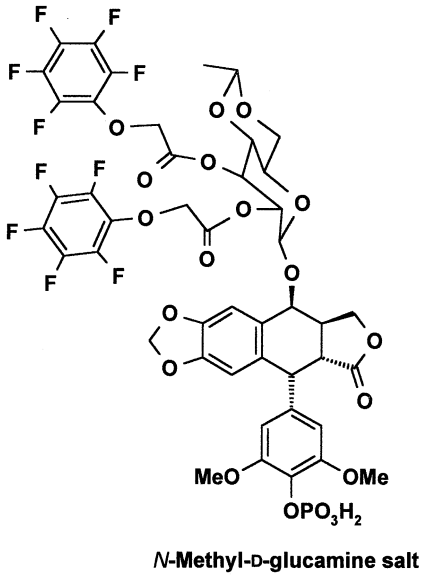

FIG. 1. Chemical structure of F 11782.

minor groove binders like distamycin A, which showed definite activities in both assays, with IC_{50} values ranging from 0.3 to 6.3 μM, associated with maximal inhibitions of 70–80% at concentrations as low as 1 μM (data not shown). However, the epipodophylloid-type compound etoposide, as well as a non-epipodophylloid type of topoisomerase II inhibitor, genistein, were found to be inactive in both displacement assays, as expected. Moreover, the known specific inhibitor of topoisomerase I, camptothecin, also proved negative in this EtBr displacement assay and was not evaluable in the bisbenzimide displacement assay due to direct interactions with the fluorochrome itself. These results indicate that F 11782 neither intercalates nor interacts with the minor groove of DNA. Subsequent studies have confirmed the absence of any detectable interaction between DNA and F 11782, as judged by melting temperature studies* and a series of biophysical techniques including UV absorption spectroscopy, thermal denaturation analyses, DNase I footprinting, as well as circular and linear dichroism† (data not shown). Overall, these generally negative findings lead to the conclusion that F 11782 presents no specific affinity for DNA.

Effects of F 11782 on Topoisomerase I-Induced DNA Relaxation and Topoisomerase II-Induced DNA Decatenation

F 11782 inhibited pBR322 relaxation induced by topoisomerase I purified from calf thymus (Fig. 2A) with an IC_{50} value of 4.2 μM, proving markedly more potent than camptothecin and topotecan, which displayed IC_{50} values of 67 and 62 μM, respectively, under the same experimental conditions (data not shown). F 11782 also inhibited the DNA relaxation activity of human topoisomerase I with a comparable IC_{50} value of 17 μM (data not shown). F 11782 inhibited DNA decatenation catalysed by either topoisomerase IIα or IIβ (Fig. 2B), with IC_{50} values of 1.8 and 1.3 μM, respectively. Thus, F 11782 appears to be a potent inhibitor of topoisomerase II, markedly more so than either etoposide (IC_{50} values of 100 μM for both enzymes), the epipodophyllotoxin GL-331 (IC_{50} values of 51 and 15 μM respectively), or genistein (respective IC_{50} values of 80 and >100 μM), which were evaluated under identical experimental conditions (data not shown). F 11782 also proved a potent inhibitor of topoisomerase II isolated from *Drosophila melanogaster*, with an IC_{50} value of 2.8 μM, confirming the overall potency of F 11782 as a catalytic inhibitor of topoisomerase II, whilst etoposide and GL-331 displayed IC_{50} values of 70 and 7.7 μM, respectively (data not shown). F 11782 inhibited the DNA relaxation mediated by topoisomerase I in crude nuclear extracts from P388 murine leukemia cells with an IC_{50} value of 5.4 μM, comparable to that of 1.7 μM, obtained with the specific and potent inhibitor, camptothecin. Similarly, in the topoisomerase II-mediated decatenation assay using crude nuclear extracts, an IC_{50} value of 3.6 μM was obtained with F 11782, which compares very favourably with the value of 87 μM obtained with the standard topoisomerase II inhibitor, etoposide (data not shown). These data therefore provide clear evidence of the inhibitory activities of F 11782 against both topoisomerases I and II in crude nuclear extracts.

Evaluation of the Ability of F 11782 To Stabilise Topoisomerase I-Induced Cleavable Complexes

F 11782 showed no stabilisation of cleavable complexes under the cleavage assay conditions employed. F 11782 appeared inactive in terms of stabilisation of topoisomerase I-induced cleavage at concentrations ≤1 mM, unlike camptothecin, where cleavage products were detected at concentrations as low as 0.32 μM (data not shown). The camptothecin synthetic derivative topotecan was also positive under these assay conditions at concentrations ≥1 μM (data not shown), whilst the epipodophylloid derivative etoposide proved negative at concentrations ≤100 μM (data not shown).

Evaluation of the Ability of F 11782 to Stabilise Topoisomerase II-Mediated Cleavable Complex Formation

F 11782, at concentrations ranging from 0.1 to 100 μM (Fig. 3, lanes 5–8), did not affect, i.e. neither increased nor inhibited, the basal rate of cleavage mediated by topoisomerase IIα revealed in the presence of the DMSO solvent alone (Fig. 3, lane 3). This contrasts with the fact that cleavage products were markedly enhanced with etoposide at 100 and 10 μM (Fig. 3, lanes 1 and 2) and even apparent at 1 μM (data not shown). This absence of stabilisation of

* Larsen AK, personal communication.
† Bailly C, personal communication.

