Decayed Nucleotide Excision Repair Activity and Alterations of Topoisomerase IIα Are Associated with the in Vivo Resistance of a P388 Leukemia Subline to F11782, a Novel Catalytic Inhibitor of Topoisomerase I and II

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ABSTRACT

Purpose: The purpose of the study was to investigate the mechanisms associated with antitumor activity and resistance to F11782, a novel dual catalytic inhibitor of topoisomerase II with DNA repair-inhibitory properties.

Experimental Design: For that purpose, an F11782-resistant P388 leukemia subline (P388/F11782) has been developed in vivo and characterized.

Results: Weekly subtherapeutic doses of F11782 (10 mg/kg) induced complete resistance to F11782 after 8 weekly passages. This resistant P388/F11782 subline retained some in vivo sensitivity to several DNA-topoisomerase II and/or I complex-stabilizing poisons and showed marked collateral sensitivity to cisplatin, topotecan, colchicine, and Vinca alkaloids, while proving completely cross-resistant only to merbarone and doxorubicin. Therefore, resistance to F11782 did not appear to be associated with a classic multidrug resistance profile, as further reflected by unaltered drug uptake and no overexpression of resistance-related proteins or modification of the glutathione-mediated detoxification process. In vivo resistance to F11782 was, however, associated with a marked reduction in topoisomerase IIα protein (87%) and mRNA (50%) levels, as well as a diminution of the catalytic activity of topoisomerase IIα. In contrast, only minor reductions in topoisomerase IIβ and I levels were recorded. However, of major interest, nucleotide excision repair activity was decreased 3-fold in these P388/F11782 cells and was more specifically associated with a decreased (67%) level of XPG (human xeroderma pigmen-
tosum group G complementing protein), an endonuclease involved in this DNA repair system.

Conclusions: These findings suggest that both topoisomerase IIα and XPG are major targets of F11782 in vivo and further demonstrate the original mechanism of action of this novel compound.

INTRODUCTION

DNA topoisomerase inhibitors represent a major class of anticancer agents with documented activities against a broad spectrum of human malignancies (1). However, the emergence of tumor cell drug resistance remains a major clinical problem and the frequent cause of failure in the long-term efficacy of cancer therapy. Therefore, one goal of experimental oncology is to understand the underlying mechanisms responsible for this phenomenon, with the hope that more effective therapies can be devised.

Extensive experimental research has shown that cellular resistance mechanisms to topoisomerase inhibitors are generally multifactorial (2–5). Three major categories have been identified: (a) pretarget events, including drug uptake, metabolism, and intracellular distribution; (b) drug-target interactions; and (c) post-target events, including macromolecular syntheses, DNA repair, cell cycle progression, and regulation of cell death (4). Earlier studies identified a novel epipodophyllotoxin dual inhibitor of topoisomerases I and II, namely, F11782 (2’,3’-bispentafluorophenoxyacetyl-4’,6’-ethyldene-β-D-glucoside of 4’-phosphate-4’-demethyllepipodophyllotoxin, N-methyl glucamine salt) with different mechanistic properties from most specific inhibitors of topoisomerases I or II (6). Indeed, F11782 exhibits distinct mechanisms of action against human topoisomerases; it appears to act by preventing the binding of topoisomerase to DNA through a preferential interaction with the enzyme (6), but it is also capable of trapping topoisomerase II in an ATP-independent noncovalent salt-stable complex that does not depend on the ability to cleave DNA (7). Furthermore, F11782 does not intercalate into DNA, and it does not stabilize DNA-topoisomerase cleavable complexes. However, F11782 was also found to create DNA damage in intact cells that differs from that mediated by classical topoisomerase poisons by increasing with time and being less efficiently repaired (8, 9). Subsequent studies provided evidence that F11782 is also a potent inhibitor of nucleotide excision repair (NER; Ref. 10). Furthermore, F11782 has major antitumor activity against a panel of experimental tumor models with different biological properties and chemosensitivities (11, 12). Therefore, this original mechanistic profile of F11782, coupled with the definite and broad spectrum of antitumor activity of F11782, prompted consideration of F11782 for clinical development.

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To investigate the mechanisms associated with antitumor activity and resistance to F11782, an F11782-resistant P388 leukemia subline (P388/F11782) has been developed in vivo and characterized. Data reported here describe the establishment of the F11782-resistant P388/F11782 subline; its in vivo profile of sensitivity/cross-resistance to a series of antitumor agents, including topoisomerase inhibitors, DNA cross-linking agents, antimetabolites, and tubulin-interacting agents; and some of its biochemical properties. More specifically, potential modification of drug uptake and resistance-related protein expression, as well as alterations in topoisomerase expression, activity, sequence and NER, were investigated in the F11782-resistant P388/F11782 subline.

MATERIALS AND METHODS

Drug Preparations. For in vivo evaluations, F11782, recently termed tafltuposide [Pierre Fabre (Fig. 1)], was solubilized in a solution containing 4.75% glucose, 5% Tween 80, and 90.25% water. The following group of drugs tested were obtained from various sources as indicated: 1,3-bis(2-chloroethyl)-1-nitroso-urea [BCNU (Bristol-Myers Squibb)]; cisplatin, colchicine, cyclophosphamide monohydrate, doxorubicin hydrochloride, and 5-fluorouracil (Sigma); mitoxantrone dihydrochloride (Lederle); and etopophos, TAS-103 dichlorhydrate or 6-[(2-dimethylamino)-ethyl]amino]-3-hydroxy-7H-indeno(2,1-c)quinolin-7-one, teniposide, topotecan acetate, vinblastine sulfate, vinflunine, or 2',20'-difluoro-3',4'-dihydrvinorelbine sulfate and vinorelbine ditartrate from Pierre Fabre were all solubilized in a saline solution containing 5% Tween 80. Solubilization of test compounds was performed immediately before use, and administration was performed at 10 ml/kg. For in vitro evaluations, F11782, etoposide, and ICRF-193 (Pierre Fabre) were solubilized in DMSO to achieve a final concentration of 1% DMSO in the final reaction volume.

