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A dual mechanism of action of the anticancer agent F 11782 on human topoisomerase II α

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Abstract

F 11782 is a novel epipodophyllotoxin that targets eukaryotic topoisomerases and inhibits enzyme binding to DNA. While F 11782 has not been found to stabilize either topoisomerase I or topoisomerase II covalent complexes, drug treatment appears to result in DNA damage. F 11782 has also been shown to inhibit the DNA nucleotide excision repair (NER) pathway. Bisdioxopiperazine-resistant small cell lung cancer (SCLC) OC-NYH/Y165S and Chinese hamster ovary (CHO) CHO/159-1 cells having functional Y49F and Y165S mutations in the topoisomerase II α isoform were both resistant to F 11782. The catalytic activity of purified human Y50F and Y165S mutant topoisomerase II α (Y50F in the human protein corresponds to Y49F in the CHO protein) was likewise resistant to the inhibitory action of F 11782. F 11782 was also found to induce a non-covalent salt-stable complex of human topoisomerase II with DNA that was ATP-independent. F 11782 thus displays a dual mechanism of action on human topoisomerase II α, reducing its affinity for DNA while also stabilizing the protein bound in the form of a salt-stable complex. Our results suggest that topoisomerase II α is a target of F 11782 *in vivo*, and that F 11782 may act as a novel topoisomerase II poison.

Keywords: Topoisomerase II; Epipodophyllotoxin; Chemotherapy; DNA damage; Surface plasmon resonance; Mutation

1. Introduction

Type II topoisomerases are nuclear enzymes that pass one DNA double helix through a transient break in another DNA molecule changing the linking number of super coiled DNA by steps of two [1]. This DNA strand passage activity is required in a number of DNA metabolic processes including DNA replication, transcription, chromosome condensation and de-condensation [2–4]. Topoisomerase I generates transient single strand breaks in DNA allowing the release of positive and negative super coils, and is also required for various DNA metabolic processes [4]. The transient intermediate of the topoisomerase reaction where topoisomerase is covalently bound to DNA and the DNA is cleaved is termed the cleavage complex, and drugs that lead to an increase in the levels of this complex have been termed topoisomerase poisons [5,6]. While different classes of structurally unrelated compounds such as the epipodophyllotoxins, anthracyclines and aminoacridines act as topoisomerase II poisons, topoisomerase I specific poisons are mainly restricted to campthothecin and its analogs [5].

Catalytic topoisomerase inhibitors act at stages in the catalytic cycle where DNA is not cleaved [7]. Catalytic inhibitors of eukariotic topoisomerase II have been described that act by several different mechanisms. Merbarone inhibits DNA cleavage by the enzyme [8]. Aclarubicin alters DNA structure and inhibits binding of

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Abbreviations: AMPPNP, 5′-adenylylimidodiphosphate; BSA, bovine serum albumin; CHO, Chinese hamster ovary; EDTA, ethylenediaminetetraacetic acid; F 11782, 2′′,3′′-bispentafluorophenoxyacetyl-4,6′-ethylidene-beta-D-glucoside of 4′-phosphate-4′-dimethylepipodophyllotoxin 2*N*-methyl glucamine salt; ICRF-187, (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane; ICRF-193, meso-4,4′-(2,3-butanediyl)-bis-(2,6-piperazinedione); kDNA, kinetoplast DNA; SPR, surface plasmon resonance.

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topoisomerase II to DNA [9,10]. Maleimide alkylates thiol-reactive cysteines in topoisomerase II, thereby inactivating the enzyme [11]. Both aclarubicin and maleimide act on diverse cellular targets, while merbarone has relatively low potency against topoisomerase II. The bisdioxopiperazine compounds, which stabilize a closed clamp intermediate of the topoisomerase II reaction, and inhibit enzymatic turnover are the most commonly used catalytic inhibitors of topoisomerase II, since these compounds are relatively potent, and specific for topoisomerase II [7]. Compounds that block topoisomerase I catalytic activity have also been identified, but catalytic inhibition of topoisomerase I by a small molecule has not been demonstrated as a cytotoxic mechanism in eukaryotic cells [12].

There has been substantial interest in compounds that may act against both type I and type II topoisomerases. Several compounds that fall into this category have been identified, including actinomycin D, intoplicine, and aclarubicin [13–16]. An interesting aspect of aclarubicin is that it acts as a topoisomerase I poison, and as a topoisomerase II catalytic inhibitor [16]. Another dual inhibitor with a unique mode of action is the epipodophyllotoxin derivative F 11782 or tafluposide (Fig. 1). This compound is a potent catalytic inhibitor of both topoisomerase I and II *in vitro*. F 11782 inhibits the binding of both topoisomerase I and II to DNA in a dose-dependent manner [17], but does not intercalate into DNA [18]. While F 11782 does not lead to detectable stimulation of topoisomerase cleavage complexes, in intact cells, it is capable of generating DNA strand breaks [19,20]. F 11782 and etoposide together act synergistically in producing DNA damage, a result that is inconsistent with F 11782 being a catalytic inhibitor of topoisomerase II *in vivo* [18]. In addition to its inhibitory activities against topoisomerase I and II, F 11782 has recently been shown to inhibit the NER pathway *in vitro* [21], and the observed synergism of F 11782 and DNA alkylating agents in the P388 murine leukemia model suggests that this may also be the case *in vivo* [22]. The action of F 11782 against multiple targets has precluded a precise identification of the major mechanisms responsible for cell killing by this agent.