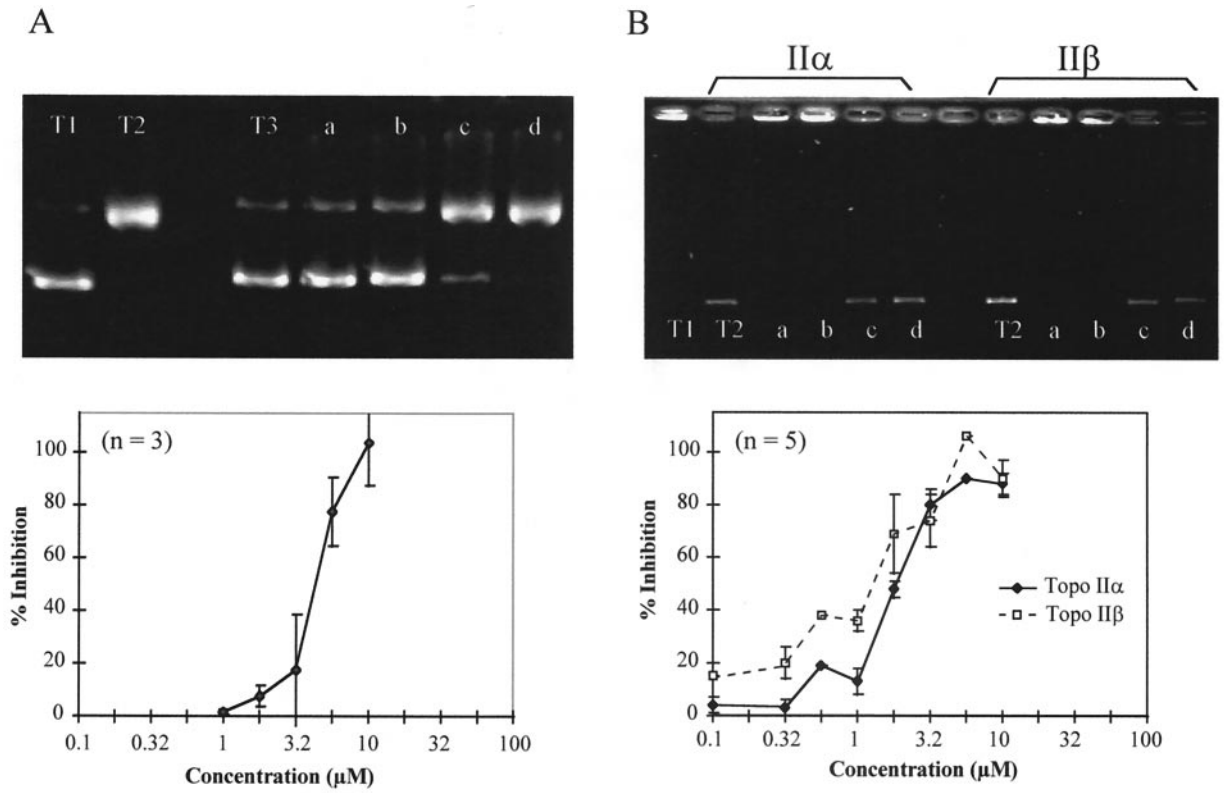

FIG. 2. Inhibitory effects of F 11782 on the relaxation of pBR322 by topoisomerase I (A) and on the decatentaion of kDNA by topoisomerase (Topo) IIα and IIβ. A representative gel is shown in each upper section. (Panel A) lane T1: pBR322, no enzyme (supercoiled form); T2: pBR322 with 1 unit of topoisomerase I (relaxed form); T3: pBR322 with 10 μM F 11782. Lanes a to d: pBR322 with 1 unit of topoisomerase I in the presence of 10, 5.6, 3.2, and 1.8 μM F 11782. (Panel B) IIα: topoisomerase IIα, IIβ: topoisomerase IIβ. Lane T1: kDNA, no enzyme (catenated form); T2: kDNA with 1 unit of topoisomerase II (decatenated form); lanes a to d: kDNA with 1 unit of topoisomerase II in the presence of 10, 3.2, 1, and 0.32 μM F 11782. Quantitation of 3 independent assays-(lower sections) are shown as the mean ± standard error.

cleavable complexes by F 11782 was also observed with human topoisomerase IIβ (data not shown).

Effects of F 11782 on the Religation Step of the Catalytic Cycle

F 11782 only interfered with the DNA religation step of the catalytic cycle of topoisomerase I with a considerably higher IC_{50} value of 33 μM (Fig. 4), relative to its potent overall inhibition of the relaxation activity of topoisomerase I (IC_{50} value of 4.2 μM). In this respect, F 11782 proved quite different from the known 'poisons' of topoisomerase I-induced religation [5], such as camptothecin and its synthetic derivative topotecan, which displayed IC_{50} values for inhibition of religation of 0.4 and 2.6 μM, respectively, under these assay conditions (data not shown). Therefore, the potent inhibition of relaxation activity by F 11782 (Fig. 2A) cannot be explained by its inhibition of the religation step. In addition, F 11782 did not inhibit the religation step of the catalytic cycle of topoisomerase II at concentrations ≤100 μM, contrasting with the two epipodophylloids, etoposide and GL-331, which proved inhibitory at concentrations as low as 10 μM, whilst genistein was inactive even at 100 μM in agreement with previous reports [52] (data not shown).

