0026-895X/07/7204-1003–1014$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2007 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 72:1003–1014, 2007

Vol. 72, No. 4
36970/3254755
Printed in U.S.A.

A Mouse Model for Studying the Interaction of Bisdioxopiperazines with Topoisomerase IIα in Vivo

Morten Grauslund, Annemette Vinding Thougaard, Annette Füchtbauer, Kenneth Francis Hofland, Peter Hansen Hjorth, Peter B. Jensen, Maxwell Sehested, Ernst-Martin Füchtbauer, and Lars H. Jensen

Experimental Pathology Unit, Department of Pathology, Copenhagen University Hospital, Copenhagen, Denmark (M.G., M.S., L.H.J.); TopoTarget A/S, Copenhagen, Denmark (M.G., A.V.T., K.F.H., P.B.J., M.S., L.H.J.); Department of Molecular Biology, University of Aarhus, Aarhus, Denmark (A.F., P.H.H., E.-M.F.); and Laboratory for Experimental Medical Oncology, Finsen Center; Copenhagen University Hospital, Copenhagen, Denmark (P.B.J.)

Received April 10, 2007; accepted July 10, 2007

ABSTRACT

The bisdioxopiperazines such as (+)-(*S*)-4,4′-propylenedi-2,6-piperazinedione (dexrazoxane; ICRF-187), 1,2-bis(3,5-dioxopiperazin-1-yl)ethane (ICRF-154), and 4,4′-(1,2-dimethyl-1,2-ethanediyl)bis-2,6-piperazinedione (ICRF-193) are agents that inhibit eukaryotic topoisomerase II, whereas their ring-opened hydrolysis products are strong iron chelator. The clinically approved analog ICRF-187 is a pharmacological modulator of topoisomerase II poisons such as etoposide in preclinical animal models. ICRF-187 is also used to protect against anthracycline-induced cardiomyopathy and has recently been approved as an antidote for alleviating tissue damage and necrosis after accidental anthracycline extravasation. This dual modality of bisdioxopiperazines, including ICRF-187, raises the question of whether their pharmacological in vivo effects are mediated through interaction with topoisomerase II or via their intracellular iron chelating activity. In an attempt to distinguish between these possibilities, we here present a transgenic mouse model aimed at identifying the contribution of topoisomerase IIα to the effects of bisdioxopiperazines. A tyrosine 165 to serine mutation (Y165S) in topoisomerase IIα, demonstrated previously to render the human ortholog of this enzyme highly resistant toward bisdioxopiperazines, was introduced at the *TOP2A* locus in mouse embryonic stem cells by targeted homologous recombination. These cells were used for the generation of transgenic *TOP2A*$^{Y165S/+}$ mice, which were demonstrated to be resistant toward the general toxicity of both ICRF-187 and ICRF-193. Hematological measurements indicate that this is most likely caused by a decreased ability of these agents to induce myelosuppression in *TOP2A*$^{Y165S/+}$ mice, highlighting the role of topoisomerase IIα in this process. The biological and pharmacological implications of these findings are discussed, and areas for further investigations are proposed.

The nuclear enzyme topoisomerase II is found in all living organisms and possesses the essential activity of cleaving and rejoining DNA by forming a transient protein concealed double-strand break, through which an intact DNA double-strand helix is transported by a gating mechanism requiring ATP hydrolysis (Roca and Wang, 1994; Baird et al., 2001). This activity assists in a number of DNA metabolic processes such as DNA replication, transcription, chromosome condensation and decondensation, and chromosome segregation (Wang, 2002). Higher vertebrates have two isoforms of this enzyme, α and β. The topoisomerase IIα isoform is nuclear, and its expression is highly cell cycle-dependent, peaking at G_2M (Falck et al., 1999), whereas topoisomerase IIβ is located both in the nucleus and cytoplasm and displays a rather constant level during the cell cycle (Austin and Marsh, 1998). Topoisomerase IIα is essential for cell proliferation (Akimitsu et al., 2003), whereas topoisomerase II β knockout mice die shortly after birth because of developmental defects in the central nervous system (CNS) (Yang et al., 2000).

The bisdioxopiperazines such as ICRF-193, ICRF-154, and ICRF-187 (dexrazoxane) are small-molecule catalytic inhibitors of eukaryote topoisomerase II. These agents target an N-terminal ATP-operated clamp in topoisomerase II, thereby trapping topoisomerase II as a closed clamp on DNA (Roca et

This work was supported by TopoTarget A/S.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.036970.

ABBREVIATIONS: CNS, central nervous system; CFU-GM, colony forming unit-granulocyte/macrophage; ES, embryonic stem cell; ICRF, Imperial Cancer Research Foundation; kDNA, kinetoplast DNA; PLT, platelet; PCR, polymerase chain reaction; RBC, red blood cell; WBC, white blood cell; bp, base pair(s); kbp, kilobase pair(s); kb, kilobase(s); wt, wild type with regard to the topoisomerase IIα gene; ICRF-187, (+)-(*S*)-4,4′-propylenedi-2,6-piperazinedione; ICRF-154, 1,2-bis(3,5-dioxopiperazin-1-yl)ethane; ICRF-193, 4,4′-(1,2-dimethyl-1,2-ethanediyl)bis-2,6-piperazinedione; ADR-925, the iron chelating hydrolysis product of ICRF-187.

al., 1994; Morris et al., 2000). This closed-clamp configuration of topoisomerase II on DNA has been found to cause cytotoxicity (van Hille and Hill, 1998; Jensen et al., 2000a) and to inhibit transcription in mammalian cells (Xiao et al., 2003). The bisdioxopiperazines have also been demonstrated to enhance the levels of DNA breaks in vitro and in cells, although this remains controversial, because this effect depends on the experimental conditions (Huang et al., 2001; Hajji et al., 2003).

