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ORIGINAL ARTICLE

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F 11782, a dual inhibitor of topoisomerases I and II with an original mechanism of action in vitro, and markedly superior in vivo antitumour activity, relative to three other dual topoisomerase inhibitors, intoplicin, aclarubicin and TAS-103

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Abstract *Purpose*: F 11782 (2″,3″-bis pentafluorophenoxyacetyl-4″,6″-ethylidene-β-D-glucoside of 4′-phosphate-4′-dimethylepipodophyllotoxin, di-N-methyl glucamine salt) is a newly synthesized dual catalytic inhibitor of topoisomerases I and II with major in vivo antitumour activity. In this study, we compared and contrasted F 11782 with three other known inhibitors of both these nuclear enzymes, namely aclarubicin, intoplicin and TAS-103, and established its novel mechanism of action. *Methods*: In vitro growth-inhibitory effects against a panel of murine and tumour cell lines were measured by cell counting, clonogenicity or tetrazolium metabolic dye (MTT) assays. In vivo antitumour activities were evaluated against two murine tumour models (i.v. P388 leukaemia and s.c. B16 melanoma). Finally, interactions with either DNA or DNA-topoisomerases were determined using various methodologies: DNA-intercalator displacement, pBR322 DNA relaxation, kDNA decatenation, topoisomerase II extractability measurements, stabilization of topoisomerase-induced cleavable complexes (CC) in vitro and in cells, and gel retardation assays. *Results*: F 11782 had a different profile of sensitivities and proved generally less cytotoxic than the other dual inhibitors tested in vitro, while showing significantly superior antitumour activity in vivo. F 11782, which did not stabilize CC either in vitro or in cells, was the only compound of this series capable of inhibiting the catalytic activity of both DNA-topoisomerases without interacting with DNA, and of completely impairing the binding of these nuclear proteins to DNA. Moreover, only cotreatment of cells in vitro with F 11782 enhanced the cytotoxic activity of etoposide. *Conclusion*: These results emphasize the novel mechanism of action of F 11782 vis-à-vis the other dual inhibitors of topoisomerases I and II and so augur well for its future clinical development.

Key words F 11782 · Topoisomerases · Dual inhibitors · In vitro · In vivo

Abbreviations *CC* cleavable complexes · *DMSO* dimethyl sulphoxide · *DTT* dithiothreitol · *EC* effective concentration · *FCS* fetal calf serum · *MCC* minimally cytotoxic concentration · *MTT* 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide · *PBS* phosphate buffered saline · *PCR* polymerase chain reaction · *RE* real effect · *SDS* sodium dodecyl sulphate · *TA* theoretical additivity

Introduction

There are two classes of DNA topoisomerases, enzymes that play an essential role in virtually every aspect of DNA metabolism (replication, transcription, recombination), known as type I and type II enzymes that alter the topological state (such as over- and under-winding, knotting and tangling) of nucleic acids by generating transient strand breaks in the sugar-phosphate backbone of DNA [27, 43]. Topoisomerase I acts by making single-stranded breaks in DNA, allowing controlled rotation about the nick, while topoisomerase II, by making double-stranded breaks, allows a separate double-stranded molecule to pass through the break.

DNA topoisomerases I and II are the cellular targets of several widely used anticancer drugs [8, 12, 32]. The only clinically active drugs that target topoisomerase I known so far are camptothecin and its derivatives [38], whereas numerous structurally diverse compounds inhibiting eukaryotic topoisomerase II are in widespread

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clinical use. These topoisomerase II inhibitors include either intercalating agents such as doxorubicin, or non-intercalating agents, such as etoposide and its derivatives [8, 18].

Most of these topoisomerase-interacting drugs, commonly referred to as "topoisomerase poisons", inhibit the enzyme by stabilizing a ternary DNA-drug-enzyme complex, the so-called "cleavable complex" (CC), whereby the DNA is cleaved but cannot readily be resealed. The cell-killing mechanism of these inhibitors is believed to be related to these enzyme-mediated DNA cleavages [8, 32]. More recently, another type of topoisomerase II inhibitor, termed catalytic inhibitors that do not stabilize CCs has been described, and these include compounds such as the bis(2,6-dioxopiperazines) and aclarubicin [1]. Aclarubicin has also proved to be highly effective in inhibiting the action of topoisomerase I in human tumour cells [7] and in stabilizing topoisomerase I covalent complexes in yeast [26], suggesting that it represents a novel class of combined topoisomerase I and II inhibitor. Indeed, a diverse family of molecules has been identified as dual inhibitors of both topoisomerases I and II, notably including intoplicin [33] and TAS-103 [2, 39], which poison both enzymes by stabilizing a covalent complex between DNA and topoisomerase I or II.

F 11782, is a novel pentafluorinated epipodophylloid characterized by marked antitumour activity in vivo [19]. Recently, Perrin et al. [29] have demonstrated that F 11782 is a dual inhibitor of the catalytic activity of both topoisomerases I and II in vitro, more potent than camptothecin and etoposide to which it was compared, and yet does not stabilize CCs formed by either enzyme. The goal of the present study was to compare F 11782 with three other known dual inhibitors, namely, intoplicin, TAS-103 and aclarubicin, in terms of their cytotoxic effects in vitro, antitumour efficacy in vivo, and mechanism of action vis-à-vis topoisomerases I and II. F 11782 showed markedly superior activity in vivo compared with the other dual inhibitors tested, and its pattern of activity in vitro was strikingly different and appears novel.

Materials and methods

Cells and compounds

L1210 murine leukaemia cells and human LoVo and T24 bladder carcinoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). P388 murine leukaemia cells originating from the National Cancer Institute (NCI) were a gift from Dr. S. Cros (Institut de Pharmacologie et de Biologie Structurale IPBS, Toulouse, France), while human testicular teratoma GCT27 cells were obtained from Dr. J.R.W. Masters (University College, London, UK). Finally, V79 Chinese hamster lung fibroblasts were purchased from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). RPMI-1640 Glutamax medium (Gibco BRL, Cergy Pontoise, France) supplemented with 10% heat-inactivated horse serum or with 10% FCS was used to grow L1210 and P388 cells, and GCT27 and V79 cells, respectively. LoVo and T24 cells were grown in MEM (Gibco BRL) supplemented with 5% FCS. The various media were supplemented with fungizone, penicillin-streptomycin and L-glutamine (4 m*M* final concentration). All cell lines were cultivated in an incubator under an atmosphere containing 5% CO_2 at 37 °C. Each line was amplified on receipt and liquid nitrogen stocks were made after confirming their negative mycoplasma status both by PCR and cell culture testing.

Aclarubicin (hydrochloride) and MTT were purchased from Sigma Chemical Co. (St Louis, Mo.), and DMSO from SDS (Peypin, France). Intoplicin (mesylate) was obtained from Rhone Poulenc (Vitry sur Seine, France) and camptothecin from Janssen (Noisy Le Grand, France). Etoposide, TAS-103 (6-[[2-(dimethylamino)ethyl]amino]-3-hydroxy-7H-indeno[2,1-*c*]quinolin-7-one) and F 11782 (2″,3″-bis-pentafluorophenoxyacetyl-4″,6″-ethylidene-β-D-glucoside of 4′-phosphate-4′-dimethylepipodophyllotoxin, diN-methyl glucamine salt) whose structure is shown in Fig. 1, were provided by Pierre Fabre Médicament (Castres, France).

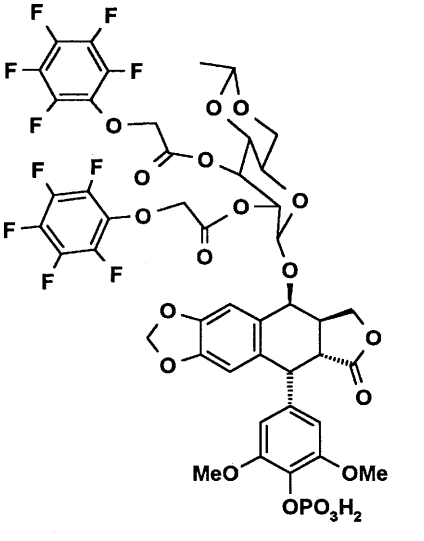
Animals

Female hybrid (CD_2F_1/CrlBR) and C57BL/6 (C57BL/6 NCrlBR) mice obtained from Charles River (St. Aubin les Elboeufs, France) were used for implanting the mouse P388 leukaemia [13] and the murine B16 melanoma [13], respectively. All mice were manipulated and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and European directive EEC/86/609, under the supervision of authorized investigators.