The present work describes experiments designed to identify mechanisms of inhibition of F 11782 against human topoisomerase II α. OC-NYH/Y165S [23] and CHO/159-1 [24] cells, which are resistant to bisdioxopiperazines due to functional mutations in the topoisomerase II α isoform were cross-resistant to F 11782. The catalytic activity of purified human topoisomerase II α carrying the corresponding mutations (Y165S and Y50F) was also resistant to F 11782. We also observed that F 11782 could trap topoisomerase II in the form of a non-covalent salt-stable complex. As previously reported, F 11782 reduced DNA binding by the enzyme. We thus found that F 11782 has two distinct mechanisms of action on human topoisomerase II α. These results suggest that topoisomerase II α is a biological target for F 11782 in mammalian cells and that F 11782-mediated cytotoxicity may be caused in part by the stabilization of topoisomerase II complexes on DNA.

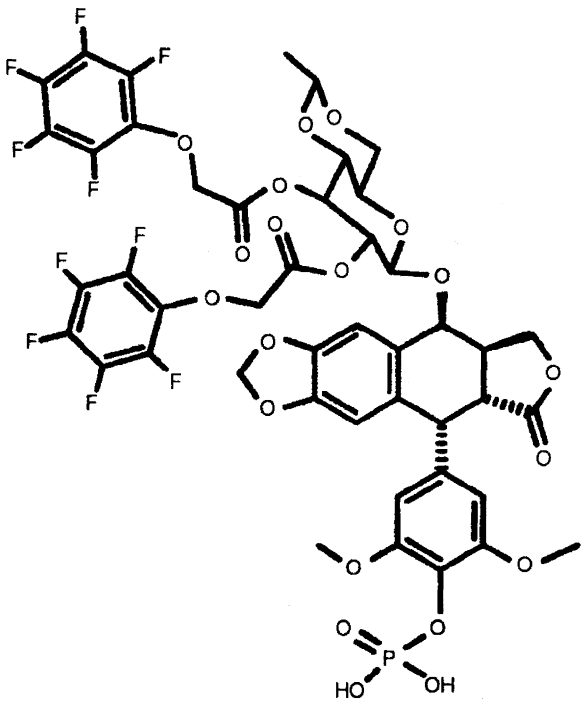

Fig. 1. Structural formula of F 11782 or 2″,3″-bispentafluorophenoxyacetyl-4,6′-ethylidene-beta-D-glucoside of 4′-phosphate-4′-dimethylepipodophyllotoxin 2*N*-methyl glucamine salt, recently renamed tafluposide.

2. Materials and methods

2.1. Drugs

ICRF-187 (Cardioxane; ICRF-187 hydrochloride salt, Chiron Corp.) and AMPPNP (Sigma) were dissolved in sterile distilled water. F 11782 provided by Pierre Fabre Medicament was dissolved in distilled sterile water when used in Biacore applications and in DMSO for all other applications.

2.2. Cell lines

The small cell lung cancer SCLC cell line OC-NYH and its bisdioxopiperazine-resistant sub-line OC-NYH/Y165S are described in [23]. The CHO cell line and its bisdioxopiperazine-resistant sub-line CHO/159-1 have been described in [24].

2.3. Clonogenic assay

Cell survival was assessed using a 3-week clonogenic assay using soft agar on a sheep red blood cell feeder layer as previously described [25].

2.4. Purification of human topoisomerase II α

The purification of wild-type and mutant human topoisomerase II α from yeast cells was carried out as described in [26] with modifications described in [27].

2.5. Decatenation assay

Tritium-labeled kDNA was isolated from *Crithidia fasciculata* as described earlier [28]. Topoisomerase II catalytic activity was measured using a kDNA decatenation assay as described in [11] with minor modifications. Briefly, 200 ng ^{3}H-labeled kDNA isolated from *C. fasciculata* were incubated with increasing concentrations of drug in 20 μL reaction buffer containing 10 mM Tris–HCl pH 7.7, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 15 μg/mL BSA and 1 mM ATP using one unit of purified wild-type or mutant topoisomerase II α for 20 min at 37° (where one unit of activity is defined as the amount of enzyme required for complete decatenation in the absence of drug in 20 min). After addition of stop buffer (5% sarkosyl, 0.0025% bromophenol blue and 50% glycerol), unprocessed kDNA network and decatenated DNA circles were separated by filtering, and the amount of unprocessed kDNA in each reaction was determined by scintillation counting. The values were finally normalized, where 100% catalytic inhibition corresponds to the radioactivity retained on the filter when no enzyme is applied.