Besides having some anticancer activity (Bakowski et al., 1979; Von Hoff et al., 1981), the bisdioxopiperazines (especially ICRF-187) are effective as pharmacological modulators of topoisomerase II poisons such as etoposide and teniposide in preclinical animal models. ICRF-187 can antagonize the effect of these drugs outside the CNS (Hofland et al., 2005b), thereby allowing for a more effective targeting of CNS tumors (Holm et al., 1998). Furthermore, ICRF-187 [dexrazoxane (Savene)] has been found to be effective as an antidote against accidental anthracycline extravasation (Langer et al., 2000; Mouridsen et al., 2007). Finally, ICRF-187 (Cardioxane, Zinecard) is also used as a cardioprotectant in anthracycline treatment (Cvetković and Scott, 2005).

We have characterized previously a tyrosine 165 to serine mutation (Y165S) in human topoisomerase IIα, originally identified in a highly bisdioxopiperazine-resistant human small cell lung cancer cell line that was heterozygous for this mutation (Wessel et al., 2002). This cell line actively transcribed both the Y165S and wt topoisomerase IIα, suggesting a dominant phenotype of this resistance-conferring mutation, which was further confirmed by functional coexpression of Y165S and wt topoisomerase IIα proteins in yeast. In addition, recombinant Y165S topoisomerase IIα was found not to bind radioactively labeled ICRF-187 (dexrazoxane) in vitro (Renodon-Cornière et al., 2003). Finally, crystallographic data obtained with yeast topoisomerase II show that the homologous residue in yeast topoisomerase II Tyr144 contributes directly to ICRF-187 binding by forming an ATPase lid interacting extensively with the dioxopiperazine rings of ICRF-187 (Classen et al., 2003).

Although the bisdioxopiperazines are known to kill cells by interacting with topoisomerase II, these agents are also strong iron chelators, and the role of this activity versus topoisomerase II inhibition has never been directly addressed in a higher vertebrate. One way of addressing this question is to engineer a transgenic mouse having altered topoisomerase II with reduced interaction with bisdioxopiperazines. Because of the strong phenotype of the topoisomerase IIα Y165S mutation, we selected this alteration in topoisomerase IIα to be engineered into the *TOP2A* locus in embryonic stem cells by homologous recombination (gene targeting) followed by blastocyst injection, generation of chimeric germ line transmitters, and back-crossing. Using this setup, we were able to develop heterozygous *TOP2A*$^{Y165S/+}$ mice carrying this dominant bisdioxopiperazine resistance conferring mutation at the correct genomic loci. We here demonstrate for the first time in the mouse that interaction with topoisomerase IIα is involved in mediating the effects of bisdioxopiperazines on general and hematological toxicity.

Materials and Methods

Drugs and Reagents. ICRF-187 [dexrazoxane (Cardioxane)] was obtained from Chiron Corporation (Amsterdam, The Netherlands); ICRF-193 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA) or synthesized. Daunorubicin (Cerubidin) was from Aventis Birkerod (Hørsholm, Denmark). Etoposide and cisplatin was from Bristol-Myers Squibb Co. (Lyngby, Denmark). Restriction endonucleases were from New England Biolabs (Medinova Scientific A/S, Glostrup, Denmark), and [^{32}P]dATP was from GE Healthcare (Hillerød, Denmark). All other reagents were from Sigma-Aldrich (Vallensbaek Strand, Denmark) unless otherwise indicated.

Construction of Gene Targeting Vector. A cDNA probe covering amino acids 131 to 325 of the mouse topoisomerase IIα coding sequence was amplified and cloned by PCR from a mouse testis cDNA library (Stratagene, AH Diagnostics, Århus, Denmark) by using the following oligonucleotide pairs: 5′-GAG CCA AAA ATG TCT TGT ATT AG-3′ and 5′-GAG ATG TCT GCC GCC CTT AGA AG-3′. The resulting PCR fragment was labeled with ^{32}P and used as probe for screening a mouse 129/SvEvTACCfBr spleen BAC library (Invitrogen A/S, Taastrup, Denmark), and 10 positive clones were obtained. From one of the isolated clones (BAC clone 182-D4), a 10.8-kb SacI-BamHI fragment (genomic position 2111–12888) containing exons 4 to 12 was subcloned into a pUC19 vector in which the HindIII site of the poly-linker had been inactivated previously. This construct is now referred to as the unmodified targeting vector. A 735-bp fragment containing SwaI-HindIII fragment of exon 6 was PCR-amplified using the oligonucleotides 5′-CTC TAT TTA AAT ATC GAT TCC TAG GTC ATT ACC AAG GCT TTT AGA ATG-3′ and 5′-TCTTTAGTGGAGCCAGCGGT-3′, in which the forward oligonucleotide contained an extra AvrII restriction site (underlined). The PCR product was cloned into the TOPO2.1 vector (Invitrogen A/S), and the Y165S mutation and a silent ScaI site were introduced by site-directed mutagenesis (Stratagene) by using the mutagenic oligonucleotide 5′-GGT CGA AAT GGC TCT GGA GCT AAA CTG TGT AAC ATA TTC AGT ACT AAA TTT ACT G-3′ and its complementary oligonucleotide (Fig. 1E). The mutated DNA fragment was then reintroduced into the unmodified targeting vector as a SwaI-HindIII fragment. A self-excision neomycin-resistance marker (neo^{R}) controlled by the phosphoglycerate kinase promoter was PCR-amplified from pPGK neo bpA (Addgene Inc., Cambridge, MA) vector using the oligonucleotides 5′-CTC TCT ATT TAA AT**A TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT** TGA TGC TCG TCA GGG GG-3′ and 5′-CTC TCC TAG G**AT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT AT**T CCA CCG CGG TGG CGG-3′ that both contained a loxP site (in boldface type) and SwaI and AvrII restriction sites in the forward and reverse primers, respectively (underlined). The neo^{R} cassette was introduced into the modified targeting vector as a SwaI-AvrII fragment. Finally a diphtheria toxin resistance marker was linked to the 3′-end of the topoisomerase IIα sequence. The diphtheria toxin marker was PCR-amplified using the oligonucleotides 5′-TTT TGA GCT CGG ATC CAC CGC TCT AGA ACT ACG ATC C-3 and 5′-TTT TGA GCT CCG AAC AAC TCC GCC GCG-3′ and ligated into the targeting vector using the SacI sites that were introduced in the oligonucleotides (underlined). All PCR-amplified DNA fragments were sequenced before being introduced into the targeting vector.