In vitro cell growth measurements

In the various assays, water (1% final concentration) was used as solvent, except for aclarubicin which was solubilized in DMSO (0.1% final concentration).

L1210 leukaemia cells were inoculated (1.0×10^5 cells/ml) into a series of 24-well plates (Nunc, Polylabo, Strasbourg, France) in the presence of solvent (control) or various concentrations of test compound. The growth of control and treated cells was determined 48 h later by cell counting using an automated Coulter counter ZM (Beckman Coulter, Villepinte, France). For the P388 murine leukaemia and the three human solid tumour cell lines used, the drug-induced growth-inhibiting effects were determined in 96-well



Structure of F 11782.

Fig. 1 Structure of F 11782

microtitre plates after a 48- or a 72-h incubation period, respectively, using a colorimetric metabolic dye-based MTT assay as described previously [11]. For each cell line the IC_{50} values, i.e. the concentration of drug required to reduce growth to 50% of control cell growth, were generated based on pooled data from at least three independent experiments.

V79 spheroids were prepared using the liquid overlay method, described by Sakata et al. [34], with slight modifications. Approximately 4×10^5 V79 cells in 4 ml medium were seeded into six-well microtitre plates (Nunc) previously coated (2 ml per well) with 2% agar solution (Gibco BRL). The plates were agitated at 37 °C for 2 h and then the cells were grown for 4 days. Spheroids with diameters of 100–190 μm were selected for pharmacological experiments using a two-step nylon mesh filtration method. Both spheroids and monolayer cells (duplicate cultures) were incubated for 24 h with appropriate concentrations of compound or solvent (control) and then washed twice with PBS before trypsinization, counting and plating for colony-forming assays. Colonies of more than 50 cells were stained (10% methylene blue in methanol) and counted after 6 days. The surviving fraction (SF) was determined for treated cells relative to control cells using the following formula in which PE (%) = (number of colonies/number of cells plated) × 100:

$$SF(\%) = (PE \text{ of treated cells/mean PE of control cells}) \times 100$$

The drug concentration required to produce a SF of 10% (IC_{10}) was determined based on pooled data from at least three separate experiments.

In vivo experimental chemotherapy and evaluation of antitumour activity

All the experiments were conducted in compliance with guidelines established in the Centre de Recherche Pierre Fabre (CRPF, Castres, France), based on the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines established for the welfare of animals in experimental neoplasia [44]. P388 cells (10^6/mouse) were implanted i.v. into CDF1 mice on day 0. For the B16 melanoma tumour model, 0.5 ml of a tumour brei made by disrupting and homogenizing tumour fragments in sterile 0.9% sodium chloride were inoculated s.c. into C57BL/6 mice. After randomization, test compounds were administered i.p. on day 1 as a single dose to mice bearing P388 tumours, or i.p. as multiple injections over 2 weeks (on days 3, 5, 7, 10, 12 and 14 following tumour grafting) to mice implanted with B16 tumours. Mice were checked daily for death and any adverse clinical reactions recorded. Tumour size was measured twice weekly with calipers and tumour volumes were estimated as $0.5 \times (\text{length} \times \text{width}^2)$ [3].

The optimal dose, i.e. that inducing the greatest increase in lifespan, reflected by the maximum T/C ratio [(median survival of treated mice/median survival of control mice) ×100] with minimum side effects, was determined for each test compound for the P388 tumour model. For the B16 melanoma tumour model, several evaluation criteria were used in parallel: survival curves of treated and control mice compared using the log-rank test [23]; tumour growth inhibition following various evaluation criteria; ratios of the median tumour volumes (T/C) of treated versus control mice; areas under the tumour growth curve (rAUC) calculated as a percentage of those of the control group, with the more active the treatment the lower the rAUC value. Comparisons of the rAUC population values of the treated and the control groups were performed using the nonparametric Mann-Whitney Rank Sum test [24].

Interactions with DNA

The procedures for the inhibition of interactions between ethidium bromide (EtBr) and DNA and between bisbenzimide and DNA have been described in detail by Perrin et al. [29]. Briefly, test compounds solubilized in DMSO (10% final concentration) were incubated for 10 min at room temperature in the presence of either 20 μ*M* calf thymus DNA (Sigma) with or without 2 μ*M* EtBr or 10 μ*M* calf thymus DNA with or without 1 μ*M* bisbenzimide (Hoechst 33258, Sigma). Fluorescence of EtBr (546 nm/595 nm) or bisbenzimide (354 nm/450 nm) was read in a Perkin-Elmer LS-50B spectrofluorimeter. IC_{50} values for DNA interactions were determined from at least three independent experiments.

Interactions with topoisomerase

DNA relaxation activity of topoisomerase I from calf thymus (Gibco BRL) was determined according to the method of Larsen et al. [22]. The reaction mixture (20 μl final volume) contained 50 m*M* Tris, pH 7.4, 60 m*M* KCl, 0.5 m*M* DTT, 0.5 m*M* EDTA, 0.2 μg pBR322 (Boehringer Mannheim, Meylan, France), the amount of topoisomerase I which resulted in 100% relaxation and either DMSO (1% final concentration) or the compound to be tested. After 30 min at 37 °C the reaction was terminated by the addition of 5 μl cold buffer (50 m*M* EDTA, 50% glycerol, 0.25 ng/ml bromophenol blue). Samples were then electrophoresed on a 1% agarose gel at 2 V/cm for 4 h in TBE buffer (90 m*M* Tris, 90 m*M* borate, 2 m*M* EDTA, pH 8.3) in the absence (neutral gel) or presence (chloroquine gel) of 5 m*M* chloroquine. Gels were stained with ethidium bromide washed in Tris-HCl and photographed by polaroid camera. For each compound the effective concentration (EC) for the inhibition of relaxation was determined on at least three separate occasions. EC represents the concentration at which a definite inhibitory effect could be detected.

The kDNA decatenation activity of topoisomerase II from *Drosophila melanogaster* (Amersham, Les Ulis, France) was determined according to the procedure of Davies et al. [10]. The reaction mixture (20 μl final volume) contained 0.2 μg kDNA (isolated from *Crithidia fasiculata*; TopoGen, Columbus, Ohio) in 50 m*M* Tris, pH 7.4, 120 m*M* KCl, 0.5 m*M* DTT, 0.5 m*M* ATP and 10 m*M* $MgCl_2$. After 30 min of incubation at 30 °C, the reaction was terminated and samples analysed as for the relaxation assay described above, except that only neutral gels were used, and EC values for kDNA decatenation were determined.

Salt extraction of nuclear topoisomerase II

Topoisomerase II salt-extractability measurements were evaluated according to the method of Nakagawa et al. [25]. Briefly, following a 6-h exposure to solvent (DMSO, 0.1% final concentration) or test compound, adherent GCT27 cells were trypsinized, washed twice in PBS containing 0.1 mg/ml protease inhibitor aminoethylbenzenesulphonyl fluoride and pelleted. The pellet was gently resuspended (50 μl/5×10^6 cells) in lysis buffer (20 m*M* Tris, 1 m*M* EGTA, 25 m*M* KCl, 5 m*M* $MgC1_2$, 250 m*M* sucrose and 0.5% Nonidet NP40, pH 7.2.), incubated for 10 min on ice and then centrifuged (6000 rpm in an Eppendorf microfuge). The pellet (containing nuclei) was resuspended in 50 μl extraction buffer (20 m*M* Tris, 1 m*M* EGTA, 2 m*M* EDTA, 2 m*M* DTT and 400 m*M* NaCl, pH 7.2), incubated for 30 min on ice and then centrifuged (14,000 rpm in a microfuge). The supernatant (containing extracted topoisomerase II) was removed and its protein content determined [6] before the addition of 3× concentrated SDS sample buffer and boiling. SDS-PAGE was carried out according to the method of Laemmli [20] on a 7.5% polyacrylamide gel. The resolved proteins were transferred onto nitrocellulose membranes (Amersham) for 2 h at 400 mA and stained with the various primary antibodies after blocking using either an anti-topoisomerase IIα antibody (TopoGen) diluted 1/6000 or an anti-topoisomerase IIβ antibody (BioTrend, Köln, Germany) diluted 1/8000, for 1 h. Incubation in the presence of the secondary antibody, goat anti-rabbit coupled to peroxidase (Jackson Immunoresearch Laboratories, West Grove, Pa.) diluted 1/6000, was then performed for 1 h. Topoisomerases were visualized by enhanced chemiluminescence (ECL) according to the instructions of the manufacturer (Pierce, Rockford, Ill.). Immunoblots were quantified using a Bio-Rad (Ivry sur Seine, France) molecular imager. The results are expressed as the percentages of control, i.e. the amount of salt-extractable topoisomerase II in drug-treated versus control (solvent-treated) cells.