Mice and Tumor Model. Female DBA/2 (DBA/2Jic; Iffa Credo, L’Arbresle, France) and hybrid CDF1 (CD2F1/CrlBR; Charles River, St Aubin-les-Elbeuf, France) mice were used for tumor model propagation and experimental chemotherapy, 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 authorized investigators. The parental P388 murine leukemia was obtained from the Division of Cancer Treatment, Tumor Repository of the National Cancer Institute (Frederick, MD).

In Vivo Establishment of the P388/F11782 Subline, Resistant to F11782. Viral-free parental P388 leukemia cells were maintained by serial weekly i.p. implantation into DBA/2 mice. For establishment of the F11782-resistant subline, the parental P388 cells were implanted i.p. at 10⁶ cells/mouse into a group of five mice on day 0. These mice were then treated with 10 mg/kg F11782 administered i.p. on day 1. When regrowth was evident, after approximately 1 week, leukemic cells from these F11782-treated mice were reimplanted in vivo, and the mice were again dosed i.p. the following day with 10 mg/kg F11782. The F11782-resistant P388/F11782 subline was thus developed over successive in vivo transplant generations.

Experimental Chemotherapy. All experiments were conducted in compliance with French regulations and Centre de Recherche Pierre Fabre ethical committee guidelines, based on the United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia, as detailed previously (13). A total of 10⁴ P388 or P388/F11782 cells were implanted i.v. into CDF1 mice on day 0 (except for merbarone evaluations, for which the i.p. implanted P388 model was used because merbarone was inactive against the i.v. implanted P388 model). After their randomization in treatment cages, test compounds were administered to mice i.p. as a single dose, the day after tumor implantation. In each chemotherapy trial, mice were checked daily, and any adverse clinical reactions were noted, and deaths were recorded. Mice were weighed twice weekly during treatment and once weekly thereafter.

Evaluation of Antitumor Activity. An increase of life span (ILS) was defined as follows: ILS (%) = T/C – 100, with the T/C (%) ratio representing (median survival of treated mice/median survival of control mice) × 100. The optimal ILS corresponds to the maximal value or to the maximal ILS achieved. According to National Cancer Institute criteria for the P388 model, 20% ≤ ILS < 75% is judged as being the minimum level for activity, and an ILS of ≥75% is judged as corresponding to a high level of antileukemic activity (14).

The areas under the survival curve as a function of dose (i.e., Areaₐ and Areaₐ₋) have been calculated using the trapezium method (Sigma Plot; Jandel Corp.) for the parental (F11782-sensitive) P388 and F11782-resistant P388/F11782 leukemic cells, respectively. The relative resistance (RR), reflecting the chemotherapeutic response of the resistant subline vis-à-vis the sensitive one, was defined as follows: RR = Areaₐ₋/Areaₐ, with a RR value of 0 representing total resistance of the F11782-resistant subline to the compound tested, a RR value of 1
corresponding to similar sensitivity of both lines, and a RR value of >1 reflecting collateral enhanced sensitivity of the F11782-resistant subline.

**Drug Uptake.** P388 and P388/F11782 cells were removed from the peritoneal cavities of tumor-bearing mice and maintained in suspension culture in RPMI 1640 supplemented with 10% heat-inactivated horse serum and 4 mM L-glutamine, all purchased from Seromed, together with 100 units/liter penicillin and 1.25 μg/ml fungizone (Invitrogen). For *in vitro* uptake studies, 10 nmol of [14C]F11782 (0.488 μCi) were added to a cell suspension of P388 or P388/F11782 cells (10^5 cells/ml) incubated at 37°C. At predetermined time intervals, aliquots were removed and subjected to rapid cooling on ice. After centrifugation (2000 g at room temperature through 0.32 M sucrose in PBS to eliminate any contaminating RBCs, and then washed twice in 10 ml of cold PBS. Then, total RNA derived from either P388 or P388/F11782 cells was isolated using TRIzol reagents (Invitrogen), according to the manufacturer’s instructions. mRNA was further isolated from total RNA using a poly(dT) bead-based extraction kit (Dynal). Northern blotting was then performed using a NorthernMax (Clinisciences) kit. Briefly, mRNA was separated in a 1% formaldehyde-dehydrated agarose gel and transferred to a positively charged nylon membrane (Clinisciences). After RNA fixation to hybrid-denaturated agarose gel and transferred to a positively charged nylon membrane (Clinisciences). After RNA fixation to membranes by UV cross-linking, membranes were hybridized at 5°C overnight using topoisomerase II and 5'-GAT-CGG-ATC-CGA-GAA-TGG-1:100; Santa Cruz Biotechnology), or an anti-actin (SC-1616; dilution, 1:100; Santa Cruz Biotechnology), or an anti-actin (SC-1616; dilution, 1:200; Santa Cruz Biotechnology) antibody, followed by a 1-h incubation with appropriate secondary antibody conjugated to peroxidase (Jackson ImmunoResearch Laboratories) before enhanced chemiluminescence detection (Pierce, Interchim, France). Membranes were scanned using a MolecularImager system (Bio-Rad).

**Catalytic Activity of Topoisomerases.** Topoisomerases were extracted from P388 and P388/F11782 cells as follows. Cells removed from the mouse peritoneal cavity were processed as described above and pelleted. The cell pellet was gently resuspended in lysis buffer [20 mM Tris, 1 mM EDTA, 25 mM KCl, 0.5 mM MgCl₂, 250 mM sucrose, and 0.5% NP40 (pH 7.2)] and incubated for 10 min on ice, followed by a 2-min centrifugation at 6000 rpm in a microfuge (Eppendorf 5804R; Merck, EuroLab). The pellet (containing nuclei) was resuspended in 50 μl of extraction buffer [20 mM Tris, 1 mM EDTA, 2 mM DTT, and 400 mM NaCl (pH 7.2)] and incubated for 30 min on ice, followed by a 15-min centrifugation at 14,000 rpm in the same microfuge. The supernatant (containing extracted topoisomerases) was removed, and its protein content was determined by the procedure of Bradford (15) before the addition of 50% (v/v) glycerol and storage at −80°C.