Antagonism by F 11782 of the Stabilisation of Cleavable Complexes Induced by either Camptothecin or Etoposide

F 11782 was shown to markedly inhibit stabilisation of topoisomerase I-mediated DNA cleavage induced by 10 μM camptothecin (Fig. 5) at equimolar or higher concentrations. Etoposide was inactive in this assay at concentrations ≤100 μM (Fig. 5). Similarly, preincubation of topoisomerase IIα with 100 μM F 11782 resulted in an inhibition of the subsequent stabilisation of cleavable complexes induced by 100 μM etoposide (Fig. 3). Quantitation, from three independent assays, of the uncleaved probe remaining at the top of the gel demonstrated an inhibition of etoposide-induced cleavage ranging from 50 to 70% by equimolar F 11782.

Influence of F 11782 on the Binding of Topoisomerases to DNA as Judged by a Gel Shift Assay

Since the potent inhibition of the catalytic activities of the topoisomerases by F 11782 cannot apparently be accounted for in terms of stabilisation of cleavable complexes and/or inhibition of religation, its effects on the first step of these catalytic cycles, namely the initial point at which the enzyme binds to the DNA, was characterised. The DNA-

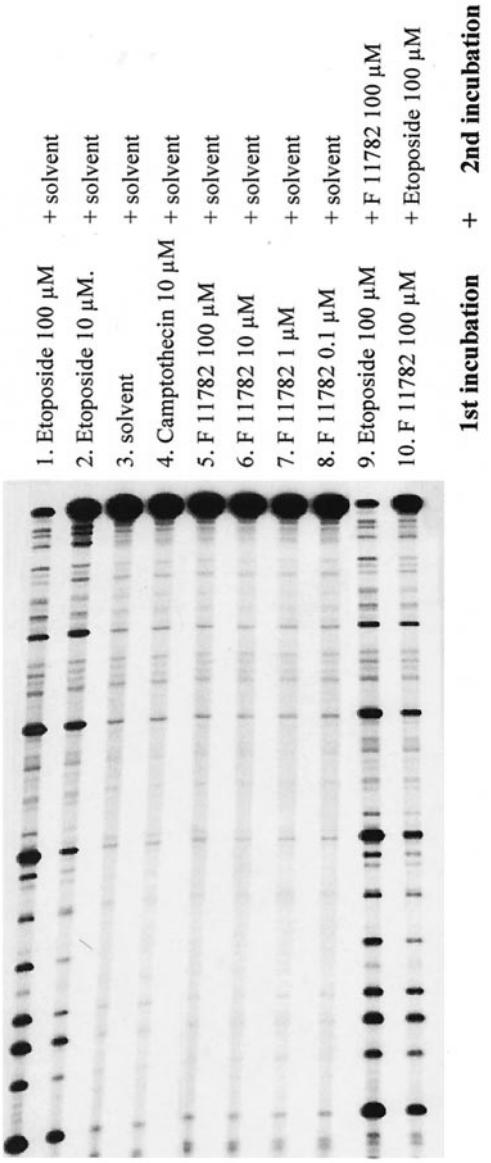

FIG. 3. F 11782 did not stabilise topoisomerase II-induced cleavable complexes and inhibited the stabilisation of topoisomerase II-induced cleavable complexes by etoposide. The cleavage pattern of a radiolabelled *Eco*RI-*Xba*I SV40 DNA fragment by topoisomerase IIα in the absence (lane 3) or presence of the indicated concentrations of either etoposide (lanes 1 and 2), camptothecin (lane 4), or F 11782 (lanes 5–8) was visualised by electrophoresis on a denaturating polyacrylamide gel and autoradiography. Whilst cleavage bands were clearly visible with etoposide at concentrations of ≤10 μM, no cleavage was detected with F 11782 at concentrations between 0.1 and 100 μM. The addition of F 11782 (lane 9) after a preincubation in the presence of etoposide did not affect the cleavage pattern of etoposide, whilst preincubating the DNA–topoisomerase II mixture with etoposide (lane 10) partially abolished the stabilisation of cleavage complexes induced by a second incubation in the presence of etoposide. The first and second incubations (15 min each) were carried out in the presence of the indicated concentrations of drug or solvent alone.