Gene Targeting by Homologous Recombination. Murine CJ7 embryonic stem cells (Swiatek and Gridley, 1993) (Fig. 1A) were electroporated with BsaBI-linearized targeting vector (Fig. 1B) and screened for resistance toward G418 at a concentration of 300 μg/ml. DNA was isolated and digested with AvrII according to Ramírez-Solis et al. (1993) and subsequently analyzed by Southern blotting using a ^{32}P-labeled 5′-end probe that binds at the genomic position 1768 to 2044 bp (Fig. 1F). Three clones that showed correct integration at the 5′-end were further confirmed with a probe that binds the 3′-end of the integration at genomic position 13240 to 13646 (Fig.

1C). Finally, the phosphotransferase expression cassette was excised from the *TOP2A* loci in correctly targeted ES clones (Fig. 1D) by transient expression of Cre recombinase, and correct excision was confirmed by PCR using the following primers: 5′-ATG AGC AAA GTC GTG TTC CC-3′ and 5′-CTT TGC TGT CCA TCC AGG TT-3′. This PCR reaction amplifies the region that contains the single loxP site, which remains after excision of the neomycin resistance cassette. Consequently, in this PCR reaction, the wild-type allele produces a 292-bp product, whereas the mutated allele produces a 326-bp product.

Generation of Transgenic Knock-In Mice. Three positive ES cell clones were individually injected into B6D2F2 blastocysts, which were then introduced into pseudopregnant NMRI female mice that gave birth to chimeric animals. Male chimeras were mated with C57BL/6 female mice, and germ line transmission was obtained from two independent ES cell clones. Heterozygous mutant mice were identified by PCR using the primer pair described above. In initial crossings between heterozygous $TOP2A^{Y165S/+}$ male and female 129B6F1 animals, no homozygous $TOP2A^{Y165S/Y165S}$ animals could be obtained. $TOP2A^{Y165S/+}$ and $TOP2A^{+/+}$ animals appeared at the ratio of 2:1 as expected in case of the $TOP2A^{Y165S/Y165S}$ allele being embryonic lethal. As a consequence, in our present studies, all in vivo and ex vivo experiments were performed using heterozygous $TOP2A^{Y165S/+}$ female B6129S hybrids obtained by crossing heterozy-