Topoisomerases I and II cleavage reactions

The procedures used for purification of calf thymus topoisomerase I, as well as for the preparation of the 5′-end radiolabelled DNA probe for cleavage assays, i.e. the nuclear matrix-associated region (MAR) of the SV40-DNA between positions 4100 and 4380 of its genome, were as described previously [40].

The protocols for the topoisomerase I-mediated cleavage assay as well as for the topoisomerase II-mediated cleavage used have been detailed elsewhere [29]. Briefly, for topoisomerase I-mediated cleavage reaction, 10 ng labelled DNA fragment and 1 U purified calf thymus topoisomerase I (the amount necessary completely to relax 200 ng of pBR322) were added to either solvent (DMSO, 5% final concentration) or compound for a 10-min incubation period at 37 °C. Denatured samples (3 min at 96 °C) were then separated by electrophoresis on a 7% acrylamide gel containing 7 *M* urea in TBE buffer. The gel was then dried and exposed against autoradiographic film (Amersham) between two intensifying screens for 16–18 h at −70 °C. For topoisomerase II-mediated cleavage assessments the reaction mixture containing 4–8 ng radiolabelled DNA probe, a final concentration of 2.5% DMSO with or without test compound and 1 µl (2 U) purified human topoisomerase IIα (TopoGen) was incubated for 15 min at 37 °C. Samples were then denatured, loaded onto a 6% polyacrylamide DNA sequencing gel and electrophoresed. Gels were then dried, exposed against an autoradiographic film and analysed as detailed above.

Formation of covalent topoisomerase-DNA complexes in intact P388 cells

The principle of this assay is to measure the amount of complexes formed between DNA and topoisomerases covalently bound to DNA in living cells by SDS-KCl precipitation [22]. Cellular DNA and protein from 3×10^5 P388 cells were metabolically labelled by incubation with both [^{14}C]leucine (0.2 µCi/ml) and [^{3}H]thymidine (0.6 µCi/ml) in the cell culture medium. Cells were then centrifuged (400*g*, 5 min) and resuspended to 8×10^5 cells/ml in sterile PBS and the indicated amounts of each test compound or solvent (DMSO 0.1% final concentration) were added to the cell suspension for an additional 30 min at 37 °C. Each sample was then divided into 0.5-ml aliquots, and reactions were stopped by adding 0.5 ml of a solution containing 2.5% SDS, 10 m*M* EDTA (pH 8.0) and 0.8 mg/ml herring sperm DNA (Boehringer Mannheim). Cell lysates were passed ten times through a 22-gauge needle and then heated to 65 °C for 10 min before the addition of KCl (250 mM final concentration) to each tube. The tubes were vortexed (10 s), put on ice for 5 min, and then centrifuged at 10,000*g* for 10 min at 4 °C. Each pellet was washed three times with 1 ml of a solution containing 10 m*M* Tris-HCl (pH 8.0), 100 m*M* KCl, 1 m*M* EDTA (pH 8.0) and 0.1 mg/ml herring sperm DNA. The pellets were then dissolved in 0.5 ml water, heated at 65 °C for 15 min and transferred to a vial for scintillation counting. The results are expressed as the ratio of [^{3}H]DNA to [^{14}C]protein, with the disintegrations per minute of the protein precipitated as the internal control for all samples.

DNA-binding activities of topoisomerases I and II

DNA-binding activity was evaluated using a gel shift assay technique, as described by Svejstrup et al. [37] and detailed by Perrin et al. [29]. Topoisomerase I (20 ng), topoisomerase IIα (2 µg) or topoisomerase IIβ (1.5 µg), and [^{32}P]-DNA probe were incubated for 30 min with the test compound in the reaction buffer (10 m*M* Tris-HCl, pH 7.6, 7.5% glycerol, 10 m*M* $MgCl_2$, and 50 m*M* KCl). Samples were then separated on polyacrylamide 5% minigels (Bio-Rad) by electrophoresis. During electrophoresis, the free DNA probe migrated through the gel, whilst the DNA/topoisomerase complex remained at the top of the gel. Gels were then dried and scanned with a Molecular Imager (Bio-Rad) and the pattern of DNA/topoisomerase complex and free DNA probe were visualized using Molecular Analyst software. Each experiment was confirmed by at least a second using identical conditions, and representative gels are shown.

Combined cytotoxic effects with etoposide in L1210 cells

L1210 cells (1.0×10^5/ml) were inoculated (5 ml/tube) into a series of 15-ml tubes (Falcon, Subra, Toulouse, France) and incubated for 2 h with increasing concentrations of etoposide or solvent (DMSO 0.1% final concentration) alone or associated with a minimally cytotoxic concentration (MCC) of the combination compound, i.e. that resulting in less than 20% growth inhibition when tested alone. The combination compound was added just prior to etoposide. Cell suspensions were then centrifuged (200 *g*, 7 min), and the pellets were washed twice with medium prior to resuspension in 5 ml drug-free medium, and then regrown for 48 h before cell counting as described above.

If the associated drugs are considered to exert their effects on cell proliferation in an independent manner, it can be postulated that the real effect (RE) of a given MCC, i.e. control cell proliferation inhibition (percent), of any combination compound remains unchanged vis-à-vis cells treated with etoposide. Thus, the effect of a defined association results from the fraction of cells affected by the etoposide itself and the fraction of cells affected by the MCC of the combination compound. Therefore, it is possible to calculate a theoretical additivity (TA) dose-response curve for etoposide combined with a given dose of associated compound, using the formula:

$$TA = EDA - (RE \times EDA/100)$$

where EDA is the effect of etoposide alone, and RE is the MCC-induced cell proliferation inhibition (percent). Based on this approach, for each of the combinations tested here, the TA dose-response curves were calculated and compared with the dose-response curves obtained experimentally. Data derived from two or three independent experiments are presented.

Results

In vitro growth inhibitory effects of F 11782 and of other dual topoisomerase inhibitors

Comparing IC_{50} values, F 11782 appeared to be 2- to 130-fold less cytotoxic than aclarubicin, intoplicin or TAS-103 against these two murine leukaemias (Table 1). F 11782 was also consistently less potent than the other three compounds against the human tumour cell lines (Table 1), except for the GCT27 cells which were more sensitive (×2.5) to F 11782 than to intoplicin. Moreover, the T24 tumour cells were identified as clearly less responsive or more 'resistant' to F 11782, and to a lesser extent also 'resistant' to intoplicin, while apparently remaining fully sensitive to both aclarubicin and to TAS-103.

Comparing cell viability of V79 cells grown either as monolayers or as spheroids revealed that monolayer cultures showed least sensitivity to F 11782 and intoplicin. Furthermore, it is clear that cells growing as spheroids showed more resistance, as exemplified by the defined ratio, to intoplicin (about 12-fold), as well as, but to a lesser extent, to F 11782 and TAS-103 (about 4-fold and about 3-fold, respectively). These data provide evidence of the proliferation-dependency of the cytotoxicity of F 11782, intoplicin and TAS-103, which was not apparent for aclarubicin.