2.6. Topoisomerase retention on DNA/streptavidin beads

When performing six reactions, 30 μL M280 streptavidin coated beads (Dynal A/S) slurry corresponding to 300 μg beads was transferred to a 1.5 mL tube that was then placed in a Dynal magnetic particle concentrator (MPC-E) rack (Dynal A/S) for 1–2 min until the beads had settled on the tube wall. The beads were then washed twice in the DNA binding solution supplied with the kilo base binding kit (Dynal A/S) by repeating this step. Finally, the beads were resuspended in 120 μL DNA binding solution. A preparation of biotin-labeled plasmid DNA containing a 5-kb super coiled circular DNA molecule carrying eight successive peptide nucleic acid (PNA) linked biotin labels at one known position (pGeneGrip biotin blank vector, Gene Therapy Systems Inc.) was made by mixing 105 μL distilled water and 15 μL biotinylated DNA. After mixing the beads and the DNA preparation, the sample was left overnight at room temperature under gentle agitation to assure optimal formation of the Dynabeads DNA complex. Next the complex was washed twice in 480 μL wash buffer (10 mM Tris–HCl pH = 7.5, 2 M NaCl, 1 mM EDTA), once in distilled water, and once in topoisomerase reaction buffer (10 mM Tris–HCl pH 7.7, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 15 μg/mL BSA). Next, the beads were resuspended in 480 μL topoisomerase II buffer and divided into six tubes. 80 μL reactions containing plasmid DNA coated Dynabeads, topoisomerase II buffer, 1 mM ATP (or 4 μM AMPPNP, Sigma), 1 μg purified human topoisomerase II α diluted to 100 ng/μL in topoisomerase II reaction buffer and drugs were incubated for 30 min at 37°. Next, each reaction mix was washed six times in 500 μL 1 M KCl containing the same drug (or AMPPNP) concentration used during the previous incubation by applying the Dynal MPC as described above. After the last wash, the tubes were centrifugated at 20,000 *g* for 1 min, and excess washing solution was removed. Next, 20 μL loading buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 5 mM EDTA) was added and the samples were boiled for 10 min and subjected to SDS–PAGE using a 7.5% separation gel with a stacking gel. As a positive control, 1 μg human topoisomerase II α was always included. As negative control a "no drug sample" was always included. After electrophoresis at 15 V/cm for 60 min the gel was washed three times in 50 mL distilled water and stained using gel code staining reagent (Pierce) as described by the manufacturer, and the gel was photographed.

2.7. Surface plasmon resonance assay

Assessment of binding of human topoisomerase II α to circular DNA in the presence of increasing concentrations of F 11782 and assessment of salt-stable complex of wild-type and mutant human topoisomerase II formed on DNA was carried out by SPR using a Biacore 3000 (Biacore Inc.). A detailed description of this assay has been published elsewhere [29]. Briefly, the pGeneGrip biotin blank vector described in the section above was allowed to bind to streptavidin coated sensor chips (Sensor Chip SA, Biacore Inc.). Next, 10 nM wild-type or mutant human topoisomerase II α in 10 mM Tris–HCl pH 7.7, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 30 μg/mL BSA, 1 mM ATP was allowed to bind DNA on the sensor chip at a flow rate of 30 μL/min at 20°. For detection of inhibition of DNA binding by F 11782 the association of topoisomerase II and DNA was followed for 2 min in the presence of different concentrations of F 11782. For the detection of salt-stable complex, the level of DNA-bound protein resistant to 1 M KCl was determined. Another flow cell containing no DNA was used to correct for refractive index changes and non-specific binding of topoisomerase II α to the sensor chip.

3. Results

3.1. Bisdioxopiperazine-resistant OC-NYH/Y165S and CHO/159-1 cells are cross-resistant towards F 11782

A bisdioxopiperazine-resistant sub-line of the SCLC cell line OC-NYH was described in [23]. OC-NYH/Y165S cells