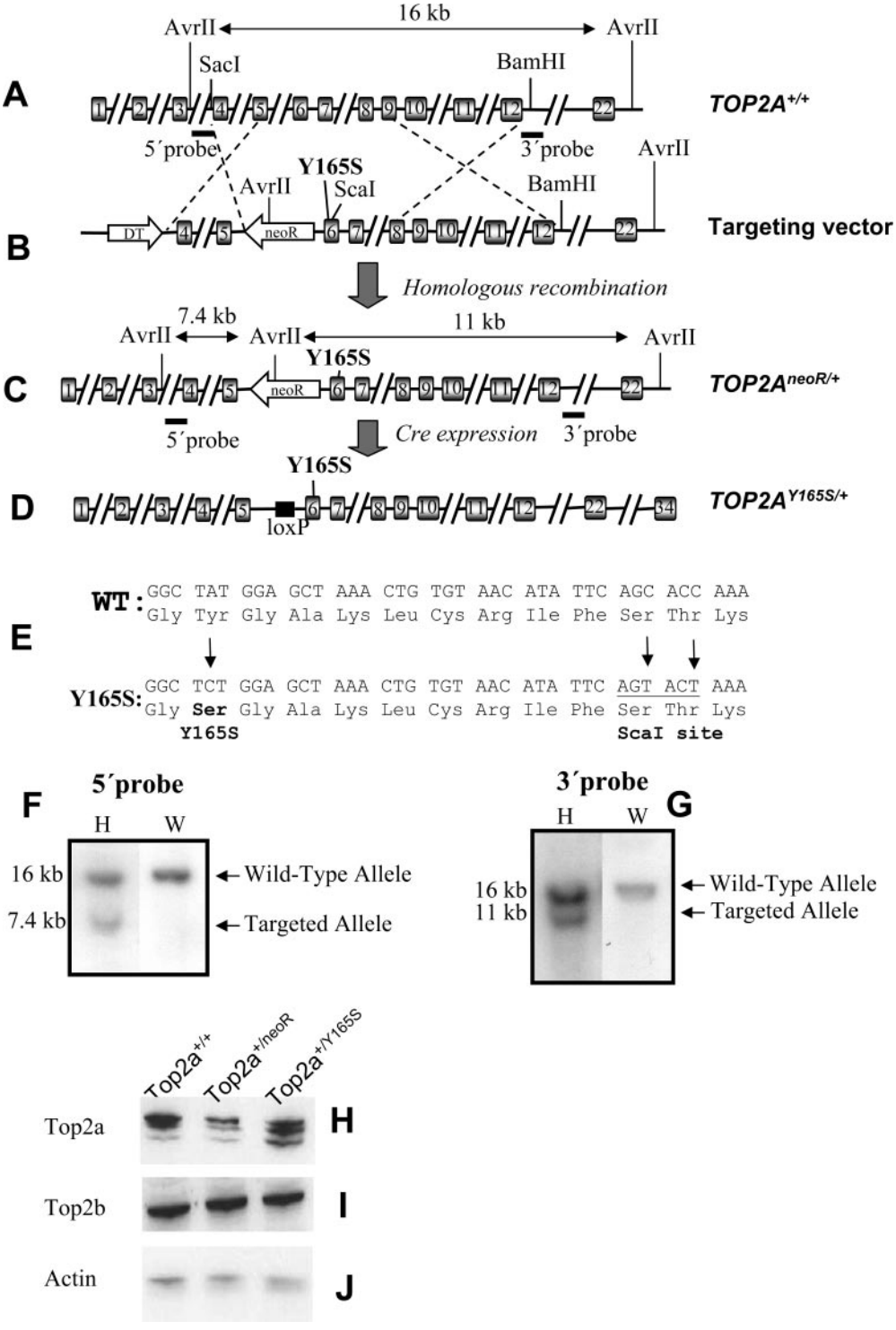

Fig. 1. Targeting strategy for the Y165S knock in into the *TOP2A* gene. A, genomic organization of the topoisomerase IIα genomic locus in the mouse. B, design of the targeting vector used for mutagenesis of the *TOP2A* gene. C, graphic presentation of the targeted *TOP2A* allele after correct homologous integration of targeting vector DNA. D, structure of the targeted *TOP2A* allele after removal of the neomycin resistance cassette by Cre-lox recombination. E, nucleotide changes introduced into the coding sequence of topoisomerase IIα at the targeted *TOP2A* allele upon homologous recombination. F, Southern blot of genomic DNA isolated from $TOP2A^{neoR/+}$ (lane H) and wt embryonic stem cells (lane W) hybridizing to the 5′-probe. G, Southern blot of genomic DNA isolated from $TOP2A^{neoR/+}$ (lane H) and wt embryonic stem cells (lane W) hybridizing to the 3′-probe. H: topoisomerase IIα levels in crude extracts isolated from $TOP2A^{+/+}$, $TOP2A^{neoR/+}$, and $TOP2A^{Y165S/+}$ embryonic stem cells. I, topoisomerase IIβ levels in crude extracts isolated from $TOP2A^{+/+}$, $TOP2A^{neoR/+}$, and $TOP2A^{Y165S/+}$ embryonic stem cells. J, actin levels in crude extracts isolated from $TOP2A^{+/+}$, $TOP2A^{neoR/+}$, and $TOP2A^{Y165S/+}$ embryonic stem cells.

gous *TOP2A*$^{Y165S/+}$ 129B6 male mice with wild-type C57BL/6 female mice.

Western Blot Analysis. Twenty micrograms of protein obtained from whole-cell extracts were denatured and reduced by boiling in SDS sample buffer containing dithiothreitol, separated by SDS-polyacrylamide gel electrophoresis in NuPAGE 4–12% Bis-Tris Gels (Invitrogen A/S), and transferred to nitrocellulose membranes (Invitrogen A/S) according to standard procedures. The following antibodies were used: Rabbit polyclonal antiactin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal antitopoisomerase IIα and IIβ (Biotrend, Copenhagen, Denmark). Detection was performed by using a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare), and an ECL plus Western Blotting Detection system (GE Healthcare).

Decatenation Assay. Topoisomerase II catalytic activity was measured by kinetoplast DNA (kDNA) decatenation by using ^{3}H-labeled kDNA isolated from *Crithidia fasciculata* (American Type Culture Collection, Manassas VA) as described previously (Wessel et al., 1999), by using a 96-well format filter-based assay (Jensen et al., 2002).

Embryonic Stem Cells Viability Assay. The sensitivity of *TOP2A*$^{+/+}$, *TOP2A*$^{neoR/+}$, and *TOP2A*$^{Y165S/+}$ embryonic stem after exposure toward increasing concentrations of ICRF-193 or etoposide was assessed as described previously (Kobayashi et al., 2001).

Ex Vivo Granulocyte-Macrophage Colony-Forming Unit Assay. Mice were sacrificed with 70% CO_2/O_2, and bone marrow cells were obtained by flushing the femoral bone cavity with Iscove's modified Dulbecco's medium with 10% fetal bovine serum at 37°C. Cells were then cooled at 1°C per minute until −180°C in a freezing solution containing 7.5% dimethyl sulfoxide. All cells were kept under liquid nitrogen and were prepared identically for use; after washing twice in 37°C medium, they were left for 1 h to rest at 37°C and then stained with 0.1% nigrosin to test for cell viability. Clonogenic CFU-GM assays were carried out as described previously (Hofland et al., 2005b).

Animals and Housing. Experiments were conducted according to institutional and national guidelines for the care and use of laboratory animals and were approved by the Danish Experimental Animal Inspectorate, Ministry of Justice. Female B6129SF1 *TOP2A*$^{Y165S/+}$ and *TOP2A*$^{+/+}$ mice, 10 to 16 weeks old and weighing 20 to 28 g, were obtained from contract breeding at Taconic (Ry, Denmark). The mice were allowed to become acclimatized for at least 1 week before being included into experiments. They were kept in standard type III plastic cages (Scanbur B-K, Koege, Denmark) on wood-chip bedding with nest material, wooden chewing blocks, and plastic houses for environmental enrichment (Brogaarden, Gentofte, Denmark). They were kept in a 12-h light/dark cycle with ad libitum access to tap water and standard laboratory diet (Altromin; Brogaarden). Treatments were given during the light-cycle period.

