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Preclinical report

Characterization of the biological and biochemical activities of F 11782 and the bisdioxopiperazines, ICRF-187 and ICRF-193, two types of topoisomerase II catalytic inhibitors with distinctive mechanisms of action

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F 11782 is a newly identified catalytic inhibitor of topoisomerases I and II, without any detectable interaction with DNA. This study aimed to establish whether its catalytic inhibition of topoisomerase II was mediated by mechanisms similar to those identified for the bisdioxopiperazines. *In vitro* combinations of F 11782 with etoposide resulted in greater than additive cytotoxicity in L1210 cells, contrasting with marked antagonism for combinations of etoposide with either ICRF-187 or ICRF-193. All three compounds caused a G_2/M blockade of P388 cells after an 18-h incubation, but by 40 h polyploidization was evident only with the bisdioxopiperazines. Gel retardation data revealed that only F 11782, and not the bisdioxopiperazines, was capable of completely inhibiting the DNA-binding activity of topoisomerase II, confirming its novel mechanism of action. Furthermore, unlike ICRF-187 and ICRF-193, the cytotoxicity of F 11782 appeared mediated, at least partially, by DNA damage induction in cultured GCT27 human teratoma cells, as judged by a fluorescence-enhancement assay and monitoring p53 activation. Finally, the major *in vivo* antitumor activity of F 11782 against the murine P388 leukemia (i.v. implanted) and the B16 melanoma (s.c. grafted) contrasted with the bisdioxopiperazines' general lack of activity. Overall, F 11782 and the bisdioxopiperazines appear to function as quite distinctive catalytic topoisomerase II inhibitors. [© 2000 Lippincott Williams & Wilkins.]

Key words: Bisdioxopiperazines, catalytic inhibitor, fluorinated epipodophylloid, *in vivo* antitumor activities, topo isomerase II.

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Introduction

DNA topoisomerase II is an essential nuclear enzyme, being implicated in topological changes in DNA, and plays a major role in DNA replication, transcription and chromosome condensation, as reviewed earlier.[1] DNA topoisomerase II has been identified as a molecular target for two distinct classes of anticancer drugs with unique modes of action. The first, termed topoisomerase II poisons, has been characterized widely, and includes the clinically important etoposide, amsacrine and doxorubicin.[2,3] These poisons are considered to exert their cytotoxic effects by stabilizing covalent complexes between topoisomerase II and DNA, which leads to irreversible DNA breaks and ultimately cell death. A second group, termed catalytic inhibitors, has been defined more recently on the basis that they do *not* stabilize the covalent intermediate of the topoisomerase II reaction.[4,5] Instead, members of this group interfere with the enzyme at another point of the catalytic cycle, thus depriving cells of their essential nuclear enzyme and resulting in cell kill. Several rather diverse chemical entities have been described as potent catalytic inhibitors of eukaryotic topoisomerases, including merbarone which inhibits DNA cleavage by the enzyme,[6,7] suramin which by interacting with the enzyme in living cells may affect its association with the nuclear matrix,[8] aclarubicin which intercalates DNA and prevents binding of the enzyme to DNA,[9,10] and the bisdioxopiperazines. These latter compounds, studied extensively,[5,11–13] have been shown to inhibit the re-opening of the closed clamp, formed by the enzyme around the DNA, thus

sequestering topoisomerase II within cells and also have the ability to block ATP hydrolysis. We have recently described a novel fluorinated epipodophyllloid which interacts with both topoisomerases I and II, without stabilizing cleavable complexes or intercalating DNA or binding to its minor groove.[14,15] This molecule F 11782 (2″,3″-bis-pentafluorophenoxyacetyl-4″,6″ethylidene-β-D-glucoside of 4′-phosphate-4′-demethylepipodophyllotoxin 2*N*-methyl glucamine salt) thus appears to act as a catalytic inhibitor of both these nuclear enzymes and is certainly unusual in this regard. The aim of this study therefore was to establish whether the mechanisms and biological characteristics identified as associated with the catalytic inhibition of topoisomerase II by the bisdioxopiperazines are shared by F 11782 or whether these structurally diverse molecules achieve their inhibition by quite different means. The data presented provide evidence of distinctive mechanisms of action of these two types of topoisomerase II catalytic inhibitors.

Materials and methods

Chemicals and test compounds

MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma (St Louis, MO) and dimethyl sulfoxide (DMSO) from SDS (Peypin, France). F 11782 and ICRF-193 [*meso*-2,3 bis(3,5-dioxopiperazine-1-yl)butane] were synthesized by Pierre Fabre Médicament (Castres, France), whilst ICRF-187 (dexrazoxane) was purchased from Chiron (Milan, Italy).

Tumor cell lines

L1210 murine leukemia cells, and the human DLD-1, LoVo, SK-OV-3 and T24 tumor cell lines were obtained from the ATCC (Rockville, MD). The MX-1 cell line was obtained from the National Cancer Institute (NCI, NIH, Bethesda, MD) and the P388 murine leukemia cells, originating from the NCI, were a gift from Dr S Cros (Institut de Pharmacologie et de Biologie Structurale IPBS, Toulouse, France). The human testicular teratoma GCT27 cells were obtained from Dr JRW Masters (University College, London, UK), the GLC4 cells were kindly provided by Dr EGE de Vries (University of Groningen, Groningen, The Netherlands) and the CEM cell line originated from Dr V Ling (Ontario Cancer Institute, Toronto, Ontario, Canada). RPMI 1640 Glutamax medium (Gibco/BRL, Cergy Pontoise, France) was used supplemented either with 10% heat-inactivated horse serum for growing L1210 and P388 cells or with 10% fetal calf serum (FCS) for growing GCT27, SK-OV-3 and CEM cells. LoVo, DLD-1, MX-1 and T24 cells were grown in MEM medium (Gibco/BRL) supplemented with 5% FCS. The various media were supplemented with fungizone, penicillin–streptomycin and L-glutamine (4 mM final). All cell lines were cultivated in a CO_2 incubator (37°C, 5% CO_2). 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.