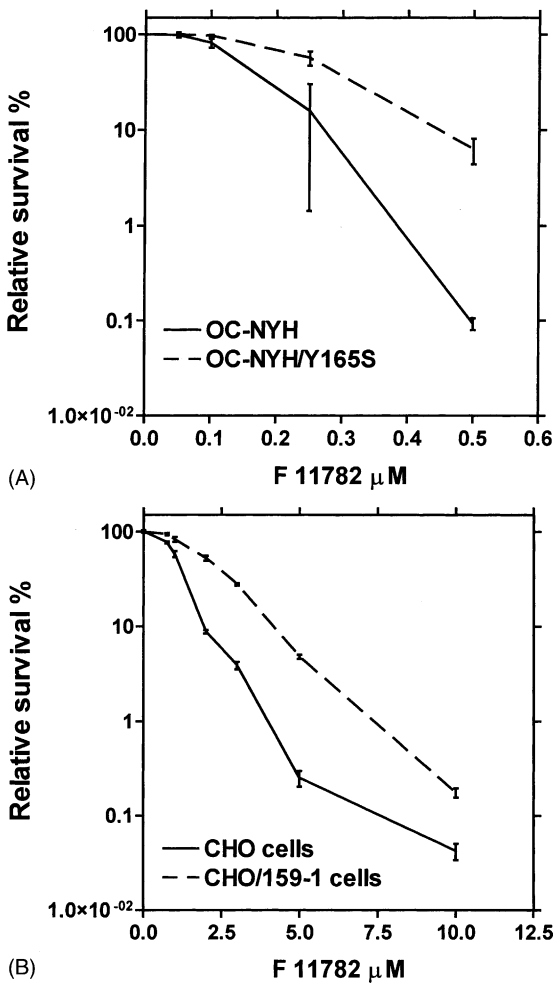

Fig. 2. Bisdioxopiperazine-resistant OC-NYH/Y165S (A) and CHO/159-1 (B) cells are cross-resistant to F 11782. A 3-week clonogenic assay using continuous drug exposure was used as described in Section 2 and references herein. Error bars represent SD of two independent experiments.

are highly resistant towards ICRF-187 showing more that 30-fold resistance towards this drug. These cells carry a heterozygous Y165S mutation in the topoisomerase II α isoform, but have no change in topoisomerase II α and β levels. Furthermore, OC-NYH/Y165S cells do not have elevated levels of multidrug resistance protein 1 (MDR1), multidrug resistance-related protein (MRP) and mitosantrone resistance protein (MXR) mRNA. The morphology and doubling time of OC-NYH/Y165S cells are identical to that of parental OC-NYH cells. OC-NYH and OC-NYH/Y165S cells were assessed for sensitivity towards F 11782 using a 3-week continuous exposure clonogenic assay. Fig. 2A shows the result of clonogenic assays. The OC-NYH/Y165S cells display a significant resistance to F 11782 being 1.7 cross-resistant in terms of IC_{50} values. Previous results strongly suggested that the bisdioxopiperazine resistance was mediated by the identified point mutation in topoisomerase II α [23]. The observed cross-resistance to F 11782 is consistent with topoisomerase II α acting as an *in vivo* target of this drug. To examine this further, a second bisdioxopiperazine-resistant cell line was also examined (Fig. 2B). This cell line, CHO/159-1 is also resistant to bisdioxopiperazines in part due to a mutation in topoisomerase II α [24] being 12.6-fold cross-resistant towards ICRF-187 and 19.6-fold cross-resistant towards ICRF-159. The CHO-159 cell line carries a mutation changing tyrosine 49 to phenylalanine (corresponds to Y50F in human topoisomerase II α). Unlike the OC-NYH/Y165S line, the CHO-159 cells also have alterations in topoisomerase II expression; with the topoisomerase II α expression level reduced 40–50%, while the topoisomerase II β expression level is elevated 10–20% [24]. As was seen with the OC-NYH/Y165S cells above, CHO/159-1 cells are also significantly cross-resistant towards F 11782 (1.9-fold in terms of IC_{50} values, see Fig. 2B). Thus, two independently selected bisdioxopiperazine-resistant cell lines are both cross-resistant towards F 11782. Although the ICRF/159-1 and OC-NYH/Y165S cell lines are clearly less resistant towards F 11782 than towards bisdioxopiperazine compounds, this result is consistent with topoisomerase II α being a *in vivo* target of F 11782.

3.2. The catalytic activity of bisdioxopiperazine-resistant human topoisomerase II α protein is resistant to F 11782

Attempts to study the effect of F 11782 on the viability of yeast cells functionally expressing wild-type, Y50F and Y165S human topoisomerase II α which could have illuminated the role of the mutations in F 11782 cytotoxicity failed, because the drug did not produce a cytotoxic response even when using drug permeable cells. We therefore instead examined the inhibitory effect of F 11782 on the catalytic activity of purified Y50F and Y165S human topoisomerase II α. Previous experiments had shown that the mutant proteins had the same catalytic activity as the wild-type enzyme, and that the decatenation activity was highly resistant towards bisdioxopiperazines. As shown in Fig. 3, Y165S protein was also resistant to F 11782. The IC_{50} for the Y165S protein for F 11782 was approximately 15–20 μM vs. less than 5 μM for the wild-type enzyme. A similar level of resistance was seen with human topoisomerase II α carrying the Y50F mutation (data not shown). The tyrosine 50 to phenylalanine mutation is homologous to the tyrosine 49 to phenylalanine mutation that occurs in the CHO/159-1 cell line described above. The level of cross-resistance towards F 11782 seen with purified Y50F and Y165S mutant proteins is comparable to the level of cross-resistance observed for the two resistant cell lines analyzed. Taken together, these results are consistent with topoisomerase II α playing a role in the F 11782 resistance observed with the two bisdioxopiperazine-resistant cell lines.