Treatment of Mice with ICRF-187 and ICRF-193. Female B6129SF1 *TOP2A*$^{Y165S/+}$ and *TOP2A*$^{+/+}$ animals were used in these experiments. Because in pilot experiments single-dose administration of the bisdioxopiperazines ICRF-193 and ICRF-187 proved insufficient to induce toxicity, these agents were administered for 3 consecutive days by the intraperitoneal route (days 0, 1, and 2) in a volume of 0.2 ml of isotonic saline for ICRF-187 or in 0.05 ml of dimethyl sulfoxide for ICRF-193. In general toxicity experiments (survival studies), the following daily doses of ICRF-187 were given: 50, 125, 250, 350, 450, 500, and 550 mg/kg body weight. For hematological studies, three daily doses of 250 or 450 mg/kg ICRF-187 were administered. This represents a nonlethal and a lethal dose, respectively. In toxicity experiments with ICRF-193, three daily doses of 10, 20, 25, and 30 mg/kg were administered in general toxicity studies. For hematological measurements 10, 20, and 30 mg/kg ICRF-193 was used, representing one partially lethal dose and two lethal doses (in wt animals).

Histopathology. Groups of *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ male and female mice were treated with a single daily dose of 550 mg/kg dexrazoxane or 30 mg/kg ICRF-193 i.p. for 3 consecutive days. The mice were sacrificed the day after last treatment by cervical dislocation, and 1-cm samples were excised in the same anatomical location in each animal from duodenum (right after the stomach), mid of jejunum, and ileum (right before colon). The small intestinal samples were immersed in phosphate-buffered 4% formaldehyde for 3 days and were then mounted in paraffin, and 5-μm longitudinal sections were cut and stained with hematoxylin and eosin stain.

Hematological Toxicity. Mice were sacrificed on day 3, and the right femur bone was dissected free from muscle and excised immediately after sacrifice. The ends of the femur bone were clipped off to expose the bone marrow cavity. The marrow cells were flushed out with phosphate-buffered saline in a syringe with a 27-gauge needle using four turns of flushing with the same fluid (fluid volume, 1 ml), two flushes from each end to ensure good recovery and dispersion of the bone marrow cells. After flushing, the emptied femur bone was weighed, and the total bone marrow cell counts normalized to the femur bone weight. The cell count of the marrow flush fluid was measured using the CA530VET hematology analyzer set up for mouse sample analysis (Boule Medonic, Stockholm, Sweden). This device uses electrical impedance for sizing and counting cells in fluid (blood), and counts obtained for cells of a size corresponding to peripheral white blood cells express the number of bone marrow stem cells recovered from the femur. Counts obtained with the hematology analyzer were found to correlate well with bone marrow cell counts performed manually in a Fuchs-Rosenthal counting chamber ($r^2 = 0.68$, $n = 15$, $p < 0.001$; data not shown).

Peripheral blood counts [white blood cells (WBCs), red blood cells (RBCs) and platelets (PLTs)] were also determined using the CA530VET hematology analyzer. Before sampling, mice were placed for approximately 2 min in a Plexiglas cage under a heating lamp to produce vasodilation and facilitate bleeding. The tail vein was then punctured using a 27-gauge needle, and a blood drop was collected into a 20-μl K_2EDTA-coated capillary tube.

In Vivo Toxicity Using Humane Endpoints. Mice were also allocated to survival studies after treatments, and the survival endpoint was defined as the number of days until the mice showed signs of toxicity according to humane criteria defined below. The mice were observed twice daily, weighed two or three times weekly, and assigned an activity score from 1 to 5 (Hofland et al., 2005a). To receive a score of 5, the mouse exhibits a normal active and curious behavior. It moves about and stands upright at the sides of the cage. For a score of 4, the mouse is not quite as active. It does not stand up as often and prefers to stay in the corners of the cage. For a score of 3, the mouse is less active, and when it moves, it often stops and sits. It stays in the nest corner. For a score of 2, the mouse moves only when touched, and only for a short distance. It preferably hides in the nest corner. Finally, for a score of 1, the mouse is moribund. Activity scores of 5 or 4 were accepted. Mice having an activity score of 3 were monitored closely, and if activity decreased below a score of 3, they were sacrificed. Mice that suffered a weight loss of more than 20%, regardless of an otherwise acceptable activity score, were euthanized. In these experiments, the number of mice in each group ranged from six to seven.

Statistical Analysis and Graphical Presentation. The graphs were produced using the Prism 4 software (GraphPad Software Inc., San Diego, CA). Statistical analysis was performed using the software SAS 9.1 (SAS Institute, Cary, NC). Hematology values and bone marrow counts were analyzed using analysis of variance and *t* tests on preplanned comparisons or when the residuals from analysis of variance were not normally distributed using nonparametric Wilcoxon test. Survival data were analyzed using log-rank statistics. The level of significance was set to a probability of $p = 0.05$ or below.

Results

Introduction of a Heterozygous Y165S Mutation at the *TOP2A* Loci in Embryonic Stem Cells. To investigate

the function of the topoisomerase IIα Y165S mutation in vivo, the mutation was introduced into mouse CJ7 embryonic stem cells by targeted homologous integration. The murine *TOP2A* gene is located on chromosome 11 and consists of 34 exons encompassing 30.1 kb of DNA (Fig. 1A). A targeting construct was designed to replace the tyrosine codon 165 with a serine (Y165S) by homologous recombination (Fig. 1, A and B). This construct contains a 10.4-kb stretch of the murine *TOP2A* gene spanning exons 4 to 12. Here, the Y165S mutation is located in codon 6 (Fig. 1B). After being linearized, this construct was introduced in mouse embryonic CJ7 stem cells as described under *Materials and Methods*. Correctly targeted ES cells were selected in medium containing G418 (Fig. 1C).