The catalytic activity of topoisomerase II-containing crude extracts was evaluated using a decatenation assay. The total reaction volume was 20 μl, containing 50 mM Tris (pH 8.0), 120 mM KCl, 0.5 mM DTT, 0.5 mM ATP, 10 mM MgCl₂, 30 μg/ml BSA, 0.2 μg kinetoplast DNA (Topogen), the required amount of topoisomerase II-containing crude extract to completely deccatenate 0.2 μg of kDNA, and either the test compound or the solvent alone. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 5 μl of a solution maintained at 4°C containing 50 mM EDTA, 50% glycerol, and 0.25 mg/ml bromphenol blue. Samples were separated through a 1% agarose gel. After staining with ethidium bromide, gels were photographed and counted under UV illumination.

The catalytic activity of topoisomerase I-containing crude extracts was evaluated using a relaxation assay. The total reaction volume was 20 μl, containing 10 mM Tris·HCl (pH 7.9), 0.15 mM NaCl, 1 mM EDTA, 0.1 mM spermidine, 0.1% BSA, 5% glycerol, 0.2 μg pBR322 (Invitrogen), the amount of topoisomerase I-containing crude extract required to completely relax 0.2 μg of pBR322, and either the test compound or the solvent alone. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 5 μl of a solution maintained at 4°C.
containing 50 mM EDTA, 50% glycerol, and 0.25 mg/ml brom-
phenol blue. Samples were separated and processed as described
above.

**Sequencing of Topoisomerase IIα Segments.** Total
RNA was isolated from P388 or P388/F11782 cells, collected
directly from the peritoneal cavities of tumor-bearing mice, and
centrifuged for 5 min at 100 \( \times \) g at room temperature through
0.32 M sucrose in PBS, using TRIZol reagent (Invitrogen). The
reverse transcription was performed in a Genius PCR System
(Techne) using a Superscript II one-step reverse transcription-
PCR kit (Invitrogen) with oligo(dT)\(_{12-18}\), according to the man-
ufacturer’s instructions. The entire topoisomerase IIα cDNA
was amplified and sequenced as follows: cDNA obtained from
5 \( \mu \)g of total RNA from P388 or P388/F11782 cells was used to
perform a PCR with 1 \( \mu \)M primer pairs, 2 units of Taq DNA
polymerase (Invitrogen) 0.2 mM deoxynucleotide triphosphate
in PCR buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], and
1.5 mM MgCl\(_2\). PCR was carried out in 50 \( \mu \)l using the Genius
PCR System (Techne), with an initial denaturation for 4 min at
94°C, annealing for 1 min at 65°C, and extension for 2 min at
72°C; followed by 34 cycles of denaturation for 30 s at 94°C,
annealing for 45 s at 65°C, and extension for 2 min at 72°C; and
finished by 10 min at 72°C. To verify that PCR products were
of the correct length, 5 \( \mu \)l of PCR products were electrophoresed
in TBE buffer (100 mM Tris, 90 mM boric acid, and 1 mM
EDTA) in a 1% agarose-0.5% ethidium bromide gel. Sequenc-
ing of the PCR products was performed using the GeneAmp
PCR System 2400 (Perkin-Elmer) and the ABI Prism BigDye
Terminator Cycle Sequencing Ready Reaction Kit (Perkin-
Elmer). Sequencing of the PCR products was performed on
two different mRNA extraction using primers, with 25 cycles
of denaturation for 10 s at 98°C, annealing for 5 s at 50°C, and
extension for 4 min at 56°C.

**NER Activity Measurement Using Unscheduled DNA
Synthesis.** The method of Bootsma et al. (16) was used to
monitor unscheduled DNA. Briefly, cells were removed from
the peritoneal cavity of tumor-bearing mice and maintained in
suspension culture, as described in “Drug Uptake.” A cell sus-
pension (3 \( \times \) 10^6 cells/ml) was incubated on histological slides
at 37°C in 5% CO\(_2\) in air for 18 h. Medium was then removed,
and after a 4-h incubation at 37°C with or without a range of
concentrations of 10^{-6} to 10^{-4} M mechlorethamine to damage
DNA, cells were incubated in culture medium containing 10 \( \mu \)M
5-fluoro-2'-deoxyuridine and 10 mM hydroxyurea to inhibit
replication and then labeled with 10 \( \mu \)Ci/ml [\( ^3\)H]thymidine.
Cells were washed three times for 20 min with culture medium
containing 200 mM nonradio-labeled thymidine and then fixed
with methanol-acetic acid (3:1) for 15 min and air dried. Slides
were dipped for 10 s into an autoradiographic emulsion (EM-1;
Amersham). After a 5-day exposure in the dark at 4°C, slides
were developed (Kodak), and the nuclei were counterstained
with Mayer’s hemalum solution (Merck). Cells were visualized
under a Zeiss Axioplan microscope combined with a KY-F50
camera (JVC) using a \( \times 40 \) objective. For each image, nuclei
were detected, and the number of nuclear silver grains, reflect-

![Fig. 2](image-url)  
**Fig. 2** Diagrammatic representation of the *in vivo* establishment of the P388/F11782 subline. Parental P388 cells were implanted i.p. into mice on
day 0. These mice were then treated with 10 mg/kg F11782, a suboptimal dose of F11782 administered i.p. on day 1. When regrowth was evident,
after approximately 1 week, ascitic cells from these F11782-treated mice were reimplanted *in vivo*, and the mice were again dosed i.p. the following
day with 10 mg/kg F11782. The F11782-resistant P388/F11782 subline was thus developed over successive *in vivo* transplant generations. During
this period, the level of resistance achieved in the F11782-treated ascitic cells was evaluated by implanting them i.v. and then administering F11782
i.p. at the therapeutic dose of 160 mg/kg to these tumor-bearing mice. Levels of the topoisomerasers were also monitored during this *in vivo*
establishment of F11782 resistance.
ing NER activity, was counted using Histolab software (Microvision).