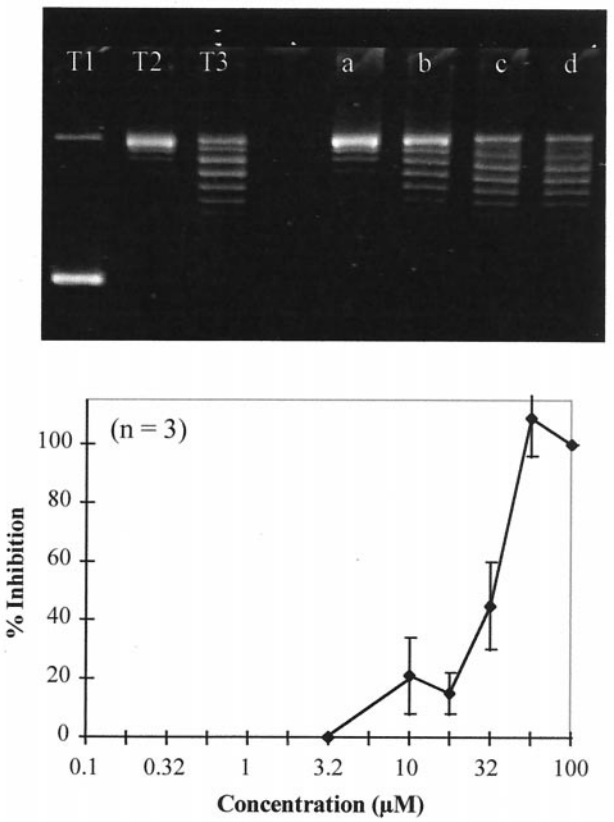

FIG. 4. Inhibitory effects of F 11782 on the religation of pBR322 by topoisomerase I. A representative gel is shown in the upper section: lane T1: supercoiled pBR322 DNA, no enzyme; T2: pBR322 plus 4 units of topoisomerase I (nicked form); T3: nicked pBR322 after 5-min religation (religated form); lanes a–d: 100, 32, 10, and 3.2 μM F 11782. Quantitation of 3 independent assays (lower section) is shown as the mean ± standard error.

binding activities of topoisomerases I, IIα, and IIβ were evaluated using a gel shift assay and the inhibitory effects of F 11782 relative to those observed with certain reference compounds studied. The data in Fig. 6 (panel A) show that F 11782 markedly inhibited the interaction of DNA and topoisomerase I under these experimental conditions at concentrations of 100 (lane 3) and 10 μM (lane 4), with a minor inhibitory activity still being observed at 1 μM (lane 5). This type of effect was not noted with any of the classical inhibitors of either topoisomerase I or topoisomerase II tested, exemplified in Fig. 6 by etoposide (lane 6) and camptothecin (lane 7). This inhibitory activity of F 11782 decreased with increasing concentrations of topoisomerase I (Fig. 7, panels A and B), with complete inhibition noted using 10 ng of topoisomerase I in the presence of 100 μM F 11782. Under these assay conditions, a DNA smear became apparent in the gel in the presence of aclarubicin, but only at concentrations of ≥100 μM. However, no shift was detectable. This type of effect was also noted with similar concentrations of other DNA intercalators, such as doxorubicin (data not shown). F 11782 also completely inhibited the interaction of DNA with either topoisomerase IIα or IIβ at concentrations of 100 and 10 μM, respectively (Fig. 6, panels B and C). Such inhibitory activities were not found with the classical