Table 1 In vitro growth inhibiting effects of F 11782 compared with those of other dual inhibitors of topoisomerases I and II

Test compound	Murine tumour cells (IC_{50}, μM)[a]		Human tumour cells (IC_{50}, μM)[b]			V79 fibroblasts (IC_{10}, μM)[c]		
	L1210	P388	GCT27	LoVo	T24	Spheroids	Monolayers	Ratio[d]
F 11782	0.20	1.30	0.20	6.00	120	6.20	1.70	3.6
Aclarubicin	0.03	0.01	0.01	0.01	0.01	0.48	0.27	1.8
Intoplicin	0.10	0.27	0.51	0.27	1.20	15.0	1.30	11.5
TAS-103	0.01	0.01	0.05	0.08	0.07	0.03	0.01	3.0

[a] Cells in logarithmic growth were exposed to test compound for 48 h. Then, for L1210 cells, cell numbers were counted electronically and for P388 cells, cell growth was determined using a standard MTT-based colorimetric assay
[b] Cells in logarithmic growth were exposed to test compound for 72 h. Then cell growth was determined using an MTT-based colorimetric assay
[c] Survival of V79 Chinese hamster lung fibroblasts exposed to test compounds for 24 h, and as logarithmically growing monolayers or spheroids were evaluated by a colony-forming assay
[d] IC_{10} spheroids/IC_{10} monolayers

Comparison of in vivo antitumour activities of F 11782 and other dual topoisomerase inhibitors

The i.v.-implanted P388 leukaemia tumour model was first used administering each of the test compounds via the i.p. route as a single dose on the day following tumour grafting. F 11782 was a very effective agent, exhibiting a high level (H) of antitumour activity, according to NCI criteria, over a range of doses from 80 to 320 mg/kg (Table 2). The optimal dose, i.e. that inducing the highest T/C value without any significant associated toxicity, was 320 mg/kg, resulting in a T/C value of 400. In contrast to F 11782, both aclarubicin and intoplicin proved inactive (T/C < 120%) via the i.p. route against this i.v.-implanted P388 leukaemia model (Table 2), while TAS-103 showed some activity at 80 mg/kg, but the resultant T/C value of 143% is indicative of only a low level (L) of activity. No deaths in the F 11782-treated mice occurred before the first death in the control group, whereas 40 mg/kg aclarubicin, 80 mg/kg intoplicin and 320 mg/kg TAS-103 induced 43%, 100% and 100% early deaths, respectively (data not shown).

The antitumour effects of F 11782 were next compared with those of TAS-103 (active against the i.v.-implanted P388 leukaemia) and intoplicin (inactive against the i.v.-implanted P388 leukaemia) against the s.c.-implanted B16 melanoma, using multiple i.p.-injections over 2 weeks, i.e. on days 3, 5, 7, 10, 12 and 14 after tumour implantation. F 11782 proved highly active against this relatively drug-refractory model, inducing marked inhibitory effects on tumour growth at doses of 20–160 mg/kg per injection as reflected by optimal T/C ratios of 0.2–24% ($P < 0.001$) and rAUC values of 1–43% (Table 3). This activity was associated with a highly significant increase ($P < 0.001$–0.01) in the survival of B16 tumour-bearing mice. Intoplicin (Table 3), tested concurrently, resulted in a significant increase ($P < 0.01$) of survival of B16 tumour-bearing mice at 5 mg/kg per injection but failed to induce any significant tumour growth inhibition. Therefore, the activity of intoplicin against this B16 melanoma model could be judged as being minimal. Finally, TAS-103 (Table 3), using the same schedule of administration, failed to produce any significant antitumour activity when tested at doses of 2.5, 5 and 7 mg/kg per injection, while major toxicity was recorded at 20 mg/kg (data not shown).

Overall these findings illustrate the fact that F 11782 exerted major antitumour activity against these two experimental tumour models, which in all cases was

Table 2 Comparison of the antitumour activities of F 11782, and the dual inhibitors of topoisomerase I and II aclarubicin, intoplicin and TAS-103 given i.p. as a single dose against the i.v.-implanted P388 murine leukaemia

Test compound	Dose range tested (mg/kg)	Optimal dose (mg/kg)	Maximal body weight change[a]		T/C (%)[b]	Activity rating[c]
			%	Day		
F 11782[d]	40–320[e]	320	−5.6	8	400	H
Aclarubicin	2.5–40	2.5–10	Gain		100	Inactive
Intoplicin	10–80	20	−1.5	4	114	Inactive
TAS-103	20–320	80	−9.5	8	143	L

[a] Body weight changes reported are maximal weight losses expressed as a percentage of the initial body weight. According to the NCI criteria, a dose is considered toxic if the induced body weight loss is greater than 15% of the initial weight [21]. No body weight loss was recorded in control animals
[b] T/C = (median survival of the drug-treated group/median survival of the control group) × 100
[c] According to the NCI standard criteria for the P388 tumour model, 120% ≤ T/C < 175% is the minimal level for activity (*L*) and T/C ≥ 175% corresponds to a high level of antileukaemic activity (*H*); a T/C value of < 120% indicates inactivity [42]
[d] Data reported here for F 11782 originated from Kruczynski et al. [19]
[e] Higher doses of F 11782 could not be tested because of its limited solubility and high viscosity

Table 3 Comparison of the antitumour activities of F 11782, intoplicin and TAS-103, given i.p. as multiple injections over two weeks, against the s.c.-implanted B16 murine melanoma

Compound	Dose (mg/kg/ injection)	Maximal body weight change[a]		Survival (*P*-value, log-rank test)	Tumour growth inhibition			
		%	Day		Optima T/C[b]		rAUC[c]	
					%	Day	%	*P*-value (Mann-Whitney test)
F 11782[d]	20	Gain		< 0.01	4	21	30	< 0.001
	40	Gain		< 0.001	24	17	43	< 0.001
	80	−4.4	14	< 0.001	3	21	4	< 0.001
	160	−27.0	21	< 0.01	0.2	21	1	< 0.001
Intoplicin	2.5	Gain		> 0.05	67	12	90	
	5	Gain		< 0.01	53	14	65	> 0.05
	10	Gain		> 0.05	69	14	100	> 0.05
	20	−16	10	Toxic deaths[e]				
TAS-103	2.5	2.4	7	> 0.05	56	12	74	> 0.05
	5	0	14	> 0.05	65	14	73	> 0.05
	7	−12	14	Toxic deaths[e]				

[a] Reported as maximal weight losses expressed as a percentage of the initial body weight. No body weight loss was recorded in control animals

[b] T/C = (median tumour volume of the drug-treated group/median tumour volume of the control group) ×100. According to NCI standard criteria for a solid tumour model, T/C ≤ 42% corresponds to a minimal level of activity [3]

[c] *rAUC* relative area under the tumour growth curve

[d] Data reported here for F 11782 originated from Kruczynski et al. [19]

[e] Treated animals died before controls

markedly superior to that shown by the other dual inhibitors tested concurrently.

Evaluations of any interactions between DNA and F 11782 or the other dual topoisomerase inhibitors

Compounds such as intercalating compounds or minor-groove binders that alter the gross structure of DNA can strongly affect the activity of topoisomerases [32]. DNA-binding properties were first assessed by measuring displacement of either EtBr or bisbenzimide from DNA. F 11782 was the only compound that was totally inactive in displacing both EtBr and bisbenzimide, even at a concentration of 100 μ*M* (Table 4). In contrast, positive interactions were noted amongst the three other dual topoisomerase inhibitors tested. TAS-103 proved the most potent DNA intercalating compound with IC_{50} values of 0.5 μ*M* in both assays, and major activity was apparent also with aclarubicin (respective IC_{50} values of 1.7 and 1.2 μ*M*) and with intoplicin (IC_{50} of 4 μ*M* in the EtBr assay). The autofluorescence of intoplicin prevented any measurement of its effects on the displacement of bisbenzimide from DNA. These findings taken together provide clear evidence that F 11782, which neither intercalates nor interacts with DNA as a minor-groove binder, clearly differed from the other identified dual topoisomerase inhibitors tested here.

Table 4 Evaluation of interaction between DNA and F 11782 relative to those of other dual inhibitors of topoisomerases I and II (IC_{50} concentration inhibiting 50% of the EtBr- or bisbenzimide-DNA interaction, *NE* not evaluable due to autofluorescence)

Test compound	Displacement of:	
	EtBr	Bisbenzimide
F 11782	None at 100 μ*M*	None at 100 μ*M*
Aclarubicin	IC_{50} 1.7 μ*M*	IC_{50} 1.2 μ*M*
Intoplicin	IC_{50} 4.0 μ*M*	NE
TAS-103	IC_{50} 0.5 μ*M*	IC_{50} 0.5 μ*M*

Comparison of the effects on topoisomerase I relaxation and on topoisomerase II decatenation of F 11782 and the other dual topoisomerase inhibitors

The effects of the various compounds on the catalytic activity of DNA topoisomerase I (pBR322 DNA relaxation) were evaluated by two methods: in the absence (neutral gel) or presence (chloroquine gel) of 5 μ*M* chloroquine. Neutral gels allow the detection of compounds inhibiting DNA relaxation by topoisomerase I, while chloroquine gels indicate whether or not the topoisomerase I-inhibiting effect of those compounds might be due to an interaction with DNA [22]. F 11782 inhibited the relaxation of pBR322 by topoisomerase I (neutral gel) with an EC value of 3.2 μ*M* (Table 5), thus proving slightly less potent than aclarubicin, intoplicin and TAS-103 (EC values of 1.8, 1 and 0.76 μ*M*, respectively). On the other hand, F 11782, as opposed to the other dual topoisomerase inhibitors tested, did not express any DNA-interacting properties, as suggested by the absence of any effect on the relaxation reaction in chloroquine-containing gels, thus confirming the lack of DNA-intercalating properties described above for this novel compound. Considering the effects of these