RESULTS

In Vivo Development of Resistance to F11782 Associated with Alterations in Topoisomerase IIα. Mice bearing P388 leukemia cells implanted in the peritoneal cavity were treated with a single suboptimal dose (10 mg/kg) of F11782 via the i.p. route. After approximately 7 days, ascites fluid from treated mice was transplanted into new hosts i.p., and the treatment process was repeated. During this period, the level of resistance achieved in the F11782-treated ascitic cells was evaluated by implanting them i.v. and then administering F11782 i.p. at the therapeutic dose of 160 mg/kg to these tumor-bearing mice (Fig. 2). The chemosensitivity of i.v. implanted P388 cells to 160 mg/kg F11782 was reflected by an ILS of 200% (Fig. 3A). Successive treatments and transplantations resulted in the progressive development of resistance to F11782, with complete resistance to this dose of 160 mg/kg being achieved in vivo after 8 weeks, as reflected by an ILS of 0% (Fig. 3A). Furthermore, these F11782-resistant cells (designated P388/F11782 cells) also proved completely resistant to a single dose of 320 mg/kg F11782, which was previously identified in mice bearing P388 cells as the maximal tolerated dose, corresponding to its optimal antitumor activity (ILS = 320%; Ref. 11). Under continuous in vivo F11782 selection pressure, this resistance has remained stable (Fig. 3A), and the in vivo growth rate of this P388/F11782 subline was judged to be similar to that of the parental line.

Because topoisomerases I, IIα, and IIβ constitute major in vitro targets for F11782 (6), their levels were monitored during this in vivo establishment of F11782 resistance. The levels of each topoisomerase in P388/F11782 whole cell lysates were calculated as a percentage of the level of expression of that expressed in P388 whole cell lysates, assessed using Western blotting. In vivo resistance to F11782 was associated with a marked and persistent decrease in the level of topoisomerase IIα protein (Fig. 3, B and C). This decreased level proved stable with time because repeated measurements over a 6-month period showed that the topoisomerase IIα level in these P388/F11782 cells remained around 20% of the P388 cell value (data not shown).
not shown). Consistent with these data, a 50% decrease in topoiso-
merase II mRNA was detected in P388/F11782 cells, using a glycer-
aldehyde-3-phosphate dehydrogenase housekeeping gene for normal-
ization (Fig. 4A).

In contrast, the development of resistance to F11782 was asso-
ciated with only an early and transient decrease in the level of topoiso-
merase II and proteins 2 weeks after resistance induction (Fig. 3, B and C). These relative levels of topoiso-
merase II and I increased again from the fourth week after resis-
tance induction to reach a value of 62% and 72%, respectively, of the control value on week 17 after resistance induction. Furthermore, neither topoiso-
merase II nor topoiso-
merase I mRNA level was modified in these stable F11782-resistant cells (i.e., on week 17; Fig. 4, B and C).

**In Vivo Evaluation of Therapeutic Responses of the F11782-Resistant P388/F11782 Subline to a Series of Antitu-
mar Agents.** The activities of several members of each of the major classes of antitumor agents were investigated in vivo against the stable P388/F11782 subline (after 17 transplant generations). The results, expressed as optimal ILS values obtained at the optimal dose of each compound, are listed in Table 1. Although the P388/F11782 cells were totally resistant to F11782, they retained some sensitivity to the topoiso-
merase II poisons etoposide, etopophos, teniposide, and mitoxantrone, with optimal ILS values ranging from 43% to 100%, judged as representative of definite antitumor activity (ILS > 20%) according to National Cancer Institute criteria (14). A high level of sensitivity of P388/F11782 cells to the three topoiso-
merase I poisons tested was also observed, with optimal ILS values of 114–186%. P388/F11782 cells also retained sensitivity to TAS-103 (optimal ILS = 43%), the dual inhibitor of topoiso-
merases I and II. In contrast, P388/F11782 cells showed complete cross-
resistance to the catalytic inhibitor of topoiso-
merase II, mer-
barone, as reflected by an optimal ILS value of 0%. Unfortu-
nately, the evaluation of the chemosensitivity of this F11782-
resistant P388/F11782 subline to dioxopiperazines was not possible because these compounds failed to show any in vivo activity against the parental P388 cell line. Furthermore, P388/ F11782 cells remained highly sensitive to the three DNA cross-
linking agents evaluated, namely, cisplatin, cyclophosphamide, and BCNU, with optimal ILS values of 200–900%, as well as to the four tubulin-interacting agents (optimal ILS of 100–129%). Some sensitivity was also expressed to both 5-fluorouracil and methotrexate (optimal ILS values of 100% and 43%). Finally, it is notable that the F11782-resistant P388/F11782 subline was also completely cross-resistant to doxorubicin, as reflected by an optimal ILS value of 0%.

**In Vivo Comparisons of the Therapeutic Responses of the P388/F11782 and P388 Leukemias to a Series of Antitu-
mar Agents.** In an attempt more accurately to compare the in vivo chemotherapeutic responses of these two leukemia models, an additional parameter taking into account the entire dose-
response (and not merely the optimal activity obtained at the optimal dose) was defined as the area under the survival curve as a function of dose. This parameter was calculated for each compound tested against both parental F11782-sensitive P388 (AreaR) and F11782-resistant P388/F11782 (AreaR) cells. The total resistance of the P388/F11782 subline to F11782 was reflected by an AreaR value close to 0 (i.e., 2), whereas the sensitivity of the parental line to F11782 was represented by an AreaS value of 159 (Fig. 5). Fig. 6 further illustrates this type of analysis with examples of the comparative therapeutic responses of the P388/F11782 and P388 cell lines to cisplatin, etopophos, and doxorubicin. To compare the responses of these two sub-
lines to this series of antitumor agents, a ratio of RR was defined as follows: RR = AreaR/AreaS. An RR value of 0 reflects total cross-resistance of the F11782-resistant subline to the test com-
pound, a value of 1 indicates that both cell lines have similar sensitivities, a value between 0 and 1 reflects partial cross-
resistance, and a value of >1 corresponds to collateral (en-
hanced) sensitivity of the F11782-resistant subline to the test compound. The results of calculating RR ratios for each of the series of antitumor agents evaluated are listed in Table 2. These analyses again highlight that, in vivo, P388/F11782 cells re-
tained some sensitivity to 17 of 19 agents tested and showed complete cross-resistance to only two compounds, namely, mer-

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**Table 1** In vivo evaluation of therapeutic responses of the F11782-resistant P388/F11782 leukemia to a series of antitumor agents, as assessed by increase of life span.