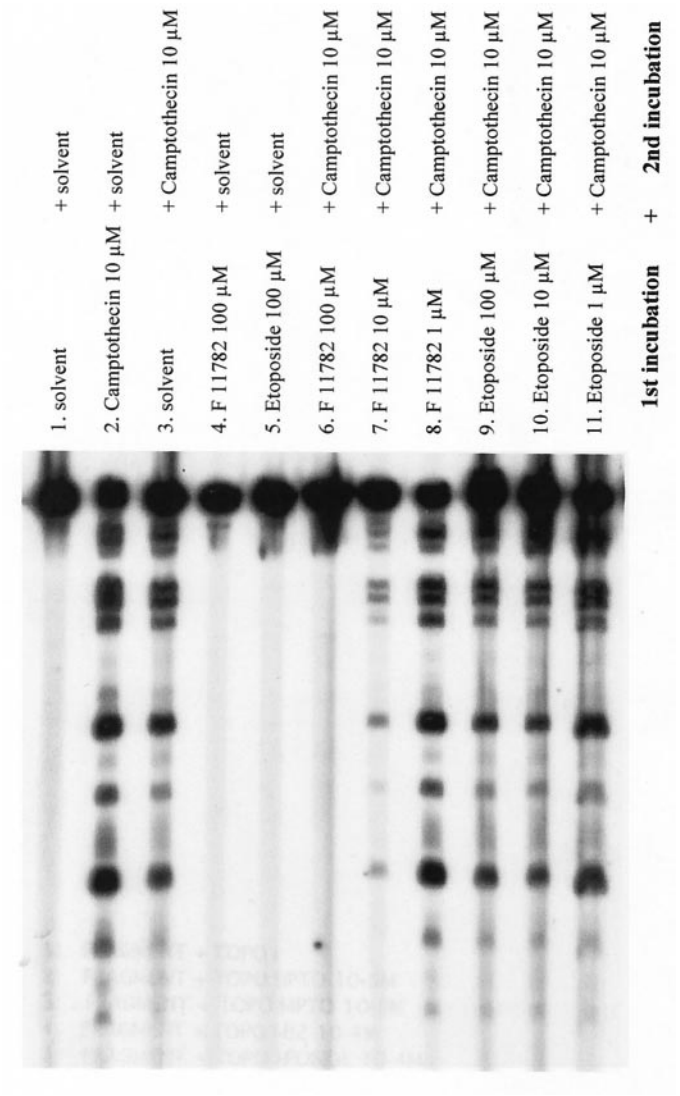

FIG. 5. F 11782 inhibited the stabilisation of topoisomerase I-induced cleavable complexes by camptothecin. The effects of F 11782 on the stabilisation by camptotecin of cleavable complexes induced by topoisomerase I was assessed by studying the cleavage pattern of a radiolabeled *Eco*RI–*Xba*I SV40 DNA fragment by topoisomerase I visualised by electrophoresis on a denaturating polyacrylamide gel and autoradiography. The first and second incubations (10 min each) were carried out in the presence of the indicated drugs or solvent alone. No cleavage was observed in the absence of drugs (lane 1) or in the presence of 100 μM F 11782 (lane 4) or etoposide (lane 5), whereas camptothecin at 10 μM (lanes 2 and 3) induced the appearance of several cleavage bands. Preincubating the mixture of DNA and topoisomerase I with the indicated concentrations of F 11782 (lanes 6–8) for 10 min before the addition of camptothecin prevented the formation of cleaved fragments in a concentration-dependent manner, but preincubation in the presence of the indicated concentrations of etoposide (lanes 9–11) had no effect on camptothecin-induced stabilisation of cleavable complexes. Similar results were obtained in three independent experiments.

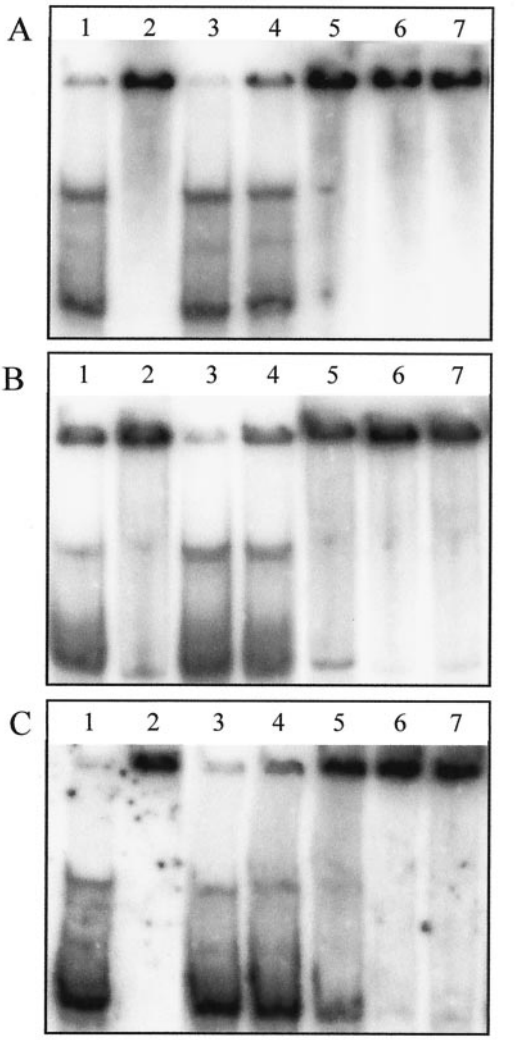

FIG. 6. The effects of F 11782 on the binding of topoisomerases to DNA, as determined by gel shift assays. Representative gels from two to three independent gel shift assays with F 11782 and topoisomerases I (A), IIα (B), and or IIβ (C) are shown. During electrophoresis, the free radiolabelled DNA probe migrated through the gel (lanes 1), whilst the DNA/topoisomerase complex remained at the top of the gel (lanes 2). Neither etoposide (lanes 6) nor camptothecin (lanes 7) at a concentration of 100 μM had any detectable effect on DNA–topoisomerase binding. (Panel A) Inhibition of the interaction between DNA and topoisomerase I was observed with 100 or 10 μM F 11782 (lanes 3 and 4), whilst only minimal inhibition was noted with 1 μM F 11782 (lane 5). (Panel B) Inhibition of the DNA/topoisomerase IIα interaction was observed with 100 or 10 μM F 11782 (lanes 3 and 4), whereas only minimal inhibitory activities were found with 1 μM F 11782 (lane 5). (Panel C) Complete inhibition of the DNA/topoisomerase IIβ interaction was observed with 10 μM F 11782 (lane 3), while partial inhibition was observed in the presence of 1 μM F 11782 (lane 4). Finally, the DNA binding of topoisomerase IIβ was only minimally inhibited by the presence of 0.1 μM F 11782 (lane 5).

inhibitors camptothecin or etoposide at concentrations ≤100 μM. Here too, the inhibitory activity of F 11782 was inversely correlated with the concentration of topoisomerase II included in the reaction mix (Fig. 7, panels C–F), with complete inhibition by 100 μM F 11782 when 40 ng of topoisomerase IIα or 20 ng of topoisomerase IIβ was