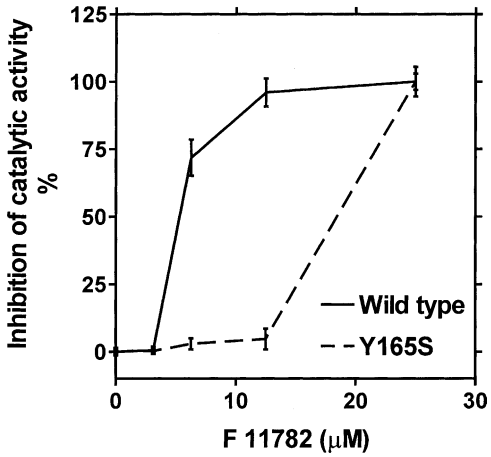

Fig. 3. The catalytic activity of Y165S mutant human topoisomerase II α is cross-resistant to F 11782. One unit of wild-type or mutant human topoisomerase II α was incubated with increasing concentrations of F 11782 and the radioactivity derived from unprocessed kDNA retained on filters was measured and used to create dose–response curves, as described in Section 2. Error bars represent SD of three independent experiments.

3.3. F 11782 induces a salt-stable complex of human topoisomerase II α on DNA in an ATP-independent manner

The results above suggest that topoisomerase II is an important *in vivo* target of mammalian topoisomerase II, and that alterations resulting in bisdioxopiperazine resistance also result in F 11782 resistance. We next addressed the nature of the inhibition of topoisomerase II by F 11782. Morris *et al*. described an assay for detecting the trapping of topoisomerase II by bisdioxopiperazines using biotinylated, covalent closed DNA bound to streptavidin beads [30]. Human topoisomerase II α protein was bound to the DNA coated beads, and in the absence of any drug, all of the binding was reversed by washing the beads with 1 M KCl (Fig. 4A, lane 1). However, binding was stable with respect to incubation with 1 M KCl when 4 μM AMPPNP or 200 μM ICRF-187 plus 1 mM ATP was included (Fig. 4A, lanes 2 and 3, respectively). F 11782 also trapped the protein as a salt-stable complex on DNA when the drug was present at a concentration of 3 μM (Fig. 4, lane 4), although the level of salt-stable complex which could be induced by F 11782 was clearly less than could be induced by ICRF-187. F 11782 concentrations above 3 μM had an inhibitory effect on the formation of the salt-stable complex (Fig. 4A, lanes 5 and 6), consistent with previous work showing that F 11782 inhibits the binding of topoisomerase II to DNA [17,18]. In order to establish a dose–response relationship for the formation of the salt-stable complex seen at 3 μM F 11782, we examined a lower range of F 11782 concentrations. A salt-stable complex was evident in the presence of 300 nM F 11782 (Fig. 4B, lanes 1–6). Interestingly, the presence of ATP had no effect on the formation of the salt-stable complex (Fig. 4C, lanes