<table>
<thead>
<tr>
<th>Antitumor agents</th>
<th>Optimal ILS (% of dose, mg/kg)</th>
<th>Activity rating</th>
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<td>P388/F11782</td>
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| DNA topoiso-
merase II poisons          |                                 |                 |
| Etopophos                 | 100 (160)                       | High            |
| Etoposide                 | 43 (160)                        | Low             |
| Teniposide                | 100 (40)                        | High            |
| Mitoxantrone              | 100 (20)                        | High            |
| Doxorubicin               | 0 (2.5–10)                      | Inactive        |
| Catalytic inhibitor of topoiso-
merase II                   |                                 |                 |
| Merbarone                 | 0 (10–80)                       | Inactive        |
| DNA topoiso-
merase I poisons          |                                 |                 |
| Camptothecin              | 157 (20)                        | High            |
| Irinotecan                | 114 (160)                       | High            |
| Topotecan                 | 186 (20)                        | High            |
| Dual inhibitor of topoiso-
merases I and II            |                                 |                 |
| TAS-103                   | 43 (80)                         | Low             |
| DNA cross-linking agents   |                                 |                 |
| Cisplatin                 | 900 (10)                        | High            |
| Cyclophosphamide          | 900 (320)                       | High            |
| BCNU<sup>d</sup>          | 200 (160)                       | High            |
| Antimetabolites            |                                 |                 |
| 5-Fluorouracil            | 100 (160)                       | High            |
| Methotrexite              | 43 (160)                        | Low             |
| Tubulin-interacting agents |                                 |                 |
| Colchicine                | 100 (5)                         | High            |
| Vinblastine               | 100 (5)                         | High            |
| Vinflunine                | 114 (40)                        | High            |
| Vinorelbine               | 129 (20)                        | High            |

<sup>a</sup> On day 0, 10⁶ leukemic cells were inoculated i.v. (i.p. for mer-
barone evaluation) into CDF1 mice. Compounds were given i.p. as a single dose on day 1.

<sup>b</sup> ILS (%) = T/C – 100, with T/C = (median survival of drug-
treated group/median survival of control group) × 100. ILS, increase in life span.

<sup>c</sup> According to National Cancer Institute criteria for the P388 tumor model, 20% = ILS < 75% is the minimum level for activity, an ILS of ≥75% corresponds to a high level of antileukemic activity, and an ILS of <20% corresponds to inactivity (11).

<sup>d</sup> BCNU, 1,3-bis(2-chloroethyl)-1-nitroso-urea.
barone and doxorubicin (RR = 0). This in itself appears to represent an unusual cross-resistance profile for this novel agent, F11782. More specifically, this P388/F11782 subline exhibited partial cross-resistance to epipodophyllotoxin-type topoisomerase II poisons, mitoxantrone, and methotrexate, as reflected by RR ratios of 0.3–0.6, yet retained sensitivity to the topoisomerase I poison camptothecin and to the dual topoisomerase inhibitor TAS-103, as reflected by RR values of 1.1 and 0.9. Interestingly, however, marked collateral sensitivity was noted to cisplatin (a DNA cross-linking agent), topotecan (a topoisomerase I poison), and vinorelbine, vinblastine, and colchicine (tubulin-interacting agents), as reflected by RR ratios of 2.2–4.5. In addition, moderate collateral sensitivity of this P388/F11782 subline was noted to cyclophosphamide, irinotecan, BCNU, vinflunine, and 5-fluorouracil, with RR values ranging from 1.3 to 1.6.

Drug Uptake and Expression of Resistance-Related Proteins in the F11782-Resistant P388/F11782 Subline. The pattern of in vitro uptake and accumulation with time of \(^{14}C\)F11782 by the P388/F11782 cells was shown to be essentially similar to that obtained with P388 cells (Fig. 7). These data suggest that altered membrane transport does not appear to be involved in resistance to F11782, although it represents one of the mechanisms of cellular resistance to epipodophyllotoxoids. To further investigate whether resistance to F11782 was associated with a classic multidrug resistance phenotype, the level of the transmembrane P-glycoprotein Pgp, as well as that of two other proteins (multidrug resistance-related protein and lung resistance-related protein) frequently overexpressed in multidrug resistance cells (4), was measured by flow cytometry, according to Broxterman et al. (17) and Fujii et al. (18). No overexpression of Pgp, multidrug resistance-related protein, or lung resistance-related protein (data not shown) was detectable in P388/F11782 cells relative to the parental, F11782-sensitive P388 cells. Furthermore, neither increased levels of cellular glutathione nor any modification in glutathione S-transferase activity was identified [performed as described by Etievant et al. (19)], suggesting that resistance to F11782 is not associated with any altered expression of this detoxification process (data not shown).

Decreased Topoisomerase II Catalytic Activity and Sensitivity to F11782 Is Associated with Resistance to F11782. The catalytic activity of topoisomerase II extracted from F11782-resistant P388/F11782 cells was reduced relative to that of P388 cells, as reflected by the amounts of topoisomerase II-containing extracts required to decatenate 200 ng of kDNA (namely, 1.8 and 0.26 µg for F11782-resistant and -sensitive cells, respectively). Furthermore, topoisomerase II extracted from F11782-resistant cells was 11-fold less sensitive to F11782 than the topoisomerase II extracted from P388 cells, whereas the sensitivity of topoisomerase II extracted from resistant cells to etoposide, a topoisomerase II poison, and ICRF-193, a topoisomerase II catalytic inhibitor, was unchanged (Table 3). This finding might explain, at least in part, the complete resistance to F11782 of these cells. The catalytic activity of topoisomerase I extracted from P388/F11782 cells, however, appeared only slightly modified and was increased 2-fold relative to the P388 cells, as reflected by the amounts of topoisomerase I-containing extracts (25 and 52 ng) required to relax 200 ng of pBR322 for F11782-resistant and F11782-sensitive cells, respectively.