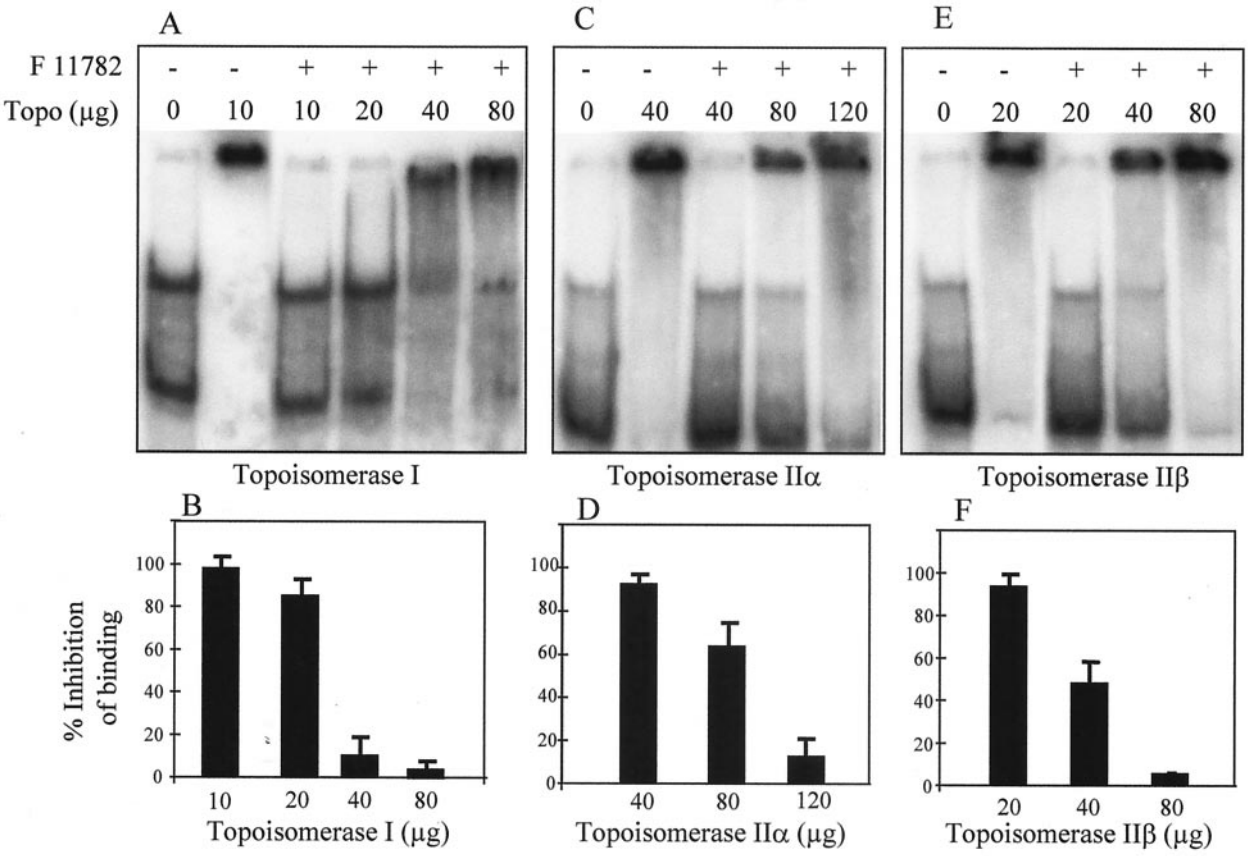

FIG. 7. Effects of increasing concentrations of topoisomerases in the gel shift assay on the inhibitory effects of F 11782 on the binding of topoisomerases to DNA. Effects of F 11782 on the DNA binding of topoisomerases I (A, B), IIα (C, D), and IIβ (E, F) were shown to depend on the quantity of topoisomerase used in the gel shift assay experiments. Panels A, C, and E: representative gels. Panels B, D, and F: quantitation of the inhibition of the shift, each determination representing the mean ± standard error of two independent experiments. The free DNA probe migrated through the gel (first lane), whilst the DNA/topoisomerase complex remained at the top of the gel (second lane) in the presence of the stated amount of topoisomerase (Topo). In the presence of 100 μM F 11782, an inhibition of the DNA/topoisomerase interaction was observed in the presence of increasing concentrations of each of the topoisomerases.

used. In controls performed with each of the concentrations of either topoisomerase I or II used, the DNA shift to the top of the gel was complete in the absence of F 11782 (data not shown).

Effects of F 11782 on the ATPase Activity of Human Topoisomerase IIα

F 11782 was found to be a weak inhibitor of the ATPase activity of human topoisomerase IIα, with an IC_{50} value of 144 μM. This activity was clearly inferior to those presented by true ATPase inhibitors, tested concurrently, such as coumermycin A1 (39 μM), suramin (0.9 μM), and Cibacron Blue (1.2 μM), yet superior to the IC_{50} value of 742 μM displayed by etoposide (data not shown).