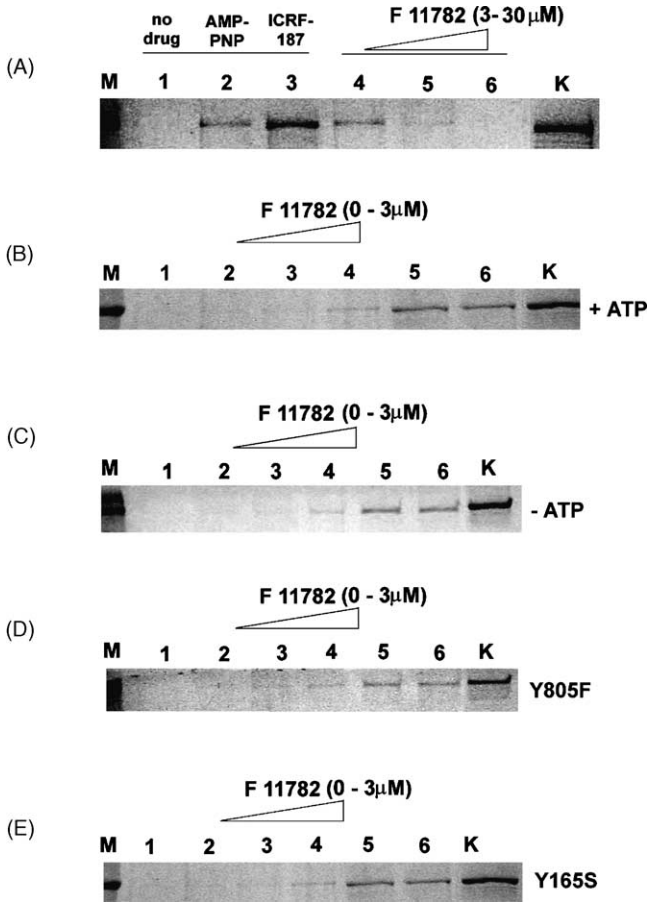

Fig. 4. F 11782 induces a salt-stable complex of human topoisomerase II α on DNA. Magnetic beads covered with circular DNA plasmid molecules were allowed to bind human topoisomerase II α in the presence or absence of drug. The amount of human topoisomerase II α retained on the beads after six washes at high salt concentration (1 M KCl) was then detected by SDS–PAGE after staining the gels using gel code staining reagent (Pierce). Contrast enhancement was used in the visualization of the protein bands (Adobe Photoshop 4.0). (A) Induction of salt-stable complex of human topoisomerase II α on DNA by AMPPNP, ICRF-187 and high concentrations of F 11782. M, molecular marker; lane 1, no drug; lane 2, 4 μM AMPPNP; lane 3, 200 μM ICRF-187; lane 4, 3 μM F 11782; lane 5, 10 μM F 11782; lane 6, 30 μM F 11782 and K, 1 μg human topoisomerase II α. (B) Induction of salt-stable complex of human topoisomerase II α on DNA by low F 11782 concentrations. M, molecular marker; lane 1, no drug; lane 2, 30 nM F 11782; lane 3, 100 nM F 11782; lane 4, 300 nM F 11782; lane 5, 1 μM F 11782; lane 6, 3 μM F 11782 and K, 1 μg human topoisomerase II α. (C) Induction of salt-stable complex of human topoisomerase II α on DNA by low F 11782 concentrations in the absence of ATP. Drug treatments are as in part B. (D) Induction of salt-stable complex of active site tyrosine Y805F human topoisomerase II α on DNA by low F 11782 concentrations. Drug treatments are as in part B. (E) Induction of salt-stable complex of mutant Y165S human topoisomerase II α by low F 11782 concentrations. Drug treatments are as in part B.

1–6), while ATP was required to induce a salt-stable complex in the presence of ICRF-187 (data not shown). In order to determine whether the salt-stable complex was due to DNA cleavage, we examined complex formation using a human topoisomerase II α mutant where the active site tyrosine involved in the transesterification reaction

had been changed to phenylalanine (Y805F). This mutant protein cannot cleave DNA, but can form a stable closed clamp in the presence of AMPPNP or bisdioxopiperazines plus ATP [29,31,32]. As shown in Fig. 4D, the Y805 mutant forms a salt-stable complex to an approximately equivalent level to that formed with the wild-type protein (Fig. 4B shows the results obtained for wild-type topoisomerase II, Fig. 4D shows the results obtained with the Y805F mutant). This result excluded DNA cleavage as being responsible for the salt-stable complex. We finally assessed the ability of the Y165S protein to form the salt-stable complex on DNA in the presence of F 11782 (Fig. 4E). No difference in the ability of F 11782 to induce the salt-stable complex was recorded between wild-type and Y165S protein despite the fact that F 11782 more efficiently inhibits the catalytic activity of the wild-type protein (Fig. 3). For all human topoisomerase II α proteins assessed, the maximal level of salt-stable complex that could be induced by F 11782 was always much less than the total amount of input protein, and also much less than could be induced by the bisdioxopiperazine ICRF-187. Apparently, the abrogation of DNA binding by F 11782 precludes the formation of high levels of the salt-stable complex, i.e. the protein can only be trapped when bound to DNA. In order to study the interaction of wild-type and mutant topoisomerase II α with DNA in the presence of F 11782 in more detail we next applied the SPR assay.

3.4. F 11782 inhibits the binding of human topoisomerase II α to DNA in a dose-dependent manner and stabilizes a salt-stable protein complex at low concentration

We recently developed a SPR assay for measuring the association of topoisomerase II with DNA [29]. We showed that this assay could detect the salt-stable complex formed by topoisomerase II on covalently closed DNA in the presence of non-hydrolysable ATP analogs, or in the presence of bisdioxopiperazines and ATP. We therefore applied the SPR assay to study the effects of F 11782. Fig. 5 illustrates a typical sensorgram obtained following incubation with human topoisomerase and increasing concentrations of F 11782, and shows that this compound inhibits the binding of human topoisomerase II α protein to DNA, and induces a salt-stable topoisomerase II complex on DNA resistant to 1 M KCl. Equal amounts of human topoisomerase II α were allowed to bind to DNA coated sensor chips in the presence of increasing concentrations of F 11782 at time zero. In the absence of F 11782 human topoisomerase II α binds to DNA increasing the RU to above 1000 at the end of the association phase. When F 11782 is also present, less protein binds to DNA during the association phase. The inhibition of binding is dose-dependent with almost complete abrogation of binding at 50 μM F 11782. The results of three independent experiments are summarized in Table 1. In the absence of drug 995 ± 75 RU of human topoisomerase II α were bound to DNA while only 70 ± 30 RU were bound in the presence of 50 μM F 11782. The concentrations of F 11782 required to inhibit binding to DNA in the present study is in agreement with published data [17], where 10 μM F 11782 was also found to inhibit efficiently the binding of human topoisomerase II α to DNA as assessed using a gel shift assay. Interestingly,