Interactions with DNA

The procedures for the inhibition of ethidium bromide (EtBr)–DNA interactions and bisbenzimide–DNA interactions have been described in detail by Perrin *et al.*[14]

Interactions with topoisomerase II

The procedures used in the production and purification of human recombinant topoisomerases IIα and IIβ from yeast are detailed in Perrin *et al.*[14] Eighteen microliters of buffer A (50 mM Tris, pH 8.0, 120 mM KCl, 0.5 mM dithiothreitol, 0.5 mM ATP and 10 mM $MgCl_2$) containing 200 ng kDNA (isolated from *Crithidia fasiculata*; TopoGen, Columbus, OH) and 1 U of the human recombinant topoisomerase II (the amount of enzyme which resulted in the complete decatenation of 200 ng kDNA) after a 30-min incubation were added to 2 μl of either solvent alone (DMSO) or a solution of the test compound.[14,16] After 30 min of incubation at 30°C, the reaction was terminated and samples analyzed on a 1% agarose gel and run at 35 mA for 2 h in TBE buffer (89 mM Tris, 89 mM borate and 2 mM NaEDTA, pH 8.3).[16] Gels were stained with EtBr and scanned under UV illumination using a BioRad molecular imager (BioRad, Ivry sur Seine, France). Assays to determine the inhibition of decatenation by quantitating the amount of decatenated DNA were carried out on three separate occasions and results are expressed as IC_{50} concentrations, i.e. those inhibiting decatentaion activity by 50%.

Crude nuclear extracts of P388 cells, prepared by the method of Nakagawa *et al.*,[17] were used in this topoisomerase II-mediated decatenation assay.

Topoisomerase II cleavage reactions

The protocol for the topoisomerase II-mediated cleavage assay used have been detailed elsewhere.[14] Briefly, the reaction mixture containing 4–8 ng radiolabeled DNA probe, a final concentration of 2.5% DMSO with or without test compound and 1 μl (2 U)

of 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 analyzed as detailed above.

Formation of covalent topoisomerase–DNA complexes in intact cultured 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 using the SDS–KCl precipitation assay according to Larsen *et al.*[16] The procedure adopted is detailed in Etievant *et al.*[15]

DNA-binding activities of topoisomerase II

DNA-binding activity was evaluated using a gel-shift assay technique, as described by Svejstrup *et al.*[18] and detailed in Perrin *et al.*[14] 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 mM Tris–HCl, pH 7.6, 7.5% glycerol, 10 mM $MgCl_2$ and 50 mM KCl). Samples were then separated on polyacrylamide 5% minigels (BioRad) 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 (BioRad) and the patterns 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 a representative gel is shown.

Combined cytotoxic effects with etoposide in L1210 cells

The assay procedure used has been detailed previously.[15] First, 10^5 L1210 cells/ml (5 ml/15 ml tube) were 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 each of the catalytic inhibitors tested, i.e. that resulting in less than 20% growth inhibition when tested alone. The catalytic inhibitor was added just prior to etoposide. Cell suspensions were then centrifuged (200 *g*, 7 min), and pellets were washed twice with medium prior to resuspension in 5 ml drug-free medium and then regrown for 48 h before cell counting using an automated Coulter counter ZM (Beckman Coulter, Villepinte, France).

If the associated drugs are considered to exert their effects on cell proliferation in an independent manner, then the real effect (RE) of a given MCC concentration, i.e. control cell proliferation inhibition (%), of any catalytic inhibitor would remain 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 catalytic inhibitor. Therefore, it is possible to calculate a theoretical additivity (TA) dose–response curve for etoposide combined with a given dose of the associated catalytic inhibitor, 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 (%)

Based on this approach, for each of the combinations tested here, the theoretical additivity dose–response curves have been calculated and then compared with the dose–response curves obtained experimentally. Data derived from two or three independent experiments are presented.

DNA damage detection

Analysis of DNA damage was carried out using a fluorometric technique recently described using a microplate format.[19] After a 24-h incubation with the test drug, GCT27 cells were trypsinized and suspended in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.4) at a cell density of 1.5×10^5 cells/ml and 25 μl aliquots of cell suspension were distributed in six replicates for each assay into wells of a black 96-well microplate. Then 25 μl lysis solution (9 M urea, 0.1% SDS and 0.2 M EDTA, pH 10.0) containing 20 μl/ml Picogreen solution was added slowly without mixing or shaking. Lysis of cells was allowed to occur on ice in the dark for 1 h. DNA unwinding at pH 12.4 was started by the addition of 250 μl 0.025 M NaOH to each sample. Fluorescence blanks contained 25 μl TE buffer instead of sample, together with 25 μl Picogreen lysis solution and 250 μl 0.025 M NaOH. Measurements of fluorescence were performed after 10 min of incubation at 480 nm excitation and 520 nm emission using a fluorescence plate reader (SpectraMax; Molecular Devices, Menlo Park, CA).

Assessment of p53 induction

Western blotting was used to detect induction of p53. Following a 24-h exposure to test compound or solvent alone, GCT-27 cells (2×10^6 cells/assay) were trypsinized, washed in phosphate-buffered saline (PBS)

and resuspended in 200 μl lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40 and 5 mM NaF) containing a cocktail of protease inhibitors (Boerhinger Mannheim, Mannheim, Germany). Cells were allowed to lyse on ice for 10 min and then pelleted by centrifugation. The protein content was determined according to Bradford[20] and 60 mg was submitted to SDS–PAGE using a 12% acrylamide gel, followed by blotting onto a nitrocellulose sheet (Amersham, Les Ulis, France). After a 1-h incubation at room temperature in blocking buffer (10% non-fat milk and 10% FCS in PBS) an anti-p52 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/1000 was added and left overnight. Extensive washing in PBS then followed before performing 1-h incubation in the presence of the secondary antibody, goat anti-mouse coupled to peroxidase (Jackson Immunoresearch, West Grove, PA) diluted 1/5000. p53 was visualized by enhanced chemiluminescence and immunoblots were quantified using a BioRad molecular imager. Results are expressed relative to controls, i.e. the amount of p53 in treated versus control cells. Each experimental point was tested in three independent experiments.

Cell cycle analyses by flow cytometry

Flow cytometric analyses of cellular DNA content by propidium iodide staining were preformed. Logarithmically growing P388 cells were incubated with test compounds for either 6, 18 or 40 h prior to centrifugation (4°C, 250 *g*, 10 min) and then being washed twice with cold PBS. The final supernatants were discarded carefully so as to leave approximately 100 μl buffer in each tube. Cells were then prepared for quantitative measurements of their DNA content using the Coulter DNA-Perp reagent kit (Coulter, Miami, FL). For each sample 15 000 cells were analyzed using a Coulter Epics XL flow cytometer, and the percentage of cells in the G_1, S and G_2/M phases calculated using MultiCycle software (Phoenix Flow Systems, San Diego, CA).