Identification of Cell Clones with Correctly Integrated Homologous Recombination. Figure 1F demonstrates the presence of the correct prognostic 7.7-kbp fragment hybridizing to the 3′-probe (lane H), whereas Fig. 1G demonstrates the presence of the correct prognostic 11-kbp fragment hybridizing to the 3′-probe (lane H). In both F and G, the expected 16-kbp fragment prognostic for the untargeted $TOP2A^{+/+}$ allele was also detected (lane W), demonstrating that only one allele has been altered by homologous recombination in these cells. Cells showing correct homologous integration were finally transfected with a Cre recombinase-expressing plasmid to excise the neomycin marker (Fig. 1D). Correct removal of the *neoR* cassette was finally assessed by PCR (data not shown).

***TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ Embryonic Stem Cells Have Comparable Expression Levels of Topoisomerase IIα.** The levels of topoisomerase IIα and IIβ expressed in normal $TOP2A^{+/+}$ CJ7 ES cell, in $TOP2A^{neoR/+}$ (before removal of neomycin resistance marker) cells, and in $TOP2A^{Y165S/+}$ cells were determined by Western blotting using actin levels as loading controls. The results of these experiments are depicted in Fig. 1, H, I, and J. It is evident that comparable expression levels of topoisomerase IIα is detected in $TOP2A^{+/+}$ and $TOP2A^{Y165S/+}$ cells (compare in Fig. 1H). This result suggests that the Y165S mutant topoisomerase IIα protein is expressed at normal levels in $TOP2A^{Y165S/+}$ cells and that Y165S and wt topoisomerase IIα protein have similar stabilities when expressed from the *TOP2A* locus in CJ7 ES cells. In contrast, the level of topoisomerase IIα was reduced to approximately 50% in