Table 1
The effect of F 11782 concentration on the binding of wild-type human topoisomerase II α to DNA

F 11782 concentration (μM)	Binding (RU) ± SD
0	995 ± 75
5	850 ± 90
10	185 ± 5
25	123 (N = 1)
50	70 ± 30

SPR measurements were performed as described in Section 2. The binding values correspond to the amount in RU of wild-type human topoisomerase II α that binds DNA after a 2 min injection of 10 nM protein in the presence of 1 mM ATP and in the absence or the presence of increasing concentration of F 11782; N = 3 except where indicated.

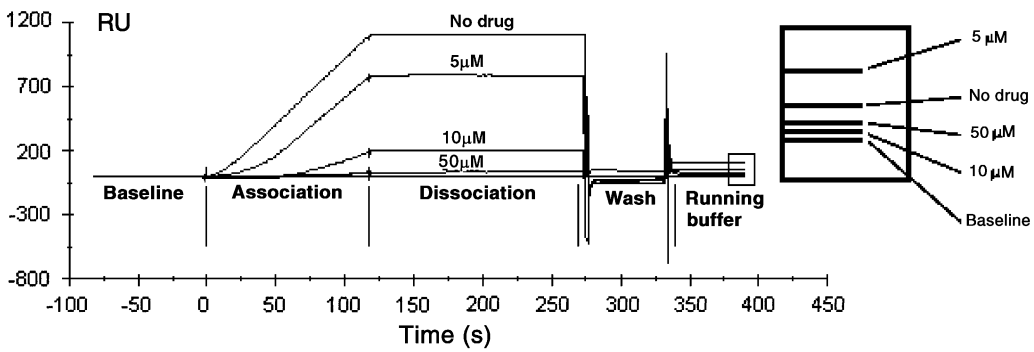

Fig. 5. F 11782 inhibits the binding of human topoisomerase II α to DNA and also stabilizes a salt-stable complex at low F 11782 concentrations. The protein was allowed to bind to DNA coated sensor chip in the absence of drug or with increasing concentrations of F 11782. A dose-dependent inhibition of binding is observed with almost complete inhibition of binding at 50 μM F 11782. The amount of protein that stays on the DNA after a 1 min wash at 1 M KCl (high salt wash) can be read from the last part of the graph which is enlarged. 5 μM F 11782 increases the total amount of protein bound while 10 and 50 μM F 11782 does not increase the total amount of protein bound above the level seen in the absence of any drug.

while the absence of ATP and the active site Y805F mutation had no significant effect on the inhibitory effect of F 11782 on protein binding to DNA (data not shown), the Y165S mutation significantly decreased the efficiency with which F 11782 inhibited protein binding to DNA in two independent experiments. Thus, 70 ± 30 RU of wild-type protein was bound to DNA at 50 μM F 11782 at the end of the association phase while the amount was 135 ± 25 concerning the Y165S protein. This result points to the possibility that the Y165S mutation causes its F 11782-resistant phenotype at least in part by decreasing the ability of F 11782 to compete with DNA binding. In Fig. 5, the association phase is followed by a dissociation phase where running buffer with no topoisomerase II or drug is applied to the DNA coated chip. The observation that human topoisomerase II α stays bound during the dissociation phase indicates that this protein binds DNA with very high affinity as previously described [29]. The amount of human topoisomerase II α protein associated with DNA increases during this phase at the high F 11782 concentration (50 μM). This may be explained by F 11782 dissociating from the protein thus allowing binding to DNA. Dissociation is terminated by a one min high salt wash (1 M KCl) that is included to determine any possible stabilization of the topoisomerase II/DNA interaction by F 11782. The actual level of salt-stable complex can be determined from the last part of the curve after 1 M KCl has been replaced by running buffer (denoted running buffer on the graph). When looking at the enlargement box it is evident that 5 μM of F 11782 induces a higher level of salt-stable complex of human topoisomerase II α on DNA than is seen in the absence of drug, although the complex stabilizing effect is relatively small, which agrees with data obtained using the bead assay in the previous section showing that complex formation is reduced above 3 μM. At higher F 11782 concentrations no increase in RU above what is seen in the absence of drug is recorded. The SPR assay thus confirms the results from the bead assay that low concentrations of F 11782 induce a salt-stable complex. We detected a F 11782-mediated increase in the total amount of salt-stable protein complex in two independent experiments.