In vitro cell growth measurements

In the various assays described, water (1% final concentration) was used as solvent, except for ICRF-193 which was solubilized in DMSO (0.1% final concentration). L1210 cells were inoculated (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. After 48 h the control and treated cell growth was determined by cell counting using a Coulter counter ZM. For the P388 leukemia cells and all the human solid tumor cell lines, except for the GLC4 cells, the growth-inhibitory effects of each test compound were determined in 96-well microtiter plates, after a 48- or 72-h incubation period, respectively, using a colorimetric metabolic dye-based MTT assay as described earlier.[15] For each cell line the IC_{50} values, i.e. the concentration of test compound required to reduce growth to 50% of that obtained for control cells, were generated based on pooled data from at least three independent experiments. For GLC4 cells, cell numbers were counted using a Coulter counter after a 72-h exposure to the test compound.

Mice and tumor models

Female hybrid CDF1 (CD2F1/CrlBR) and C57BL/6 (C57BL/6 NCrlBR) mice (Charles River, St Aubin-les-Elbeuf, France) were used for implanting the murine P388 leukemia and the murine B16 melanoma (Division of Cancer Treatment, Tumour Repository, NCI, Frederick, MD), respectively. Animals were handled 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 authorised investigators.[21]

Experimental chemotherapy

All experiments were conducted in compliance with French regulations and CRPF ethical committee guidelines, based on the UKCCCR guidelines for the welfare of animals in experimental neoplasia, as detailed previously.[21] First, 10^6 P388 cells/mouse were implanted i.v. into C2DF1 mice on day 0. For the B16 model, 0.5 ml of a tumor brei at 1 g/ml made by disrupting and homogenizing tumor fragments in sterile 0.9% sodium chloride were inoculated s.c. into mice on day 0. After randomization in treatment cages, test compounds were administered either as single doses to mice bearing the P388 leukemia or as multiple doses over 2 weeks (on days 3, 5, 7, 10, 12 and 14 following tumor graft) to mice implanted with the B16 melanoma. In each chemotherapy trial, mice were checked daily, with any adverse clinical reactions noted and deaths recorded. Mice were weighed 2–4 times weekly during treatments and once weekly thereafter. B16 tumors were measured by callipers twice weekly and tumor volumes (mm^3) were estimated as=0.5 (length × $width^2$). Results are presented for experiments involving seven to 15 mice per experimental group according to the model used.

The optimal dose, i.e. that inducing the greatest increase of 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 tumor model. For the B16 melanoma tumor model, several evaluation criteria which were detailed earlier[21] were used in parallel: survival curves of treated and control compared using the log-rank test, tumor growth inhibition following various evaluation criteria: ratios of the median tumor volumes (T/C) of treated versus control, areas under the tumor 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 non-parametric Mann–Whitney rank-sum test.

Results

Comparison of the effects of these catalytic inhibitors on topoisomerase II-induced DNA decatenation and on stabilization of topoisomerase II-mediated cleavable complex formation

F 11782 is a potent inhibitor of DNA decatention catalyzed by either topoisomerase IIα or IIβ as reported previously.[14] Data relating to topoisomerase IIα are reproduced in Figure 1 and the IC_{50} value calculated as 1.8 μM provides clear evidence that F 11782 is a far more potent inhibitor than either ICRF-193 or ICRF-187, whose respective IC_{50} values were calculated as 13 and 48 μM. Comparable date were obtained relating to topoisomerase IIβ.

Evidence has been presented earlier[14] showing that F 11782 proved active in inhibiting the catalytic activities of both topoisomerase IIα and IIβ in a