Table 5 Comparison of the effects of F 11782 with those of other dual inhibitors on topoisomerase I relaxation or topoisomerase II decatenation activities

Test compound	Topoisomerase I[a] relaxation		Topoisomerase II[b] decatenation (EC[c], μ*M*)
	Neutral gel (EC[c], μ*M*)	Chloroquine gel (EC[c], μ*M*)	
F 11782	3.2	None at 100 μ*M*	0.76
Aclarubicin	1.8	1.0	5.6
Intoplicin	1.0	1.0	5.6
TAS-103	0.76	1.0	10.0

[a] From calf thymus
[b] From *Drosophila melanogaster*
[c] EC corresponds to the effective drug concentration as defined in Materials and methods

compounds on the catalytic activity of DNA topoisomerase II (Table 5), aclarubicin and intoplicin induced comparable inhibitory effects against topoisomerase II-induced kDNA decatenation with EC values of 5.6 μ*M*, TAS-103 proved less inhibitory with an EC value of 10 μ*M*, whilst F 11782 proved significantly most potent with an EC value of 0.76 μ*M*.

Evidence has been reported previously [28] that F 11782 actively inhibits the catalytic activities of both topoisomerase I and topoisomerase II in a mixture of nuclear enzymes in extracts prepared from the highly sensitive human tumour cell line GCT27. In a second set of experiments, using the same cell line, the effects of F 11782 were compared with those of the other dual topoisomerase inhibitors vis-à-vis the extractability of topoisomerase IIα and IIβ after a 6-h drug exposure. Figure 2 illustrates the data obtained relative to topoisomerase IIα. The concentration-dependent inhibition of topoisomerase IIα extractability observed with F 11782 was also noted with intoplicin, with respective IC_{50} values of 27 and 10 μ*M*. On the other hand, neither TAS-103 nor aclarubicin appeared able to inhibit the extractability of topoisomerase IIα even at high concentrations (32 μ*M*). Comparable effects were obtained with topoisomerase IIβ, and also with another tumour cell line, namely P388 murine leukaemia cells (data not shown).

Effects of F 11782 and other dual topoisomerase inhibitors on the stabilization of topoisomerases I- and II-induced DNA cleavage in vitro

Figure 3 illustrates topoisomerase I-induced DNA cleavage formation in the absence (a,b; lane 1) or in the presence of 10 μ*M* camptothecin (a,b; lane 2) used as a positive control, or of various concentrations of each test compound. As shown in Fig. 3a, TAS-103 (lanes 3 to 5) and intoplicin (lanes 6 to 8) induced cleavage products both at 1 μ*M* (lanes 5 and 8) and 10 μ*M* (lanes 4 and 7). Certain of the cleavage products seem to be lost with increasing concentrations of TAS-103 or intoplicin, i.e. at 100 μ*M* (lanes 3 and 6), a phenomenon termed a bell-shaped response already described for intercalating topoisomerase-interacting agents [31]. In contrast (Fig. 3b), neither F 11782 (lanes 3 and 4) nor aclarubicin

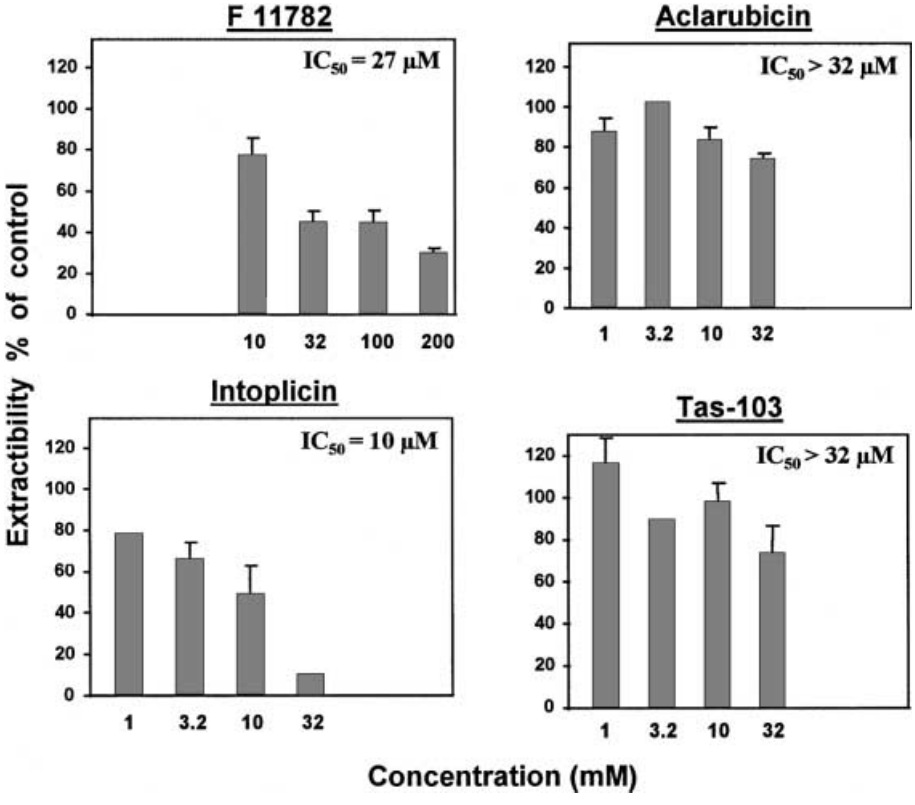


Fig. 2 Effects of F 11782 and other dual inhibitors of topoisomerases I and II on the extractability of topoisomerase IIα from GCT27 nuclei. Nuclear extracts were prepared from GCT27 cells treated for 6 h with increasing concentrations of each test compound. The extracted topoisomerase IIα was then measured by Western blot analysis using an anti-topoisomerase IIα primary antibody followed by a peroxidase-conjugated secondary antibody. After an ECL detection procedure, immunoblots were quantified using a Molecular Imager and the amount of salt-extractable topoisomerase IIα in treated cells was expressed as a percentage of the salt-extractable topoisomerase IIα in control cells

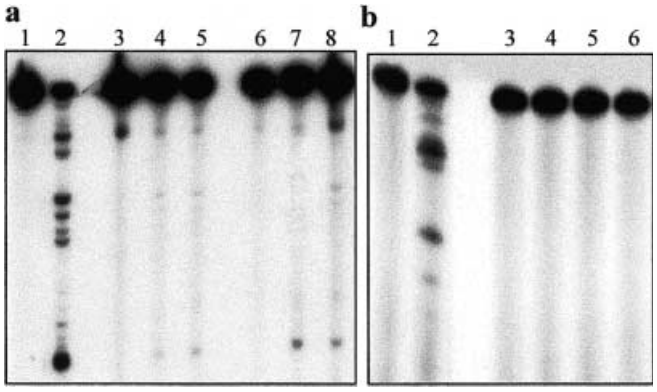


Fig. 3a,b Stabilization of topoisomerase I-induced DNA cleavage in vitro. The radiolabelled EcoRI-XbaI SV40 DNA fragment was incubated with topoisomerase I in the absence (**a**,**b**; *lane 1*) or presence of 10 μ*M* camptothecin (**a**,**b**; *lane 2*) used as a positive control, or of the indicated concentrations of (**a**) TAS-103 (100, 10, 1 μ*M*; *lanes 3, 4, 5*) and intoplicin (100, 10, 1 μ*M*; *lanes 6*, *7*, *8*), and (**b**) F 11782 (100, 10 μ*M*; *lanes 3*, *4*) and aclarubicin (100, 10 μ*M*; *lanes 5*, *6*). Cleavage patterns were visualized by electrophoresis on a denaturing polyacrylamide gel and analysed by autoradiography