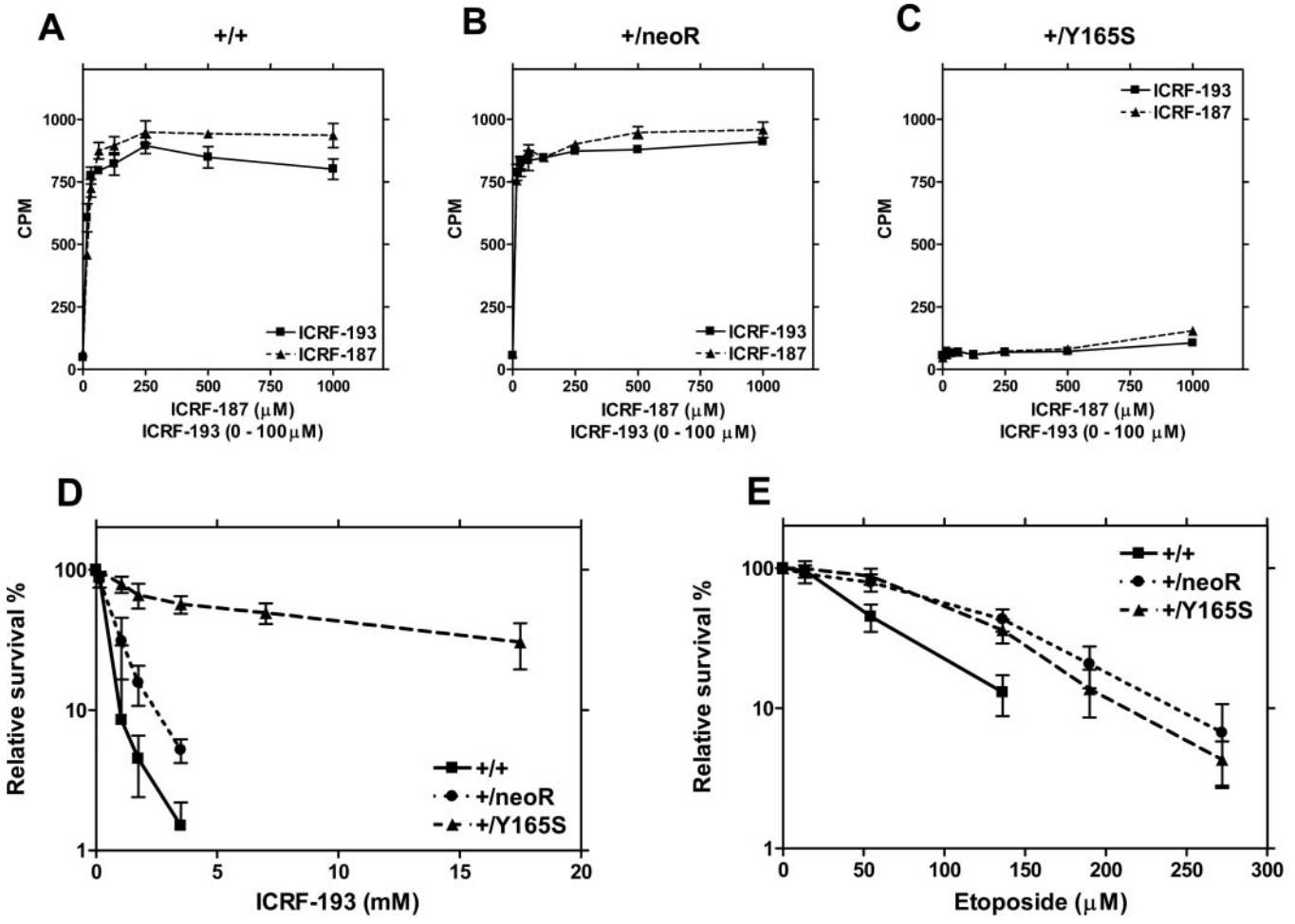

Fig. 2. Results of survival assays and decatenation experiments performed with $TOP2A^{+/+}$, $TOP2A^{neoR/+}$, and $TOP2A^{Y165S/+}$ embryonic stem cells and crude extracts of these. A, inhibition of topoisomerase II catalytic activity in crude extracts from $TOP2A^{+/+}$ embryonic stem cells by increasing concentrations ICRF-187 and ICRF-193. B, inhibition of topoisomerase II catalytic activity in crude extracts from $TOP2A^{neoR/+}$ embryonic stem cells by increasing concentrations ICRF-187 and ICRF-193. C, lack of inhibition of topoisomerase II catalytic activity in crude extracts from $TOP2A^{Y165S/+}$ embryonic stem cells by increasing concentrations ICRF-187 and ICRF-193. D, ES cell survival assays of $TOP2A^{+/+}$, $TOP2A^{neoR/+}$, and $TOP2A^{Y165S/+}$ embryonic stem cells toward ICRF-193. E, ES cell survival assay of $TOP2A^{+/+}$, $TOP2A^{neoR/+}$, and $TOP2A^{Y165S/+}$ embryonic stem cells toward etoposide. In A to C, error bars represents S.E.M. of three experiments. In D and E, error bars represents S.E.M. of 3 to 10 experiments.

TOP2A$^{neoR/+}$ cells (Fig. 1H). Apparently, the neoR allele represents a *TOP2A* null allele. The levels of topoisomerase IIβ were similar in the three cell lines as expected.

The Topoisomerase II Catalytic Activity of Crude Extract Isolated from *TOP2A*$^{Y165S/+}$ Embryonic Stem Cells Is Resistant to Inhibition by Bisdioxopiperazines. To functionally assess whether the heterozygous TOP2A$^{Y165S/+}$ allele affected the sensitivity of topoisomerase II toward bisdioxopiperazines in CJ7 ES cells, we isolated crude extract from three different cell lines *TOP2A*$^{+/+}$, *TOP2A*$^{neoR/+}$, and *TOP2A*$^{Y165S/+}$. In this assay unprocessed kDNA network is retained on a filter, whereas processed kDNA minicircles pass through when washed (Jensen et al., 2002). Because the kDNA is radioactively labeled, the amount of radioactivity on the filter represents the degree of inhibition of topoisomerase II catalytic activity. The results of these experiments are depicted in Fig. 2, A to C. It is evident that the decatenation activity of extract from *TOP2A*$^{+/+}$ (Fig. 2A) and *TOP2A*$^{neoR/+}$ (Fig. 2B) cells is sensitive to inhibition by both ICRF-187 and ICRF-193. In contrast, the decatenation activity of crude extract from *TOP2A*$^{Y165S/+}$ cells is highly resistant toward inhibition by both of these bisdioxopiperazines (Fig. 2C). When another catalytic topoisomerase II inhibitor, aclarubicin, was used, there was no difference between these three cell lines (data not shown). This result confirms that the Y165S mutation is dominant and specific with regard to bisdioxopiperazine resistance as demonstrated previously in OC-NYH small cell lung cancer cells (Wessel et al., 2002). This result also points to the notion that the Y165S protein is not expressed in *TOP2A*$^{neoR/+}$ cells or that it is expressed at very low levels.

The Sensitivity of *TOP2A*$^{Y165S/+}$ Embryonic Stem Cells toward ICRF-193 Is Greatly Reduced. We next turned toward assessing whether these decatenation results could be translated into cellular sensitivity toward bisdioxopiperazines. We exposed *TOP2A*$^{+/+}$, *TOP2A*$^{neoR/+}$, and *TOP2A*$^{Y165S/+}$ cells toward increasing concentrations of the bisdioxopiperazine compound ICRF-193 (Fig. 2D) and the unrelated topoisomerase II targeting compound etoposide (Fig. 2E) and assessed cell survival. *TOP2A*$^{Y165S/+}$ cells were highly resistant toward ICRF-193 compared with *TOP2A*$^{+/+}$ cells, thus confirming the decatenation results and attesting