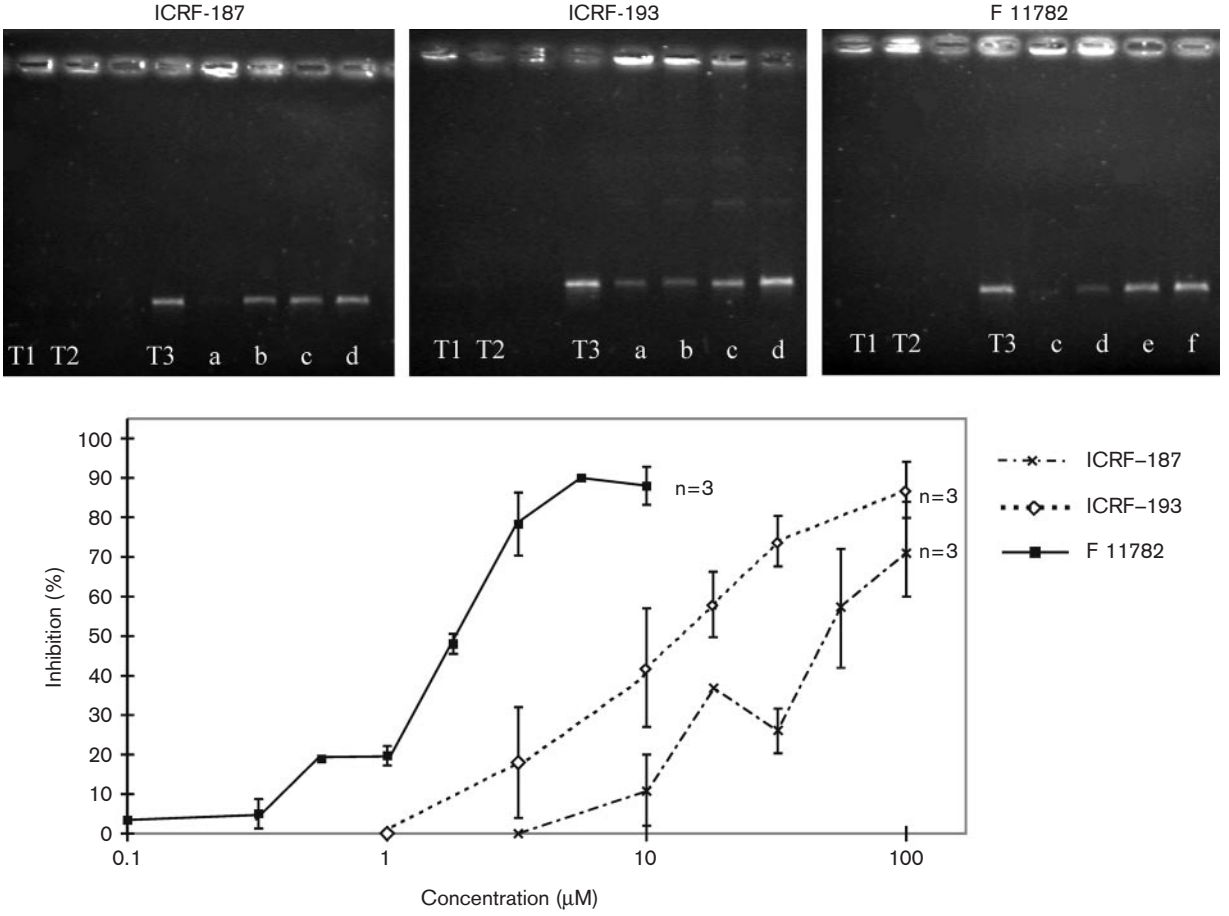

Figure 1. Inhibitory effects of F 11782, ICRF-187 or ICRF-193 or on the decatenation of kDNA by topoisomerase IIα. A representative gel, obtained with each compound, is shown in the upper section. Lane T1, kDNA, no enzyme (decatenated form); lane 2, kDNA plus highest concentration of test compound; lane T3, kDNA with 1 U of topoisomerase IIα (decatenated form); lanes a–f, kDNA with 1 U of topoisomerase IIα in the presence of 100, 32, 10, 3.2, 1.0 or 0.32 μM test compound. Quantitation of three independent assays (lower section) are shown as the mean ± standard error.

mixture of nuclear enzymes in extracts prepared from P388 murine leukemia cells. Comparable inhibitory results were obtained with ICRF-193 and ICRF-187 in the topoisomerase II-mediated decatenation assay using crude nuclear extracts (Table 1).

Evaluation of topoisomerase II-induced cleavage formation confirms previously published negative data relating to F 11782[14] and to the ICRF compounds.[11,22] F 11782 concentrations ranging from 0.1 to 100 μM had no effect, i.e. neither increased nor inhibited, the basal rate of cleavage mediated by either topoisomerase IIα or IIβ and essentially comparable negative data were obtained with 100 μM or lower ICRF-187 and 10 μM or lower ICRF-193.

In addition, and in confirmation, measurements of the effects of these compounds on the stabilization of topoisomerase–DNA cleavable complexes in P388 leukemia cells after a 30 min *in vitro* incubation period, using the SDS–KCl precipitation assay,[16] confirmed essentially negative effects for F 11782 at 100 μM or lower, as well as for ICRF-187 and ICRF-193 at respective concentrations of 100 and 10 μM or lower.

Overall, therefore, these data summarized in Table 1 emphasize that all three test compounds are effective catalytic inhibitors of topoisomerase II, which do *not* stabilize cleavable complexes induced by topoisomerase II.

Influence of F 11782, ICRF-193 or ICRF-187 on the binding of topoisomerase II to DNA, as judged by gel retardation assays

Since inhibition of the catalytic activities of topoisomerase II by these three test compounds cannot be accounted for in terms of stabilization of cleavable complexes it was considered that it might result from an interaction with the binding of the enzyme to DNA.

Earlier published data[14] led to the conclusion that F 11782 presents no specific affinity for DNA. Assessment of any potential DNA-binding properties of these two ICRF compounds by measuring the displacement of either ethidium bromide or bisbenzimide from DNA also proved negative at concentrations of 100 μM or below (data not shown).

The influence of these three catalytic inhibitors on the interactions between DNA and topoisomerase IIα and IIβ has been evaluated using gel retardation assays. As shown in Figure 2, 100 μM F 11782 completely inhibited any interaction between DNA and topoisomerase IIα (Figure 2, lane 3), confirming earlier published data which had provided evidence of a clear dose–response for this effect.[15] In contrast, absolutely no interference was observed with ICRF-187 (Figure 2 lane 4) or ICRF-193 (Figure 2, lane 5) even at concentrations of 100 μM. Comparable results were obtained with topoisomerase IIβ (data not shown). Overall these data show that only F 11782 and *not* either of the ICRF compounds is capable of completely inhibiting the DNA-binding activity of topoisomerase II.

In vitro cytotoxic effects of combinations of either F 11782 or each of the bisdioxopiperazines with etoposide

Jensen and Sehested[23] pointed out that any compound interfering with the catalytic cycle of topoisomerase II, so as to reduce the amount of available targets of cleavable complex-stability drugs, such as etoposide,

Table 1. Comparative effects of these catalytic inhibitors on topoisomerase II-induced DNA decatenation and on stabilization of topoisomerase II-mediated cleavable complex formation

Assay procedure	Test compound (μM)		
	F 11782	ICRF-193	ICRF-187
Decatenation inhibition[a] (IC_{50})			
Catalyzed by			
topoisomerase IIα	1.9	12	48
topoisomerase IIβ	1.3	34	44
Inhibition of catalytic activity[b] using nuclear extracts of P388 cultured cells (IC_{50})	3.6[c]	3.4	18
Evaluation of topoisomerase II-mediated cleavage formation *in vitro*[d]	none at $\leqslant$100	none at $\leqslant$100	none at $\leqslant$100
Stabilization of topoisomerase–DNA cleavable complex in P388 cultured cells[e]	none at $\leqslant$100	none at $\leqslant$100	none at $\leqslant$100

[a]Decatenation of kDNA by recombinant human topoisomerase IIα or IIβ. The IC_{50} values are the mean of three independent assays.
[b]Decatenation of kDNA using crude nuclear extracts.
[c]Data taken from Perrin *et al.*[14]
[d]The protocol for assessing topoisomerase II-mediated cleavage is detailed in Materials and methods.
[e]The protocol for assessing the stabilization of cleavable complex in cultured P388 cells is described in Materials and methods.

has the potential to antagonize the cytotoxicity of these drugs. Using the L1210 murine leukemia cells *in vitro*, the cytotoxic effects of etoposide alone or in combination, for a 2-h drug exposure, with a single minimally cytotoxic concentration, i.e. that resulting in less than 20% growth inhibition, of ICRF-193 (4.2 μM) or ICRF-187 (320 μM) were quantitated. A comparison of the experimental and calculated theoretical additivity curves is shown in Figure 3. These data provide clear evidence of such antagonism by both ICRF-193 and ICRF-187, with IC_{50} values for the association being 5.7 and 7.4 μM, respectively, relative to the etoposide IC_{50} value of 2 μM. In contrast, as reported earlier,[15] F 11782 (1.8 μM) significantly enhanced the cytotoxic effects of etoposide alone, with a greater than additive effect represented by an IC_{50} value of 1.1 μM for the association. Clearly these data are suggestive of a distinctive mechanism of action for F 11782 relative to these ICRF topoisomerase II catalytic inhibitors.