Effects of F 11782 on Various Nuclear Enzymes and Processes

To examine the possibility that F 11782 nonspecifically inhibits a number of DNA-targeting enzymes, its effects on DNAse I, T4 DNA polynucleotide kinase, sequenase, and Taq polymerase were investigated. F 11782 showed no inhibitory activity against DNAse I, T4 polynucleotide kinase, or sequenase at concentrations up to 100 μM, with some marginal activity identified against Taq polymerase, another DNA polymerase, but only at relatively high concentrations of ≥32 μM. Neither did F 11782 at concentrations of ≤100 μM interfere with the hybridisation of two complementary oligonucleotides (data not shown). Therefore, F 11782 was shown clearly to inhibit both topoisomerases I and II at concentrations well below those that affected the other DNA-targeting enzymes examined.

DISCUSSION

F 11782 appears to represent the first of a new class of topoisomerase-interacting agents, a dual catalytic inhibitor of topoisomerases I and II without detectable DNA intercalative or minor groove-binding properties. F 11782 was unable to displace either bisbenzimide or EtBr from DNA

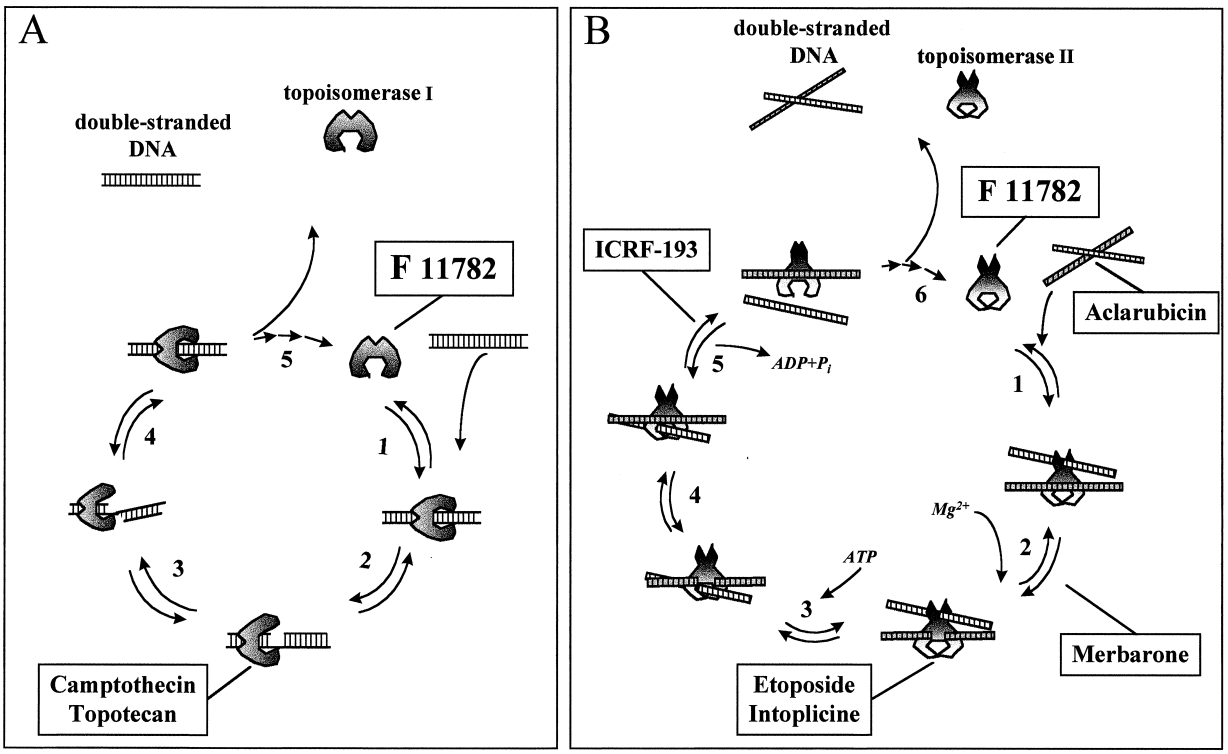

FIG. 8. Proposed site of action of F 11782 on the catalytic cycle of topoisomerases I and II [adapted from 30]. The catalytic cycle of topoisomerases I (A) and II (B) is characterised by a succession of discrete steps: binding of the enzyme to DNA (step 1), cleavage of DNA (step 2), strand passage (step 3), religation of the cleaved strand(s) (step 4), and for topoisomerase I separation of the enzyme from DNA (step 5), whilst for topoisomerase II ATPase hydrolysis permits the opening of the protein clamp (step 5) and separation of the enzyme from DNA (step 6). Several drugs which specifically affect discrete steps of the catalytic cycle have been described. Camptothecin and topotecan stabilise cleavable complexes between DNA and topoisomerases I [1] by blocking the religation step [5]. The DNA-intercalating agent aclarubicin [28] inhibits the binding of DNA to the enzyme, whilst merbarone [30] prevents the cleavage step. Furthermore, etoposide and intoplicine [7], amongst others, stabilise the cleavable complex via blockade of the religation step, yet ICRF-193 [29] has been reported to block the enzyme in a closed clamp conformation, post religation. F 11782, as detailed in this manuscript, inhibits the binding of the enzyme to DNA, most probably through a direct interaction between F 11782 and the topoisomerase enzymes.

and was shown to be a potent inhibitor of the catalytic activity of topoisomerase I, superior to both camptothecin and its derivative topotecan. However, whilst both camptothecin and topotecan were 25- to 170-fold more potent in inhibiting religation compared to the concentrations required to inhibit DNA relaxation, F 11782 inhibited relaxation more efficiently than religation by a factor of approximately 8. These data indicate that the inhibition of the catalytic cycle of topoisomerases I by F 11782 occurs at a step distinct from the religation step. Such results seem to point to a different mechanism of inhibition of topoisomerase I-induced DNA relaxation for F 11782 compared to the other known topoisomerase I inhibitors, namely camptothecin and derivatives, which affect the religation step of the catalytic cycle resulting in stabilisation of cleavable complexes [5]. In agreement with this finding, F 11782 did not induce the formation of cleavable complexes with topoisomerase I.