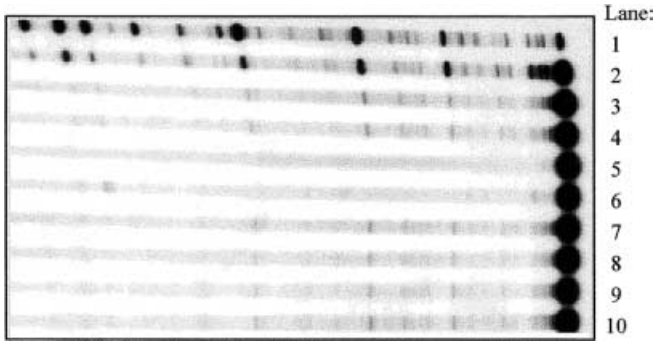


Fig. 4 Stabilization of topoisomerase IIα-induced DNA cleavage in vitro. The radiolabelled EcoRI-XbaI SV40 DNA fragment was incubated with topoisomerase IIα in the absence (*lane 3*) or presence of the indicated concentrations of etoposide (100, 10 μ*M*; *lanes 1*, *2*) used as a positive control, aclarubicin (100, 10 μ*M*; *lanes 5*, *6*) and F 11782 (100, 10, 1, 0.1 μ*M*; *lanes 7–10*). Camptothecin (100 μ*M*; *lane 4*) was used as a negative control. Cleavage patterns were visualized by electrophoresis on a denaturing polyacrylamide gel and analysed by autoradiography

(lanes 5 and 6) induced any cleavage formation at concentrations up to 100 μ*M*.

Figure 4 illustrates topoisomerase II-induced cleavage formation. As previously reported [29], F 11782 (Fig. 4, lanes 7 to 10) neither inhibited nor increased CC stabilization at concentrations ranging from 0.1 to 100 μ*M*. Aclarubicin (Fig. 4, lanes 5 and 6) induced a reduction of cleavage product formation at both 10 and 100 μ*M*. Such a reduction of CC stabilization has been described previously with intoplicin when higher concentrations were used, while the compound was described as inducing formation of topoisomerase II-

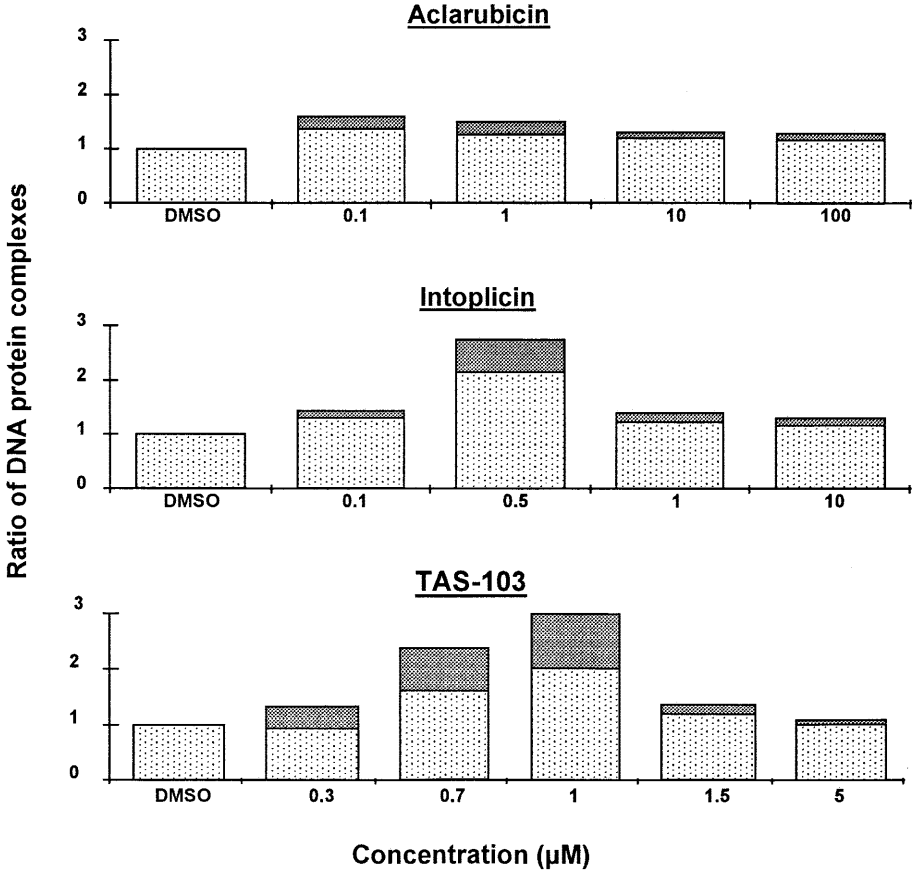


Fig. 5 Effects of the dual topoisomerases I and II inhibitors on DNA-protein complex formation in intact P388 cells. [^{3}H]thymidine- and [^{14}C]-leucine-labelled cells were incubated for 30 min with the indicated concentrations of test compound and protein complex formation was measured using the SDS-KCl precipitation assay [22]. Results are expressed as the mean ratio (±SEM) of DNA protein complexes (^{3}H dpm/^{14}C dpm) in treated versus control cells, obtained from at least three independent experiments

mediated DNA cleavage at low concentrations [30]. No conclusive data were obtained from our attempts to monitor the effects of TAS-103 on topoisomerase-II-induced cleavage formation in vitro, since gel analyses provided only uninterpretable smears.

As an extension of these studies, the effects of these compounds on the stabilization of topoisomerase-DNA CCs in P388 cells after a 30-min incubation period were next evaluated. The data obtained, illustrated in Fig. 5, clearly show that both intoplicin and TAS-103 stimulated the formation of CCs with topoisomerases in cells, thus confirming previous findings with both compounds [30, 39]. The effects were bell-shaped, with an increase at the intermediate concentrations of 0.5 μ*M* and 1 μ*M* for intoplicin and TAS-103, respectively. Aclarubicin, induced no significant or real dose-dependent stabilization of CCs, in a similar manner to the essentially negative effects reported elsewhere with F 11782 under the same experimental conditions [28].

Effects of F 11782 and other dual topoisomerase inhibitors on the binding of topoisomerases I and II to DNA, as evaluated by gel retardation assays

Amongst the various dual inhibitors of topoisomerases I and II tested here, while all inhibited the catalytic activity of both enzymes, only intoplicin and TAS-103 stabilized CCs in vitro or in cells, providing evidence for a mechanism for their inhibitory effects. However, an interaction with the binding of topoisomerases to DNA could result in an inhibition of their overall catalytic activity. Thus the influence of F 11782, and the other dual inhibitors, on the interactions between DNA and each of the topoisomerases, using gel retardation assays, were evaluated.

As shown in Fig. 6a, F 11782 (lane 3) completely inhibited the interaction between DNA and topoisomerase I at 100 μ*M*, with no DNA probe remaining at the top of the gel, thus confirming previous findings for this novel compound [29]. In contrast (Fig. 6a), no inhibitory effects were noted with TAS-103 (lane 4), intoplicin (lane 5) or aclarubicin (lane 6) at 100 μ*M*.

Similarly, 100 μ*M* F 11782 completely inhibited the interactions between DNA and topoisomerase IIα (Fig. 6b, lane 3) and II*β* (Fig. 6c, lane 3). When considering the other dual inhibitors tested at 100 μ*M*, only TAS-103 showed clear interference with the interactions of DNA and topoisomerase IIα (Fig. 6b, lane 4) and II*β* (Fig. 6c, lane 4), but to a far lesser an extent than that noted with F 11782, while marginal or no interference was observed with aclarubicin and intoplicin.