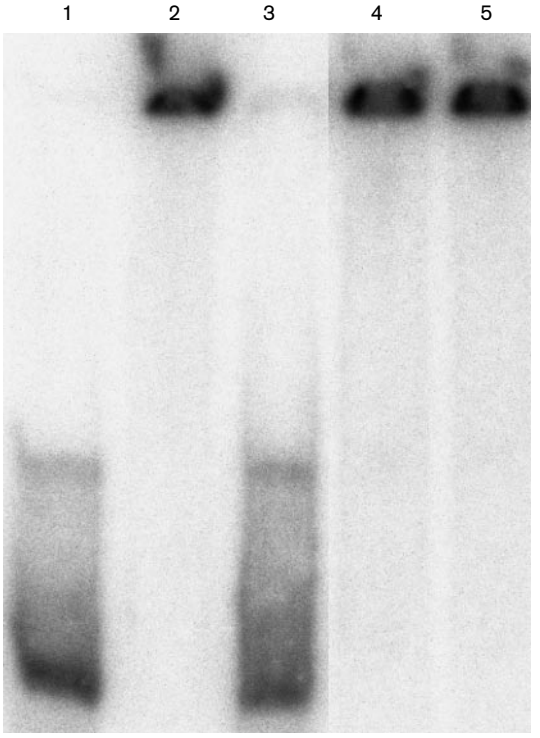

Figure 2. The effects of F 11782, ICRF-187 or ICRF-193 on the binding of topoisomerase IIα to DNA, as determined by gel shift assays. ^{32}P-labeled DNA probe (lane 1) was incubated with topoisomerase IIα in the absence (lane 2) or in the presence of F 11782 (lane 3), ICRF-187 (lane 4) or ICRF-193 (lane 5) at 100 μM.

Examination of DNA damage induction

The DNA-damaging activities of these three catalytic inhibitors of topoisomerase II were first evaluated using GCT27 human cells and a fluorescence enhancement assay. Data shown in Figure 4 clearly identify DNA damage induction by F 11782 at concentrations ranging from 0.1 to 10 μM, following a 4-h incubation, confirming our earlier preliminary report.[24] In contrast, no significant DNA damage was detected with the ICRF compounds, except at the highest concentration tested of 100 μM, where there was some evidence of a slightly increased rate of unwinding, perhaps though merely reflecting cell death.

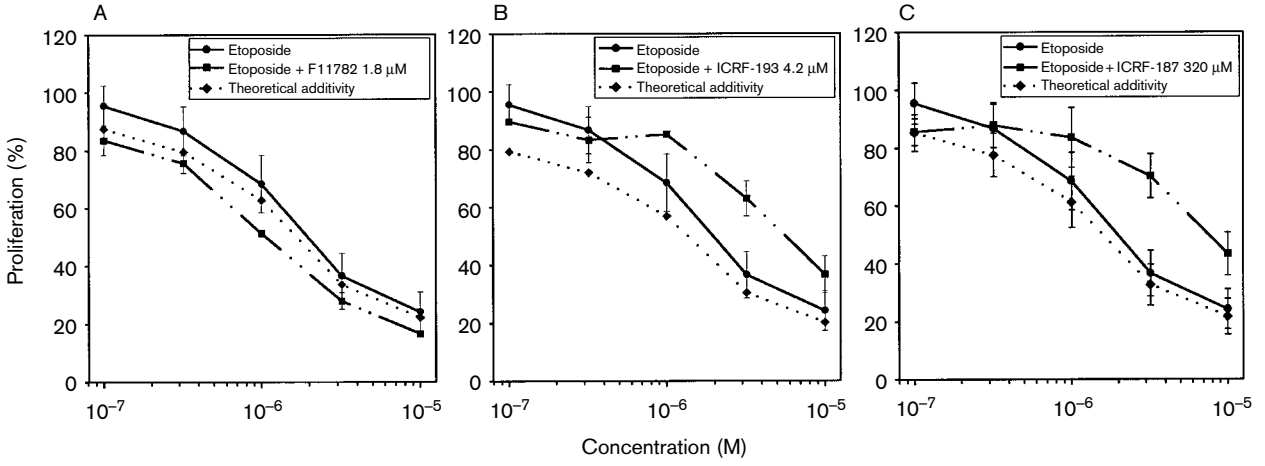

Figure 3. Relative effects of F 11782, ICRF-187 or ICRF-193 on the etoposide-induced inhibition of proliferation of L1210 leukemia cells *in vitro*. Cells were incubated for 2 h with increasing concentrations of etoposide alone (●) or associated (■) with a minimally cytotoxic concentration, i.e. that resulting in less than 20% growth inhibition when tested alone, of the associated test drug: (A) F 11782 at 1.8 μM, (B) ICRF-187 at 320 μM and (C) ICRF-193 at 4.2 μM. Cell proliferation was determined after a 48-h growth period without drug(s) by cell counting. TA curves (dotted line) are included for comparison. TA curves were calculated using the formula detailed in Materials and methods. Bars, standard deviation from at least three independent experiments. Data relating to F 11782 are taken, with permission, from Etiévant *et al.*[15]

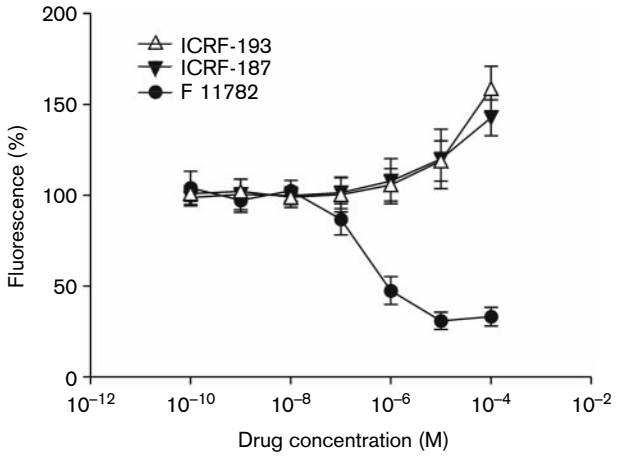

Figure 4. Evaluation of the induction of DNA damage in cultured GCT27 human teratoma cells using a Picogreen fluorescence enhancement assay. Data shown were obtained following a 24-h incubation at 37°C in the presence of F 11782 (●), ICRF-187 (▼) or ICRF-193 (△). Each point represents the mean ± standard error of at least three independent experiments.

Consistent with these observations there was an induction of p53 in these GCT27 cells after a 24-h incubation with F 11782, but this was not apparent following exposure to either ICRF-187 or ICRF-193 (see Table 2). This induction of p53 by F 11782 reached a plateau at a level 3-fold higher than that of the controls.

These data provide evidence that the cytotoxic effects of F 11782 are mediated, at least in part, via DNA damage induction. In this respect, F 11782 differs

Table 2. Evaluation of DNA damage induction in GCT27 cells, exposed to test compounds *in vitro* for 24 h.