Similar results were obtained with topoisomerases IIα and IIβ, with F 11782 proving a potent inhibitor of the catalytic activity of these enzymes, superior to etoposide and its derivative GL-331, yet neither stabilising cleavable complexes nor affecting the basal religation rate. Furthermore, F 11782 was found to antagonise the stabilisation of cleavage both between DNA and topoisomerase I by camptothecin, and between DNA and topoisomerase II by etoposide. Since etoposide [53] and camptothecin [54] are considered to interact primarily with these target enzymes, these results point to an interaction between F 11782 and the topoisomerase enzymes at sites overlapping, at least partially, those binding sites of camptothecin and etoposide. These results are in line with recently described structural similarities between topoisomerases I and II, notably in their DNA-binding and catalytic domains [55]. Moreover, in agreement with the results obtained with purified enzymes, F 11782 proved active in inhibiting the catalytic activities of topoisomerases I and II in a mixture of nuclear enzymes. These results are indicative of the efficacy of F 11782 as an inhibitor of the topoisomerases under conditions closer to those prevailing in the cell nucleus than those associated with studies involving purified enzymes. In terms of mechanism of action, F 11782 proved a

weak inhibitor of the ATPase activity associated with topoisomerase II, with an IC_{50} value of 144 μM compared to the inhibition of the catalytic activity of topoisomerase IIα (IC_{50} value of 1.8 μM) in the presence of the same concentration of enzyme in both assays. Therefore, the effect of F 11782 on topoisomerase II appears not to be mediated by an inhibition of ATPase activity.

The effect of F 11782 on the binding of the topoisomerases to their DNA substrate was studied using a gel shift assay. The results clearly demonstrated an inhibition of the direct interaction between DNA and each of the topoisomerase enzymes. This inhibitory effect could be suppressed by increasing the amount of enzyme in the assay. These results provide evidence of what appears to be an original mechanism of action amongst topoisomerase-interacting agents, namely preventing the binding of the enzyme to DNA through a preferential interaction with the enzyme (Fig. 8), resulting in an overall inhibition of the catalytic activity.

Chemotherapeutic agents that target topoisomerases I and II can set in motion a series of biochemical changes that culminate in cell death, but only under certain conditions [56]. The realisation that stabilisation of covalent topoisomerase–DNA complexes was insufficient to insure this ultimate fate has prompted major research in this area. Whilst a range of signalling molecules have been implicated in cell death mediated by topoisomerase-interacting agents, generally their roles remain undefined [56]. The basis of the cytotoxicity of the topoisomerase catalytic inhibitors is even less well understood [27], although molecules such as aclarubicin and a number of the bis(dioxopiperazines) have shown definite cytotoxicity both *in vitro* and *in vivo*. In terms of F 11782, its *in vitro* cytotoxicity profile against a panel of murine and human tumour cell lines has been defined recently and its concentration-dependent apoptotic-inducing properties described [57]. Moreover, F 11782 has shown potent antitumour activity *in vivo* against a series of experimental murine and human tumours [58]. One might speculate, therefore, that the abilities of these catalytic inhibitors to compromise the overall catalytic activities of these topoisomerases impact on their cytotoxic potential via some as yet to be defined activation of an apoptotic program.

In conclusion, F 11782 is a novel epipodophylloid which is a dual inhibitor of the catalytic activity of both topoisomerases I and II and yet does not stabilise cleavable complexes formed by either enzyme. From a mechanistic point of view, F 11782 has been found primarily to prevent the binding of the enzyme to its DNA substrate without evidence of binding to DNA and with only marginal inhibition of the ATPase activity of topoisomerase IIα. The data presented here favour a direct interaction between F 11782 and the topoisomerase enzymes. This mechanism is not shared by other known catalytic inhibitors of topoisomerase II, with the DNA-intercalating agent aclarubicin postulated to prevent the binding of DNA to the topoisomerase enzyme [28], whilst ICRF-193 has been shown to trap yeast topoisomerase in a non-covalent DNA–topoisomerase complex [29], therefore acting at the post-religation step of the catalytic cycle. More recently, merbarone has been shown to inhibit the cleavage step of the catalytic cycle of topoisomerase II [30]. Therefore, F 11782 appears to display an original mechanism of action in terms of its inhibition of both topoisomerases I and II. Structure–activity relationship studies with derivatives of F 11782 are underway to further characterise the molecular basis of the activity of F 11782 against these two essential nuclear enzymes.

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