A K155N Mutation of Topoisomerase IIα Is Associated with Resistance to F11782. Sequencing of the entire topoisomerase IIα cDNA in P388/F11782 cells revealed a homozygous G→T point mutation at nucleotide 514, leading to a K155N conversion in the ATP binding domain of topoisomerase IIα (Fig. 8). Sequencing of the corresponding region in parental P388 cDNA showed the wild-type sequence only.

Decreased NER Is Associated with Resistance to F11782. F11782 has been identified as a potent inhibitor of NER (10). Furthermore (see above), P388/F11782 cells expressed marked collateral (enhanced) sensitivity to cisplatin, a DNA cross-linking agent inducing DNA damage repaired mainly by NER (20). Comparative measurements indeed revealed a 3-fold decrease of the NER activity of these P388/F11782 cells (Fig. 9). Furthermore, Barret et al. (10) demonstrated that F11782 preferentially inhibited the incision step, rather than the repair synthesis step, of NER. This incision step includes three main activities, namely, DNA damage recognition and helicase and endonuclease activities (21).
ence with either the binding of XPA-RPA (XPA, human xeroderma pigmentosum group A complementing protein; RPA, DNA replication protein) complex to DNA damage or SV40 large T-antigen helicase activity has been shown with F11782 (10), suggesting that the inhibitory activity of F11782 on NER might involve an effect on XPG or ERCC1 (the human DNA excision repair gene cross-complementing Chinese hamster ovary mutant cell lines of complementation group 1)-XPF endonuclease activity. In support of this hypothesis, a decrease of the XPG protein level was identified in P388/F11782 cells, as reflected by a residual XPG level 33% of that of the P388 cells (Fig. 10A). In contrast, no modification of the XPF protein level was observed in F11782-resistant cells (Fig. 10B).

DISCUSSION

F11782 is a novel dual catalytic inhibitor of topoisomerases I and II that exhibits DNA repair-inhibitory properties and does not intercalate into DNA (6, 10). This compound has marked in vivo antitumor activity in a series of experimental tumor models (11, 12) and is now under consideration for Phase I clinical development, as tafluposide. To attempt to identify in vivo the F11782 targets mainly involved in or associated with the antitumor activity of F11782, a P388 leukemia subline resistant to F11782 has been established in vivo and characterized.

The present study has shown that P388 cells treated in vivo by repeated subtherapeutic doses of F11782 developed total resistance to F11782 after eight transplant generations. The resultant P388/F11782 subline exhibits a markedly reduced level of topoisomerase IIα protein and mRNA, which was associated with a decrease of topoisomerase IIα catalytic activity, yet only minimal modifications in the levels of topoisomerase IIβ and I. Furthermore, P388/F11782 cells exhibited a K155N mutation in the topoisomerase IIα isoform, and topo-

![Fig. 6 Comparative in vivo responses of P388 (A) and P388/F11782 (B) cell lines to cisplatin (A1 and B1; relative resistance (RR) AreaR/AreaS ratio = 4.5), etopophos (A2 and B2; RR ratio = 0.6), and doxorubicin (A3 and B3; RR ratio = 0.0). See the Fig. 5 legend.](image)
The fact that F11782-resistant cells showed modifications in the level of expression, sequence, catalytic activity, and drug sensitivity of topoisomerase IIα suggests that, in vivo, this constitutes the major/preferential target of F11782. Indeed, these findings were supported by the recent study of Jensen et al. (7), which demonstrated that cell lines resistant to bisdioxopiperazines, due to topoisomerase IIα mutations, were also resistant to F11782. This research group also showed that F11782 displays a dual mechanism of action on human topoisomerase IIα, reducing its affinity for DNA while also stabilizing the protein bound in the form of an ATP-independent salt-stable complex. Levels of topoisomerase are commonly decreased in topoisomerase poison-resistant cell lines, and these generally show cross-resistance to other topoisomerase poisons (2, 4, 5). The resistance phenotype of non-complex-stabilizing catalytic inhibitors of topoisomerase IIα has more recently been reviewed and revealed certain common features and also differences relative to the complex-stabilizing topoisomerase IIα poisons (2). For example, whereas a leukemia cell line resistant to the catalytic topoisomerase II inhibitor merbarone showed a decreased level of topoisomerase IIα and proved cross-resistant to topoisomerase IIα poisons (21), an increase in topoisomerase IIα

### Table 2: In vivo comparison of the therapeutic responses of P388/F11782 and P388 leukemias to a series of antitumor agents, as assessed by areas under the survival curves (AreaR and AreaS)

<table>
<thead>
<tr>
<th>Antitumor agents</th>
<th>P388/F11782</th>
<th>P388</th>
<th>Relative response</th>
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<tbody>
<tr>
<td></td>
<td>AreaR</td>
<td>AreaS</td>
<td>AreaR/AreaS</td>
</tr>
<tr>
<td>F11782</td>
<td>2</td>
<td>159</td>
<td>0.0*</td>
</tr>
<tr>
<td>DNA topoisomerase II poisons</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Etopophos</td>
<td>49</td>
<td>80</td>
<td>0.6</td>
</tr>
<tr>
<td>Etoposide</td>
<td>48</td>
<td>156</td>
<td>0.3</td>
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<tr>
<td>Mitoxantrone</td>
<td>64</td>
<td>178</td>
<td>0.4</td>
</tr>
<tr>
<td>Teniposide</td>
<td>61</td>
<td>117</td>
<td>0.5</td>
</tr>
<tr>
<td>Doxorubicin</td>
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<td>0.0</td>
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<tr>
<td>Catalytic inhibitor of topoisomerase II</td>
<td></td>
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<tr>
<td>Merbarone</td>
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<tr>
<td>DNA topoisomerase I poisons</td>
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<td>Camptothecin</td>
<td>117</td>
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<tr>
<td>Irinotecan</td>
<td>151</td>
<td>112</td>
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<td>Topotecan</td>
<td>134</td>
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<td>TAS-103</td>
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<td>Antimetabolites</td>
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<td>5-Fluorouracil</td>
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<tr>
<td>Methotrexate</td>
<td>52</td>
<td>92</td>
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<td>Vinblastine</td>
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<tr>
<td>Vinflunine</td>
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<tr>
<td>Vinorelbine</td>
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<tr>
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<th>P388</th>
<th>Relative response</th>
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<tr>
<td></td>
<td>AreaR</td>
<td>AreaS</td>
<td>AreaR/AreaS</td>
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<tr>
<td></td>
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<td>Topoisomerase II</td>
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<tr>
<td>F11782</td>
<td>63.7</td>
<td>5.8</td>
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</tr>
<tr>
<td>Etoposide</td>
<td>85.2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ICRF-193</td>
<td>2.7</td>
<td>3.4</td>
<td></td>
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* On day 0, 10⁶ leukemic cells were inoculated i.v. (i.p. for merbarone evaluation) into CDF1 mice. Compounds were given i.p. as a single dose on day 1.