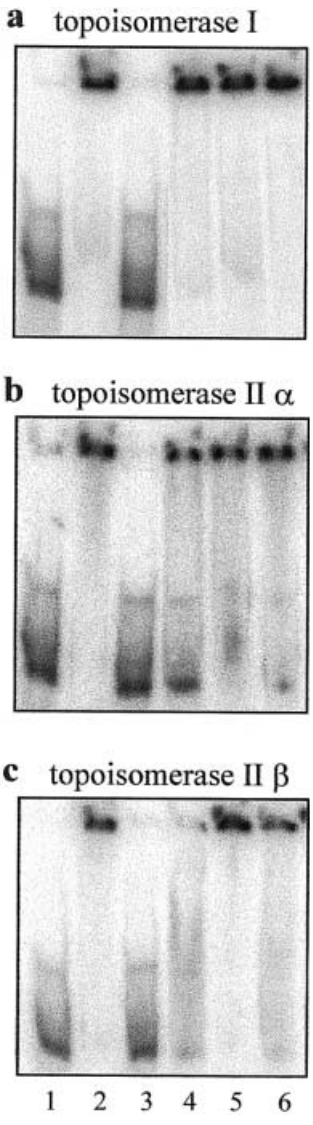


Fig. 6a–c The effects of F 11782 compared to those of other dual inhibitors of topoisomerases I and II on the binding of topoisomerases to DNA, as determined by gel-shift assays. [^{32}P]-DNA probe (*lane 1*) was incubated with topoisomerase I (**a**), IIα (**b**) or II*β* (**c**) in the absence (*lane 2*) or in the presence of 100 μ*M* F 11782 (*lane 3*), TAS-103 (*lane 4*), intoplicin (*lane 5*) and aclarubicin (*lane 6*). During electrophoresis, the free radiolabelled DNA probe migrated through the gel (*lane 1*), whilst the DNA/topoisomerase complex remained at the top of the gel (*lane 2*). When the DNA binding of the topoisomerase was inhibited, the electrophoretic profile looked like that of the free DNA probe. Representative gels from two or three independent assays are presented

Combined in vitro cytotoxic effects of F 11782 and other dual topoisomerase inhibitors with etoposide

As suggested previously [16], any compound interfering with the catalytic cycle of topoisomerase II in such a way as to reduce the amount of available target for CC-stabilizing drugs, such as etoposide, has the potential to antagonize the cytotoxicity of these drugs. Using the L1210 murine leukaemia cell line in vitro (Fig. 7), we examined the cytotoxic effects of a 2-h exposure to etoposide, a specific topoisomerase II inhibitor, used alone or in combination, with a single MCC, i.e. that resulting in less than 20% growth inhibition, of F 11782 (1.8 μ*M*), intoplicin (0.06 μ*M*), aclarubicin (0.01 μ*M*) or TAS-103 (0.01 μ*M*). A comparison of the experimental

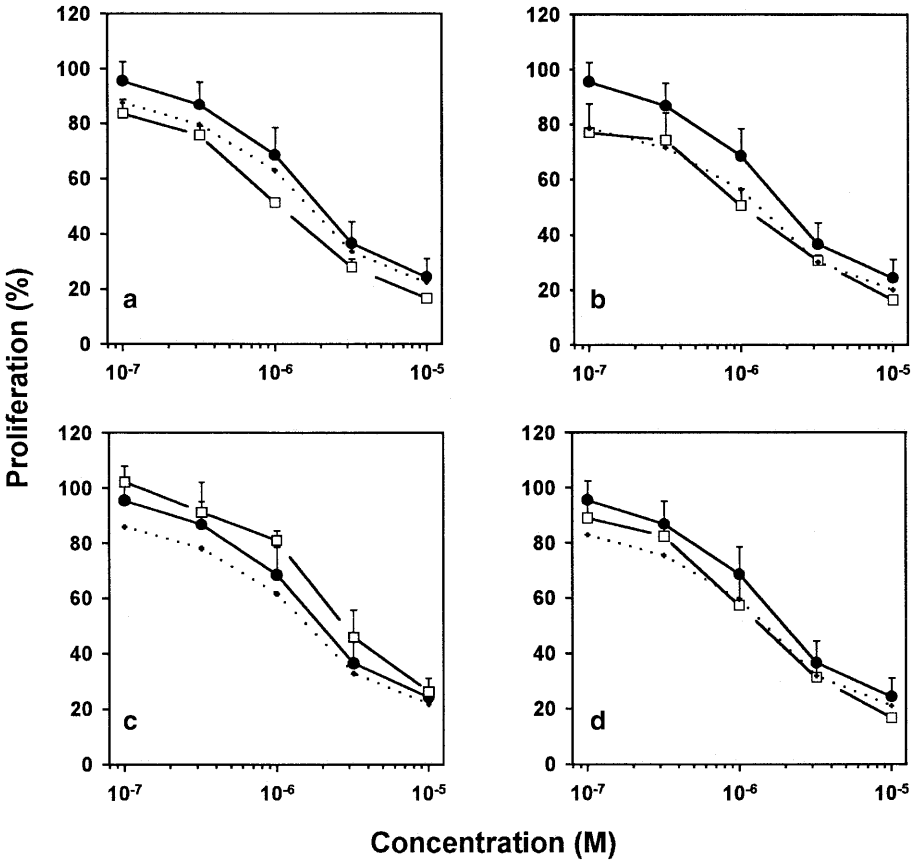


Fig. 7a–d Effects of F 11782 compared to those of other dual inhibitors of topoisomerases I and II on the etoposide-induced inhibition of L1210 cell proliferation. Cells were incubated for 2 h with increasing concentrations of etoposide alone (*black circles*), or associated (*open squares*) with a MCC, i.e. that resulting in less than 20% growth inhibition when tested alone, of the associated test drug: **a** F 11782 1.8 μ*M*; **b** intoplicin 0.06 μ*M*; **c** aclarubicin 0.01 μ*M*; **d** TAS-103 0.01 μ*M*. Cell proliferation was determined after a 48-h cell growth period without drug by cell counting. Theoretical additivity (TA) curves (*dotted line*) are included for comparison. TA curves were calculated using the formula TA = EDA − (RE × EDA/100) where EDA is the effect (percent proliferation) of etoposide alone and RE is the real effect (percent proliferation) of the MCC of the associated test drug. *Bars* indicate standard deviation from at least three independent experiments

and the calculated TA curves showed that F 11782 (Fig. 7a) enhanced the cytotoxic effects of etoposide alone, with a greater than additive effect, as opposed to aclarubicin which clearly inhibited etoposide cytotoxicity (Fig. 7c). On the other hand, the effects of both intoplicin (Fig. 7b) and TAS-103 (Fig. 7d) appeared merely additive. F 11782 thus differs from the catalytic inhibitor aclarubicin, and from the CC-stabilizing agents, intoplicin and TAS-103.

Discussion

F 11782, a novel epipodophylloid, is a potent catalytic inhibitor of both topoisomerases I and II in vitro [29] and has shown markedly superior antitumour activity in vivo compared to etoposide [19]. In the study presented here, we compared the properties of F 11782 with those of other well-known dual inhibitors of both enzymes which have reached clinical trials, i.e. aclarubicin, intoplicin and, more recently, TAS-103, in terms of their in vitro and in vivo antitumour effects, as well as inhibitory activities vis-à-vis topoisomerases I and II.

F 11782 proved generally least potent relative to the other dual topoisomerase inhibitors when tested in vitro against a panel of two murine leukaemias and three human tumour cell lines, whilst in turn aclarubicin and TAS-103 proved considerably more potent than intoplicin. When considering the relative chemosensitivities of the human tumour cell lines vis-à-vis the various dual inhibitors tested, their profiles differed in certain respects. The GCT27 cell line expressed the highest level of sensitivity to F 11782. In contrast, T24 cells proved strongly resistant to F 11782, and to a lesser extent to intoplicin, whilst remaining fully sensitive to TAS-103 and aclarubicin. In studies with a large series of topoisomerase inhibitors (data not shown) we have noticed that a high level of sensitivity of GCT27 cells and the relative resistance of T24 cells are two characteristics shared by inhibitors of topoisomerase II, but not generally by the camptothecins, topoisomerase I inhibitors. Thus F 11782 appeared to share a profile comparable to that expressed by specific topoisomerases II inhibitors, and in this respect was like intoplicin, but quite different from TAS-103 and aclarubicin.

We also investigated the comparative cytotoxic effects of F 11782 and these three other dual inhibitors against V79 Chinese hamster cells grown either as two-dimensional monolayer cultures or as three-dimensional multicellular spheroids. Growing spheroids have been shown to have a relatively high proportion of noncycling or nonproliferating cells compared with monolayers, and this has permitted their use for evaluating the proliferation dependency of any cytotoxicity exerted by anticancer drugs in vitro [36]. Our data clearly indicate that V79 cells growing as spheroids were more resistant to intoplicin, and to a lesser extent to F 11782 and TAS-103, while no significant resistance was expressed to

aclarubicin. Thus, these cytotoxic effects of F 11782, intoplicin and TAS-103, but not those of aclarubicin, were proliferation-dependent. These results are in accordance with previous results showing that the cytotoxicity of aclarubicin is independent of the proportion of cells in S-phase at the time of drug exposure, while generally topoisomerase II inhibitors [17], as well as topoisomerase I inhibitors [5], are more toxic to proliferating cells.