Test compound	p53 expression level relative to control [concentration tested (μM)]			
	1	10	32	100
F 11782	1.9 ± 0.1	3.0 ± 0.5	2.8 ± 0.2	NE
ICRF-187	NE	1.1 ± 0.4	NE	1.4 ± 0.4
ICRF-193	1.1 ± 0.3	1.6 ± 0.4	NE	1.6 ± 0.3

NE, not evaluated.

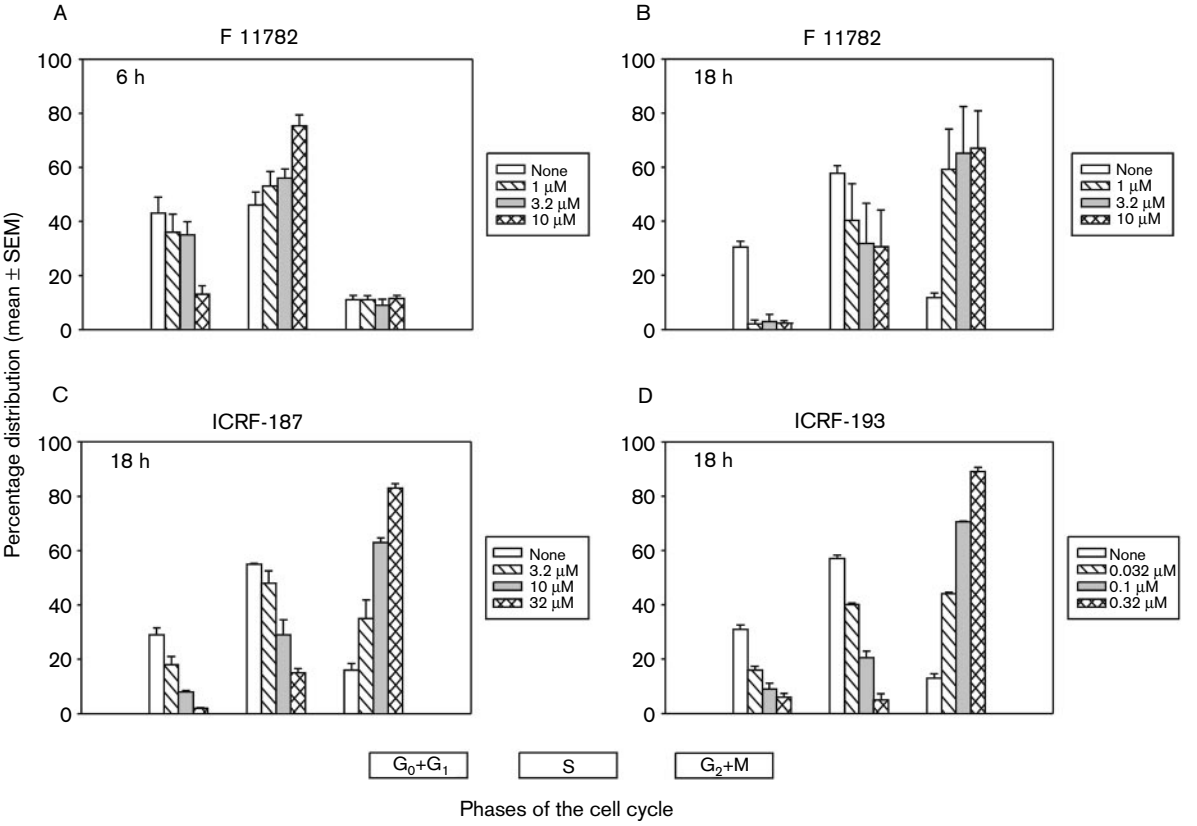

Figure 5. Cell cycle distribution of P388 murine leukemia cells after either 6 or 18 h of incubation *in vitro* with test compound. Cells were exposed to the specified drug concentration for the indicated time: (A) 6 h of exposure to F 11782, (B) 18 h of exposure to F 11782, (C) 18 h of exposure to ICRF-187 and (D) 18 h of exposure to ICRF-193. Then, the percentage of cells in the various phases of the cell cycle was determined by flow cytometry as detailed in Materials and methods. Each point represents the mean of at least three independent experiments.

quite clearly from the bisdioxopiperazine catalytic inhibitors of topoisomerase II.

Effects of catalytic inhibitors on cell cycle distributions

The results of flow cytometric analyses of the cellular DNA contents of P388 leukemia cells indicated that a 6-h incubation with F 11782 induced a limited dose-dependent accumulation of P388 cells in S phase, as reflected by the proportions of F 11782-treated cells of 53–75% in this phase, compared to 46% of the controls, representing a 163% increase relative to the control value (Figure 5A). However, this effect proved transient since on prolonging the exposure duration to 18 h, cells accumulated in the G_2/M phases of the cycle again in a dose-dependent manner (Figure 5B), with 50–59% of F 11782-treated cells versus only 11% of controls being distributed in the G_2/M phases, representing a maximal increased proportion of 536% over the control value. This increased proportion of cells in the G_2/M phases was associated with corresponding decreases in the G_0/G_1 and S phase populations (Figure 5B), the latter being especially marked, consistent with a definite G_2/M blockade. In contrast, similar experiments with ICRF-193 and ICRF-187 revealed evidence of merely a G_2/M block following an 18-h incubation period (Figure 5C and D). Such effects have been reported earlier[11,25–28] for a number of bisdioxopiperazine derivatives, although the precise location of the blockade within these cell cycle phases appears debatable, depending at least to some extent on the cell type studied.

Prolongation of the exposure duration times to 40 h and use of the lower concentrations tested, so as to minimize cell death evident by the apoptotic population identified, provided evidence of polyploidization resulting with ICRF-193 and ICRF-187 (Figure 6) in terms of the appearance of a 8*N* subpopulation, confirming earlier published data.[11] However, no such effects were noted with F11782 (Figure 6).