* Relative response = AreaR/AreaS, with Area being the area under the survival curve, calculated using the trapezium method (Sigma Plot; Jandel Corp.).

* Relative response ratio calculated for F11782 (2/159 = 0.0125) was approximated to 0.

* BCNU, 1,3-bis(2-chloroethyl)-1-nitroso-urea.

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* Catalytic activity of topoisomerase II-containing extracts was assessed by decatenation assay, as described in “Materials and Methods.” Topoisomerase II-containing extracts and 0.2 μg of kDNA were incubated in the presence or absence of test compound at 37°C for 30 min. The reaction was then stopped, and samples were separated on a 1% agarose gel.

* Topoisomerase II was extracted from either F11782-resistant P388/F11782 or parental P388 cells, as described in “Materials and Methods.”

* IC₅₀ = concentration of test compound required to inhibit topoisomerase II-mediated decatenation by 50%.
II poisons etoposide, etopophos, teniposide, and mitoxantrone. They do not interfere with topoisomerase catalytic activity by the same mechanisms, and thus different resistance mechanisms might well be expected to operate. P388/F11782 cells, which are resistant to F11782, a catalytic inhibitor of topoisomerases, in vivo retain some sensitivity or show only partial cross-resistance to the topoisomerase II poisons etoposide, etopophos, teniposide, and mitoxantrone.

This reduced sensitivity to these topoisomerase poisons might be due to decreased complex formation as a consequence of the decreased topoisomerase IIα level in these F11782-resistant cells. Furthermore, it has been postulated that topoisomerase IIβ could be a pharmacological target for etoposide (23) and mitoxantrone (24). In this case, mitoxantrone, etoposide, and its derivatives, etopophos and teniposide, could retain some activity against the F11782-resistant cells by targeting topoisomerase IIβ, which appears little modified in these cells. Furthermore, resistance of P388/F11782 cells was also found to be associated with a K155N mutation in the topoisomerase IIα isoform, and whereas the catalytic activity of topoisomerase II extracted from F11782-resistant cells was resistant to F11782, it retained sensitivity to etoposide. Indeed, this mutation conferring resistance to F11782 occurred in the ATP binding domain of the topoisomerase IIα isoform, at sites distinct from those mainly identified as responsible for resistance to complex stabilizing agents, such as etoposide or amsacrine, i.e., around the Walker B or catalytic domains of topoisomerase IIα (25, 26). Therefore, overall, these data suggest that although F11782 is structurally related to etoposide and has topoisomerase IIα as a common target, its resistance profile and associated mechanisms appear quite different. In contrast, the functional mutations Y49F and Y165S in topoisomerase IIα, identified in bisdioxopiperazine-resistant small cell lung cancer and Chinese hamster ovary cells, respectively, proved to confer resistance to F11782 (7). This suggests that F11782 might have an interaction site in a topoisomerase IIα domain also targeted by the dioxopiperazine compounds. However, whereas the catalytic activity of topoisomerase II extracted from P388/F11782 was resistant to F11782, it retained sensitivity to the dioxopiperazine compound ICRF-193. Therefore, these various mutations seem to induce different alterations of the interaction of dioxopiperazine compounds and F11782 with topoisomerase II. These findings are in agreement with previous studies suggesting that, despite showing some similarities, F11782 and dioxopiperazines appear to function as quite distinctive catalytic inhibitors of topoisomerase II (7, 27).

One of the most critical differences is that F11782-induced salt-stable complexes were observed even in the absence of a nucleotide triphosphate (7). Therefore, as suggested by Jensen et al. (7), F11782 might induce a conformational change resembling the ATP-bound form of the enzyme, which could also partly explain the inhibition of DNA binding by F11782. However, because the level of salt-stable complexes that can be obtained with F11782 is much less than the level of complexes
Characterization of Resistance to F11782, reduced level and/or mutation of topoisomerase II, i.e. P388/H9251. Reduced level of topoisomerase II, analog (a catalytic inhibitor of topoisomerase II) and doxorubicin. Alterations of topoisomerase IIα from P388/F11782 cells (i.e., reduced level and/or mutation of topoisomerase IIα) might contribute to this resistance. However, in the case of doxorubicin, if its major recognized mode of action involved intercalation into DNA, resulting in impairment of topoisomerase II activity, other mechanisms, including free radical formation, direct membrane effects, or DNA ligase impairment, have also been postulated (28). Therefore, resistance induction in P388 cells by F11782 might have modified other parameters important for these other aspects of doxorubicin activity. Overall, these data suggest that F11782 interferes with topoisomerase II in a manner different from that of the majority of topoisomerase II-targeting agents. P388/F11782 cells also showed some collateral sensitivity to the topoisomerase II poison topotecan and irinotecan. Although only minor alterations in topoisomerase II were noted in these cells, NER activity was decreased. Because it has been suggested that NER would be involved in topoisomerase I poison-induced DNA damage repair (5, 29), it can be postulated that the decreased NER activity of these P388/F11782 cells contributes to enhanced sensitivity to topoisomerase I poisons.

The increased sensitivity of P388/F11782 cells to certain Vinca alkaloids has not been elucidated. It can only be postulated that F11782 might have induced modifications of certain cellular signals critical for the transformation of Vinca alkaloid-induced microtubule damage into antitumor activity.