Next a comparison was made of the in vivo antitumour activities of F 11782 with those of aclarubicin, intoplicin and TAS-103, given as a single i.p-injected dose against the i.v.-implanted P388 murine leukaemia. F 11782 demonstrated a high level of activity, as shown by optimal T/C values, markedly superior to the minimal level of antitumour activity shown by TAS-103 and in turn to that shown by both aclarubicin and intoplicin, which were inactive under these experimental conditions. Previous findings have indicated some activity for intoplicin against the P388 murine leukaemia, but using quite different experimental conditions, i.e. repeated i.v. injections of the highest nontoxic dose of the test compound against the i.p.-implanted tumour [4]. F 11782, given i.p as multiple injections (2-week schedule) against the relatively drug-refractory s.c.-implanted B16 melanoma tumour model [41], was also greater than that recorded for TAS-103, both in terms of survival and of tumour growth inhibition. Intoplicin, previously reported to show some activity against this same tumour [4], using a 1-week schedule of repeated i.v injections, proved only marginally active under our experimental conditions, increasing the survival of tumour-bearing mice, yet without inhibiting their tumour growth. Thus the results presented here provide evidence of the very definite superiority of F 11782 over the other dual topoisomerase inhibitors evaluated in both these experimental animal tumour models.

Moving on to the more mechanistic studies and considering interactions with DNA, F 11782 proved the only compound showing no activity in these DNA-intercalator displacement assays, providing evidence that it does not intercalate DNA. Using chloroquine-containing gels, again no evidence was obtained of any interaction between F 11782 and DNA, in contrast to aclarubicin, intoplicin and TAS-103. As detailed previously [15, 17, 33], aclarubicin, intoplicin and TAS-103 were all shown to intercalate DNA, with TAS-103 being the most potent in this respect. Moreover, more extensive studies with F 11782, employing melting temperature data and a series of biophysical techniques including thermal denaturation, footprinting and circular and linear dichroism, have confirmed these negative findings (as discussed in reference 29). Finally, molecular studies using purified topoisomerases and isolated DNA showed that F 11782, as well as the other dual inhibitors tested, inhibited the catalytic activities of both topoisomerases I and II, with topoisomerase II being more sensitive to F 11782 than topoisomerase I, while the reverse was observed with the three other compounds evaluated. Thus F 11782 appears a unique dual inhibitor of topoisomerases I and II having been shown to inhibit the catalytic activities of both enzymes without interacting with DNA.

It has been clearly demonstrated that incubation of cells with topoisomerase II inhibitors, such as etoposide which stabilizes topoisomerase II-DNA CCs, or the bisdioxopiperazine derivative ICRF-187, acting on topoisomerase II without CC formation, reduces the amount of extractable topoisomerase II enzyme [35]. The proposed mechanism for such an inhibition has been associated with a covalent trapping of the enzyme in the CCs in the case of etoposide, and in the post-DNA-religation closed clamp form in the case of ICRF-187. Using the technique of Western blotting, etoposide and ICRF-187 were shown to induce a concentration-dependent decrease in the amount of extractable topoisomerase IIα and β from GCT27 human teratoma cells in culture (data not shown). Moreover, it has been shown here that F 11782, as well as intoplicin, also markedly reduced, in a concentration-dependent manner, the amount of extractable enzyme of both the α and β isoforms from the GCT27 cells. In contrast, no such effect was recorded with either aclarubicin or TAS-103. Regarding aclarubicin, Sehested and Jensen [35] have reported that in the human small cell cancer cell line OC-NYH aclarubicin actually increases the amount of extractable topoisomerase II. An explanation may be that aclarubicin competes with the DNA binding of topoisomerases, thus displacing a portion of the cellular enzymes which are normally crosslinked as a consequence of transcription and replication [7]. Thus the data presented here demonstrate that F 11782 affects its putative target topoisomerase II in cultured cells, preventing its extractability, a property shared by intoplicin, but not by aclarubicin nor by TAS-103.

In a second set of experiments the effects of F 11782 and the other dual topoisomerase inhibitors on the topoisomerase I- and topoisomerase II-mediated DNA cleavage activities were quantified both in vitro and in cells. Neither F 11782 nor aclarubicin stabilized the CCs formed by topoisomerases I and II on DNA, and aclarubicin, but not F 11782, inhibited the topoisomerase II DNA cleavage activity in vitro, thus confirming previously reported data relating to these two compounds [17, 29]. Our data also confirmed the well-described stabilization of topoisomerase I CC induced by intoplicin [30] and TAS-103 [39] in vitro, while in our hands no clear effect on their stabilization of topoisomerase II CC was noted. One explanation could be that the concentrations of compounds that we selected for comparative studies (10 and 100 μM) were too high. Indeed, we noted that intoplicin as well as TAS-103, which induced topoisomerase-I mediated DNA cleavage at low concentrations, tended to suppress cleavage at high concentrations (Fig. 3). Moreover, Poddevin et al. [30] have already demonstrated that the effects of intoplicin on topoisomerase II-mediated DNA cleavage show the same type of bell-shaped concentration

dose-response curve in vitro. Finally, the results presented here on the stabilization of topoisomerase-DNA complexes in P388 cells, using the SDS-KCl precipitation assay, confirmed that both intoplicin and TAS-103 stabilized covalent DNA-topoisomerase complexes, following a bell-shaped dose-response curve, while aclarubicin, like F 11782, induced no stimulation of complex formation.

In previous experiments [29], F 11782 has been found to inhibit the stabilization of DNA-topoisomerase CC induced by either camptothecin or etoposide, and has proved a potent inhibitor of the DNA-binding activities of topoisomerases I and of both isoforms of topoisomerases II, using mobility-shift or gel-shift assays. Here, using the same assay methodology, the effects of aclarubicin, intoplicin and TAS-103 were investigated and compared to those of F 11782. Our data demonstrate that F 11782 was the only dual inhibitor tested capable of completely inhibiting the DNA-binding activities of topoisomerases I and II, as shown by the total recovery of the mobility of the DNA probe in the gel (Fig. 6) in the presence of F 11782. When such interactions were noted with the other dual inhibitors, only a partial recovery and (or) a smear of the DNA probe was observed, suggesting that the intercalating properties of such compounds may influence the DNA-binding activities of topoisomerases in this in vitro system. Thus, F 11782 seems to be the only dual inhibitor amongst those tested clearly impairing the DNA-binding activities of both DNA topoisomerases, whilst not having any direct DNA-binding properties.

Topoisomerase II catalytic inhibitors, such as aclarubicin and ICRF-187, impair the availability of the target for CC-stabilizing drugs such as etoposide either by inhibiting the binding of topoisomerase II to DNA or by trapping the enzyme fixed to DNA and they have been shown to inhibit the cytotoxic activities of such drugs [14, 16]. Since anticancer drugs are used widely clinically in combination with other drugs, generally improving their effectiveness, it was of interest to compare the cytotoxic effects of MCCs of either the novel compound F 11782 or one the other dual inhibitors, either catalytic inhibitors or CC-stabilizing agents, combined with increasing concentrations of etoposide. Cotreatment of cells with F 11782, together with etoposide, resulted in a supraadditive effect on the cytotoxicity exerted by etoposide alone. F 11782, in this respect, appears to differ from the other non-CC-stabilizing inhibitor aclarubicin, which clearly inhibited etoposide cytotoxicity, while TAS-103 and intoplicin, both CC-stabilizing agents, induced only additive cytotoxicity in combination with etoposide. Evaluation of further drug combinations involving F 11782 and other potent anticancer agents, using a more standardized methodology, such as that described by Chou and Talalay [9], are now under consideration.

In conclusion, our results indicate that F 11782, a novel dual inhibitor of topoisomerases I and II, whilst proving generally less cytotoxic in vitro, showed a higher level of antitumour activity in two experimental models in vivo than the three other dual inhibitors which are already undergoing clinical testing, i.e. aclarubicin, intoplicin and TAS-103. This novel inhibitor does not stabilize CCs induced by either enzyme, either in vitro or in cells. Furthermore, F 11782 was the only compound of this series to inhibit the catalytic activity of both DNA-topoisomerases without showing any interaction with DNA, and to strongly interfere with the DNA binding of these nuclear proteins. Finally, in combination in vitro with etoposide, only F 11782 was capable of enhancing the cytotoxicity of etoposide, thus suggesting that the concomitant use of F 11782 with other antitumour agents may prove beneficial. Overall the results presented here reinforce the idea that F 11782 represents a new class of antitumour agents with definite potential for further preclinical development.

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