In vitro cytotoxicities against a panel of murine and human tumor cell lines

The data in Table 3 indicate that, generally, an intermediate sensitivity was expressed to F 11782 relative to either ICRF-187, to which cells were least responsive, or to ICRF-193, to which cells proved most sensitive. Thus overall, the three leukemia cell lines

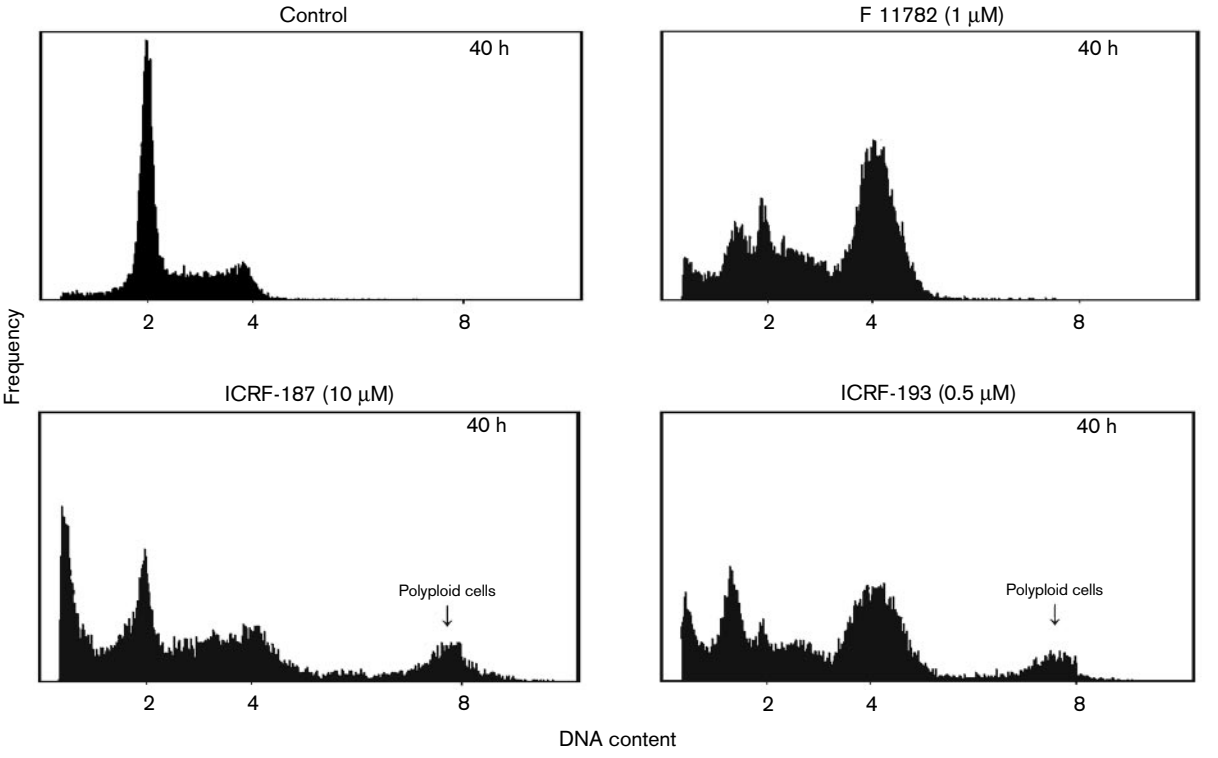

Figure 6. Effects of F 11782, ICRF-187 or ICRF-193 on the cell cycle distribution of P388 leukemic cells after an *in vitro* incubation of 40 h. Cells were exposed to solvent (control) or to the specified concentration of each test compound for 40 h. Samples were taken and DNA analyzed as described in the legend to Figure 2. Peaks of polyploid populations are indicated.

Table 3. Cytotoxicity of F 11782 against a panel of murine and human tumor cell lines: comparison with ICRF-187 and ICRF-193

Cell line tested	Origin/tumor histology	IC_{50} values[a] (μM)		
		F 11782	ICRF-187	ICRF-193
Leukemia cell lines[b]				
L1210	murine	0.20	5.90	0.06
P388	murine	1.30	9.50	0.58
CEM	human	6.90	150	2.30
Human 'solid' tumor cell lines[c]				
DLD-1	colon/adenocarcinoma	160	~1000[d]	1.60
GCT27	testicular/teratoma	0.21	100	0.64
GLC4	lung/small cell carcinoma	3.80	17.0	0.02
LoVo	colon/adenocarcinoma	5.90	31.0	0.82
MX-1	breast/carcinoma	39.0	87.0	3.20
SK-OV-3	ovary/adenocarcinoma	190	~1000[d]	10.0
DU145	prostate/adenocarcinoma	130	43	~100[d]
T24	bladder/transitional cell carcinoma	120	140	~100[d]

[a]IC_{50} values represent concentrations required to inhibit cell growth by 50% and were derived from pooled data obtained from at least three independent experiments.
[b]Cells were exposed to test compound for 48 h. Then, for L1210 or CEM cells, cell numbers were counted (Coulter counter), and for P388 cells, cell growth was determined using a standard MTT-based colorimetric assay.
[c]Cells were exposed to test compound for 72 h. Then cell growth was determined using a standard MTT-based colorimetric assay, except for the GLC4 cells for which cell numbers were counted (Coulter counter).
[d]Concentrations are only approximate since the quantity of compound available for testing was limited.

tested and five of the 'solid' tumor cell lines proved more sensitive to F 11782 than to ICRF-187 by a factor ranging from 2- to 30-fold and yet showed considerably less sensitivity than to ICRF-193 (2- to 100-fold). Exceptionally, GCT27 human teratoma cells proved most sensitive to F 11782, whilst DU145 (prostate) and T24 (bladder) carcinoma cells showed relatively comparable sensitivities to all three agents, under these experimental conditions.

Comparison of *in vivo* antitumor activities of F 11782 and the ICRF bisdioxopiperazines using the murine P388 leukemia and B16 melanoma experimental models

The marked *in vivo* antitumor activity of F 11782 has been well documented.[29,30] In these present studies, we have confirmed the high level of activity of F 11782 over a range of single doses administered by the i.p. route against the i.v.-implanted P388 murine leukemia (Table 4). In contrast, no activity was recorded for either ICRF-187 or ICRF-193, under identical test conditions. In the s.c.-grafted B16 murine melanoma, F 11782 showed significant activity both in terms of prolonging overall survival and in inhibiting tumor growth, following administration i.p. as multiple injections over a 2-week period at doses of 40, 80 and 160 mg/kg/injection.[30] Evaluation of one of these bisdioxopiperazines, ICRF-187, concurrently using an identical treatment schedule, provided no evidence of any survival benefit. Even at the maximal tolerated dose of 160 mg/kg/injection of ICRF-187, the optimal T/C ratio achieved was only 46%, failing to reach significance according to NCI criteria for a solid tumor.[33]

Therefore, whilst F 11782 shows marked *in vivo* antitumor activity as a single agent in these experimental murine models, this contrasts with the overall lack of activity of these bisdioxopiperazines tested under comparable experimental conditions.