Of major interest, P388/F11782 cells were shown to exhibit a decreased level of XPG protein, an endonuclease involved in NER, reflected by a global decrease of NER activity in these F11782-resistant cells. Furthermore, it has been shown that F11782 was a potent inhibitor of NER and, more specifically, of the DNA incision step (10). Therefore, these results suggest that in vivo, in addition to topoisomerase IIα, XPG also appears to be a target of F11782. The decreased NER activity in P388/F11782 cells was associated with a marked collateral sensitivity to cisplatin, which is known to induce DNA damage repaired mainly by NER (20). Furthermore, F11782-resistant P388/F11782 cells also showed collateral sensitivity to two other DNA cross-linking agents, cyclophosphamide and BCNU. Consistent with these results, synergistic antitumor activity had been identified in vivo in the parental P388 model, when single i.p. doses of F11782 were combined with either cisplatin or mitomycin C, another DNA cross-linking agent (30). Certain components of the NER system have been shown to play a role in cellular sensitivity to DNA cross-linking agents. More specifically, overexpression of XPA or the endonuclease ERCC1 was identified in patient ovarian cancer tissues resistant to cisplatin (31). On the other hand, cells deficient in ERCC1 or XPF were found to be hypersensitive to cisplatin, cyclophosphamide, melphalan, or mitomycin C (32–34). More recently, it has been shown that cellular resistance to eteclinisadin 743, a DNA-damaging agent, resulted in only acquired XPG deficiency, whereas deficiency in any of the other XP proteins led to eteclinisadin 743 resistance (35). These XPG-deficient cells did not overexpress multidrug resistance transporters and did not show cross-resistance to doxorubicin, Taxol, and etoposide (36).

However, thus far, XPG deficiency has not been associated with resistance to topoisomerase inhibitors, although resistance to topoisomerase inhibitors has already been associated with modifications of cellular response to alkylating agents. For example, increased topoisomerase II activities have been shown to contribute to the resistance of both nitrogen mustard- and cisplatin-resistant cells, and similarly, cells with decreased topoisomerase II levels show increased sensitivity to cisplatin, carmustine, mitomycin C, and nitrogen mustard (4). Therefore, it would be interesting to investigate whether resistance to topoisomerase in such cell lines could be associated with modifications of XPG or other components of the NER system. The XPG endonuclease appears to have multiple functions that are not completely elucidated. It is a phosphoprotein that is hyperphosphorylated on DNA damage (37). In addition to the endonuclease activity of XPG in NER, XPG has nonenzymatic functions via protein-protein or protein-DNA interactions. Indeed, XPG appears to coordinate the function of several proteins implicated in different DNA repair mechanisms (37). More specifically, XPG has an essential nonenzymatic role in transcription-coupled repair, a process aimed at removing, by either NER or base excision repair, DNA lesions that blocks transcription (38). Additional research studies are therefore expected to advance our under-
standing of the multiple functions of XPG and its involvement in response to anticancer treatment.

As discussed above, previous studies have suggested a causal relationship between topoisomerase II expression and resistance/sensitivity to DNA cross-linking agents (4). Indeed, it is considered that DNA topoisomerase might influence the formation of interstrand cross-links through regulation of DNA structure. Cells with lower topoisomerase activities are likely to have a higher degree of superhelical density, and it has been shown that bifunctional cisplatin lesions are formed at a higher rate on supercoiled plasmids than on relaxed ones (39). Therefore, the markedly decreased level of topoisomerase II shown that bifunctional cisplatin lesions are formed at a higher degree of superhelical density, and it has been shown that bifunctional cisplatin lesions are formed at a higher rate on supercoiled plasmids than on relaxed ones (39). Therefore, the markedly decreased level of topoisomerase II in P388/F11782 cells might also contribute, at least in part, to the enhanced sensitivity of these cells to DNA cross-linking agents.

Furthermore, recent studies tend to suggest that certain DNA endonucleases and topoisomerases may have structural homology and might evolve from a few common ancestors (40). Interestingly, comparison of the amino acid sequences between various topoisomerases and human or murine XPG endonuclease showed that the K155N mutation identified in the P388/F11782 cells occurred in a highly conserved domain of these enzymes (Fig. 11). In addition, site-directed mutagenesis studies have revealed that this domain is critical for XPG functions (41). Therefore, these findings support our data indicating that topoisomerase IIs and the endonuclease XPG are two major targets of F11782, as initially identified in vitro (6, 10) and subsequently shown in vivo.

Ongoing studies aimed at further characterization of the F11782-resistant P388/F11782 subline include evaluations of the expression of the other components of the NER system, namely, XPA, the helicases XPB and XPD, and the endonuclease ERCC1, as well as the sequencing of XPG. It would also be interesting to assess the effects of F11782 against cells resistant to DNA cross-linking agents or deficient in the NER factors XPA, XPB, XPD, XPF, or XPG. Further work will also attempt to understand the total cross-resistance to doxorubicin of these P388/F11782 cells and their collateral sensitivity to tubulin-interacting agents.

In conclusion, this study has shown that P388/F11782 cells selected in vitro for resistance to F11782 either showed no cross-resistance or proved only partially cross-resistant to other topoisomerase-targeted agents while exhibiting collateral sensitivity to a range of other antitumor agents, including DNA cross-linking agents, topoisomerase I poisons, antimitabolites, and tubulin-interacting agents. Furthermore, alterations of topoisomerase IIs and XPG in these F11782-resistant cells suggest that these two enzymes are major targets of F11782 in vivo. Therefore, these findings further demonstrate the original mechanism of action of this novel dual catalytic inhibitor of topoisomerases with DNA repair-inhibitory properties. These data also suggest that resistance to F11782 might be overcome by cotreatment with several compounds from different pharmacological classes and provide useful background information vis-à-vis the potential incorporation of F11782 into combination chemotherapy regimens.

ACKNOWLEDGMENTS

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REFERENCES


Decreased Nucleotide Excision Repair Activity and Alterations of Topoisomerase II α Are Associated with the \textit{in Vivo} Resistance of a P388 Leukemia Subline to F11782, a Novel Catalytic Inhibitor of Topoisomerases I and II

Anna Kruczynski, Jean-Marc Barret, Benoît van Hille, et al.