4. Discussion

In the present study, we have investigated the role of topoisomerase II α as a potential target for the epipodophyllotoxin F 11782. Our results demonstrate that cell lines that are resistant to bisdioxopiperazines due to topoisomerase II α mutations are also resistant to F 11782. The catalytic activities of the topoisomerase II α expressed in bisdioxopiperazine-resistant cell lines were also resistant to F 11782, suggesting that the mechanism of resistance to both bisdioxopiperazines and F 11782 was due to the topoisomerase II mutation.

Bisdioxopiperazines trap topoisomerase II as a salt-stable closed clamp on DNA [29,30,33]. The closed clamp form of topoisomerase II occurs during the normal topoisomerase II reaction cycle. Clamp closure occurs when the enzyme binds a nucleotide triphosphate, and clamp opening is coincident with hydrolysis and release of the nucleoside di-phosphate and inorganic phosphate. Inability of the enzyme to hydrolyze the nucleotide triphosphate results in trapping of the enzyme as a closed clamp. The closed clamp form of the enzyme is quite stable as seen by the ability to purify this form of the enzyme in the presence of high salt concentrations. Our results reported here with F 11782 resemble what is observed with bisdioxopiperazines, with several important differences. The critical difference is that a stable complex can be seen even in the absence of a nucleotide triphosphate. A possible explanation for this observation is that F 11782 induces a conformational change resembling the ATP bound form of the enzyme. A drug-induced conformational change into the closed clamp form could partly also explain the inhibition of DNA binding by F 11782. Roca and Wang showed that topoisomerase II could form a closed clamp in the absence of DNA [34]. If the closed clamp forms, then the enzyme can subsequently bind to linear but not circular DNA. However, since Hill and co-workers examined F 11782 using linear oligonucleotide substrates [17], formation of a closed clamp is unlikely to be the only explanation for inhibition of DNA binding by F 11782.

An alternate explanation for the occurrence of a salt-stable complex in the presence of F 11782 is that the complex is a covalent complex, with topoisomerase II covalently bound to DNA. Hill and co-workers failed to observe any stimulation of topoisomerase II-mediated DNA cleavage by F 11782. In addition, high salt typically reverses the topoisomerase II DNA covalent complex, although there are notable exceptions, including etoposide [35]. To test whether DNA cleavage could partly explain the observed salt-stable complex, we examined complex formation using a human topoisomerase II mutant protein that is unable to cleave DNA due to a mutation in the active site tyrosine (Y805). Since the levels of salt-stable complex with this mutant protein in the presence of F 11782 were similar to those seen with the wild-type protein, we can conclude that DNA cleavage plays no role in the complex we observe.

Although we favor a model in which F 11782 traps topoisomerase as a closed clamp, we cannot exclude the possibility that the ternary complex of drug, DNA, and enzyme is some different complex, perhaps one that does not normally occur during the enzyme reaction cycle. We also cannot unequivocally conclude that the complex formation plays an important role in cell killing. Firstly, the level of salt-stable complex that can be obtained with F 11782 is much less than the level of salt-stable complex that can be formed by the bisdioxopiperazines as assessed in both the bead assay (this study) and SPR assay (this study and [29]), which is probably due to the fact than F

11782 inhibits the binding of topoisomerase II to DNA leaving less protein available for complex formation. Secondly, the finding that the presence of catalytically F 11782-resistant mutant topoisomerase II α in cells is associated with cellular drug resistance suggests that inhibition of topoisomerase II catalytic activity in cells contribute to cytotoxicity. On the other hand, the detection of DNA damage in cells treated with F 11782 [36], and the finding that the drug is cytotoxic at much lower concentrations than is required for inhibition of binding of topoisomerases (I, II α and β) to DNA and inhibition of topoisomerase catalytic activity (I, II α and β) *in vitro* ([17] and present study), suggests that the complex could well play a role in mediating the cytotoxic effect of F 11782, despite the fact that we detected no reduction in the level of F 11782-induced salt-stable complex with the Y165S mutant *in vitro*. Our results suggest that F 11782 targets human topoisomerase II *in vivo*, and that cell killing depends on the action of this drug against topoisomerase II. We have observed that F 11782 targeting of topoisomerase II appears to be distinct from previously identified topoisomerase II inhibitors. While F 11782 inhibits binding of the enzyme to DNA, the drug can also stimulate the formation of a complex that does not depend on the ability to cleave DNA. The formation of complexes in an ATP independent fashion distinguishes this agent from bisdioxopiperazines. The promising activity of this agent as seen in pre-clinical models [37] suggests that there may be several distinct ways that a drug can interfere with topoisomerase II and generate an antitumor response.

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