Discussion

This novel epipodophylloid, F 11782, has been reported earlier[14] as a potent inhibitor of the catalytic activity of topoisomerase II, being considerably more potent than etoposide and its derivative GL-331, as well as other dual topoisomerase inhibitors including aclarubicin, intoplicin and TAS-103.[15] This study confirms the superior catalytic inhibitory properties of F 11782, relative to both ICRF-193 and ICRF-187, against both topoisomerase IIα and IIβ (Table 1 and Figure 1). In agreement with these results obtained with purified enzymes, all three of these catalytic inhibitors also proved active in inhibiting the catalytic activities of topoisomerase II in a mixture of nuclear

Table 4. Comparison of the antitumor activities of F 11782, ICRF-187 and ICRF-193, given i.p. in a single dose against the i.v.-implanted P388 murine leukemia

Test compound	Dose range tested (mg/kg)	Optimal dose (mg/kg)	Body weight change[a] (%) [day]	Presumed drug-related deaths[b] (%)	Optimal T/C[c] (%)	Optimal activity rating[d]
F 11782[e]	40–320	320	−5.6 [8]	0	400	H
ICRF-187	10–160	–	gain	0	100	inactive
	640	–	–	100		toxic
ICRF-193	40–160	–	–9.0 [4]	0	100	inactive

[a]Body weight changes reported are maximal weight losses expressed as a percentage of the initial body weight. According to NCI criteria, a dose is considered toxic if the induced body weight loss is greater than 15% of the initial weight.[31] No body weight loss was recorded for the control animals.
[b]A death was presumed drug-related if it preceded the first death in the control group.
[c]T/C = (median survivial of the drug-treated group/median survival of the control group) × 100.
[d]According to the NCI standard criteria for the P388 tumor model, 120% ⩽ T/C < 175% is the minimal level for activity (L) and T/C ⩾ 175% corresponds to a high level of antileukemic activity (H); '0' represents a T/C value of < 120%.[32]
[e]Data reported here for F 11782 originated from Kruczynski *et al.*[30]

enzymes extracted from cultured P388 leukemic cells. Confirming earlier published data,[11,12,22,26] and consistent with their role as catalytic inhibitors of topoisomerase II, F 11782, ICRF-193 and ICRF-187 neither induced cleavage formation using isolated purified enzyme nor stabilized topoisomerase II–DNA cleavable complexes in P388 cultured cells (Table 1).

This overall inhibition of the catalytic activity of topoisomerase II by F 11782 has been ascribed previously[14] to what appears to be an original mechanism, i.e. an inhibition of the direct interaction between DNA and this nuclear enzyme. Under identical experimental conditions, using a gel shift assay, absolutely no such interference was observed with the bisdioxopiperazines (Figure 2). Similar negative data have been reported earlier for etoposide,[14] as well as for the dual catalytic inhibitor aclarubicin.[15]

Topoisomerase II catalytic inhibitors such as aclarubicin and ICRF-187 are considered to reduce the amount of available target of complex-stabilizing inhibitors, such as etoposide, either by interfering with the DNA and the binding of topoisomerase II or by trapping the enzyme fixed to DNA, thus having the potential to antagonize the cytotoxic activities of such drugs.[16,34–36] A clear confirmation of this antagonism was obtained when the *in vitro* cytotoxic effects of etoposide on L1210 cells were evaluated in the presence of ICRF-187 and similar antagonistic effects resulted when ICRF-193 was tested (Figure 3). An earlier study using this same test system had also revealed antagonism with the combination of aclarubicin and etoposide.[15] In contrast, however, the combination of F 11782 and etoposide had produced significantly enhanced cytotoxicity relative to etoposide alone.[15] These contrasting data therefore appear suggestive of distinctive mechanisms of action for F 11782 as opposed to other known catalytic inhibitors of topoisomerase II, and so caution against generalizing any concept for combining catalytic inhibitors and topoiosmerase II poisons, as suggested earlier.[37]

Another clear difference between F 11782 and the bisdioxopiperazines was shown in terms of their abilities to induce DNA damage. Evidence that the cytotoxic effects of F 11782 are mediated, at least in part, via DNA damage induction is presented in Figure 4, confirming an earlier preliminary report,[24] and this is backed up by its induction of p53 (Table 2), while no such effects resulted with either of the ICRF compounds at concentrations of 100 μM or less. The induction of p53 is one of the major cellular events in response to DNA-damaging agents,[38] yet has not been identified with the bisdioxopiperazines.[11,39] Indeed, bisdioxopiperazines are generally considered to be non-DNA-damaging agents[40] that sequester topoisomerase II in the closed clamp formation and inhibit enzyme activity inside the cell.[41] More recently, it has been argued that the closed clamp form of the enzyme, induced by these ICRF compounds, when trapped on DNA is sufficient to interfere with DNA metabolism and cause cell killing.[42]

Flow cytometry studies also provided evidence of differing effects on cell cycle distributions resulting from exposure to F 11782 or to either of the ICRF compounds. A transient dose-dependent accumulation of F 11782-treated cells in S phase preceded a general G_2/M blockade (Figure 5), which was maintained even when prolonging the exposure duration to 40 h (Figure 6). Similar experiments with ICRF-193 and ICRF-187, though, confirmed the G_2/M blockage reported by others[11,25,26,28] and provided evidence of polyploidization after prolonged 40-h exposures (Figure 6), as described in earlier publications.[25–28]

Finally, whilst a panel of murine and human tumor cell lines *in vitro* generally showed a relative intermediate sensitivity to the cytotoxic effects of F 11782, proving more resistant to ICRF-187 and most sensitive to ICRF-193 (Table 3), this same pattern was not reflected in terms of their *in vivo* antitumor activities against the two experimental murine tumors tested. As described earlier,[29,30] F 11782 exhibited a high level of antitumor activity against both the i.v.-implanted P388 murine leukemia, when administered as a single i.p. dose, and the s.c.-grafted B16 murine melanoma, following multiple injections over a 2-week period.These two experimental tumors are generally considered as relatively refractory to standard cytotoxic agents. Indeed, ICRF-187 tested concurrently showed an overall lack of any significant antitumor activity in either model.

In conclusion, overall these data are consistent with our earlier claim[14] that F 11782 is a novel catalytic inhibitor of topoisomerase II with an original mechanism of action. Its precise mode(s) of interaction with this nuclear enzyme remain to be defined, but this appears to be quite distinctive from that elucidated for the bisdioxopiperazines. These interesting mechanistic properties, coupled with the major *in vivo* experimental antitumor activities identified for F 11782, have provided the impetus for its current preclinical development.

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