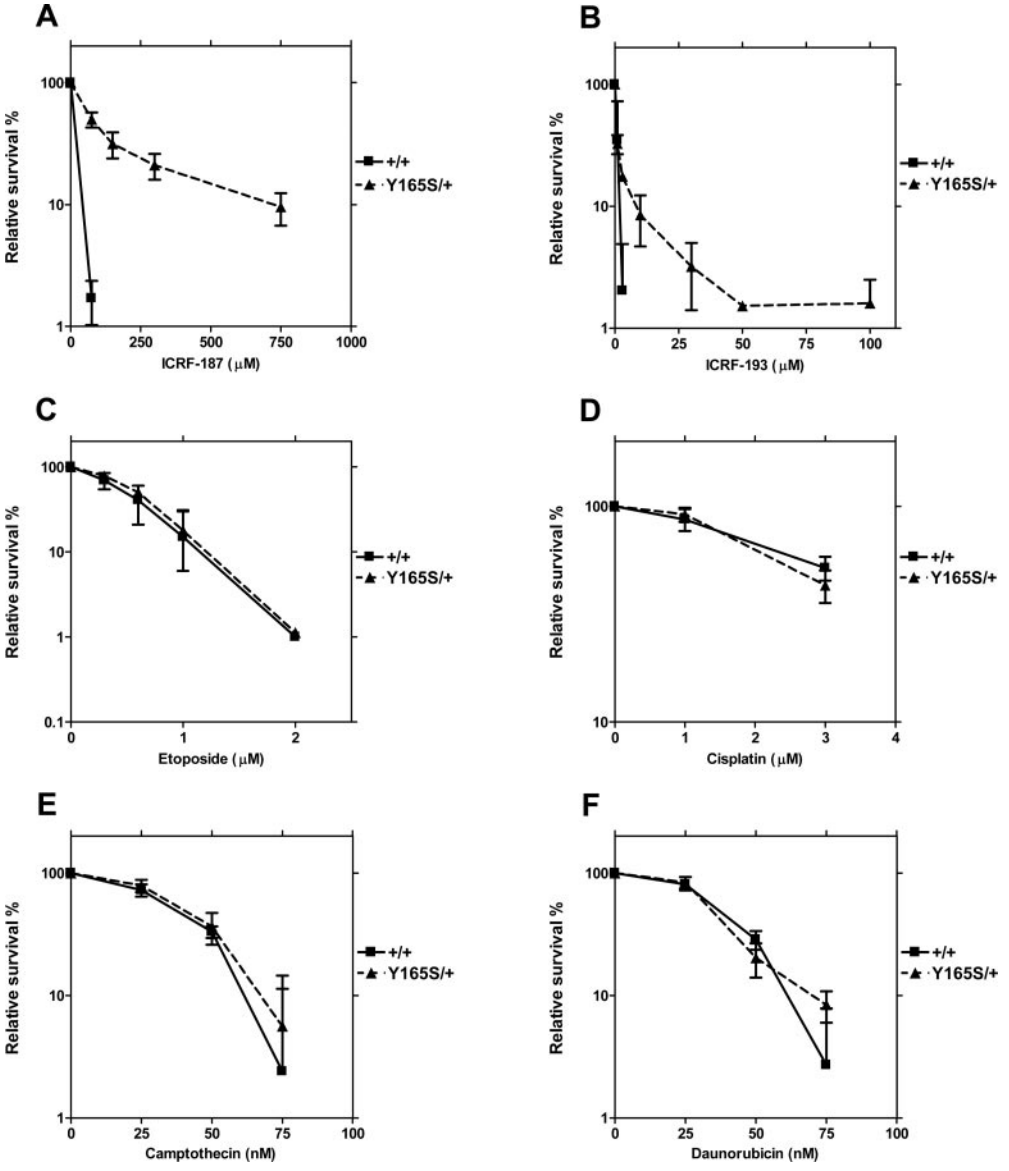

Fig. 3. Clonogenic sensitivity of bone marrow stem cells isolated from *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ mice toward different drugs as assessed in CFU-GM assay. A, ICRF-187; B, ICRF-193; C, etoposide; D, cisplatin; E, camptothecin; F, daunorubicin. Error bars represent S.E.M. of two determinations.

to the fact that the Y165S mutation is dominant with regard to bisdioxopiperazine resistance (Fig. 2D). The *TOP2A*$^{neoR/+}$ cells were also less sensitive than *TOP2A*$^{+/+}$cells. This result can be explained by the fact that *TOP2A*$^{+/neoR}$ cells contain lower levels of topoisomerase IIα than *TOP2A*$^{+/+}$cells. A similar correlation between topoisomerase IIα content and bisdioxopiperazine sensitivity has been reported previously for mouse embryonic stem cells (Kobayashi et al., 2001). In contrast, *TOP2A*$^{Y165S/+}$ cells were only moderately resistant toward the topoisomerase II poison etoposide that interacts with topoisomerase IIα in a different way (Fig. 2E). Together, these results establish that the Y165S mutation is dominant and specific for bisdioxopiperazine resistance. Finally, the observation that *TOP2A*$^{neoR/+}$ cells are slightly resistant to etoposide is also in line with their 50% reduction of topoisomerase IIα levels (Kobayashi et al., 2001). In our hands, the CJ7 embryonic stem cells were generally quite insensitive toward ICRF-193. Although the exact reason for this is not known, the ICRF-193-resistant phenotype of the *TOP2A*$^{Y165S/+}$ cells is still evident.

Bone Marrow Stem Cells Isolated from Heterozygous *TOP2A*$^{Y165S/+}$ B6129SF1 Hybrids are Highly Resistant toward Bisdioxopiperazines. Ex vivo experiments were performed with bone marrow cells isolated from the femur of *TOP2A*$^{Y165S/+}$ and *TOP2A*$^{+/+}$ female B6129SF1 hybrids. CFU-GM clonogenic assays were carried out as described under *Materials and Methods*. The results of these experiments are depicted in Fig. 3, A to F. From Fig. 3, it is evident that bone marrow stem cells from *TOP2A*$^{Y165S/+}$ female B6129SF1 hybrids are highly resistant toward the bisdioxopiperazines ICRF-187 (Fig. 3A) and ICRF-193 (Fig. 3B) compared with similar *TOP2A*$^{+/+}$ cells. In contrast, *TOP2A*$^{Y165S/+}$- and *TOP2A*$^{+/+}$-derived cells showed similar sensitivity toward etoposide (Fig. 3C), cisplatin (Fig. 3D), camptothecin (Fig. 3E), and daunorubicin (Fig. 3F).

***TOP2A*$^{Y165S/+}$ Mice Are Resistant to the General Toxicity of Bisdioxopiperazines.** The bisdioxopiperazines including ICRF-187 (dexrazoxane) are known to target eukaryotic topoisomerase II in vitro and in cells, and the latter has been directly associated with cytotoxicity, because cell lines containing mutations in topoisomerase IIα isoform (Y50F, R162Q, and Y165S) have shown high-level resistance toward these compounds (Sehested et al., 1998; Wessel et al., 1999, 2002). However, little is known about the role of this interaction for the mechanism of action of bisdioxopiperazines in mammalians. To study this, we exposed *TOP2A*$^{Y165S/+}$ and *TOP2A*$^{+/+}$ mice to high doses of ICRF-187 given i.p. using groups of mice ranging from six to seven in each experiment, and we looked at the survival in accordance with the humane endpoint evaluation criteria outlined under *Materials and Methods*. The doses were given on days 0, 1, and 2. In these experiments, we used daily ICRF-187 doses ranging from 50 to 550 mg/kg. Doses of less than 350 mg/kg body weight did not cause any toxicity and are not discussed further. Daily doses of 350, 450, 500, and 550 mg/kg ICRF-187 were effective in exerting toxicity, and the outcome of these experiments is depicted in Fig. 4. At the two lowest dose levels,

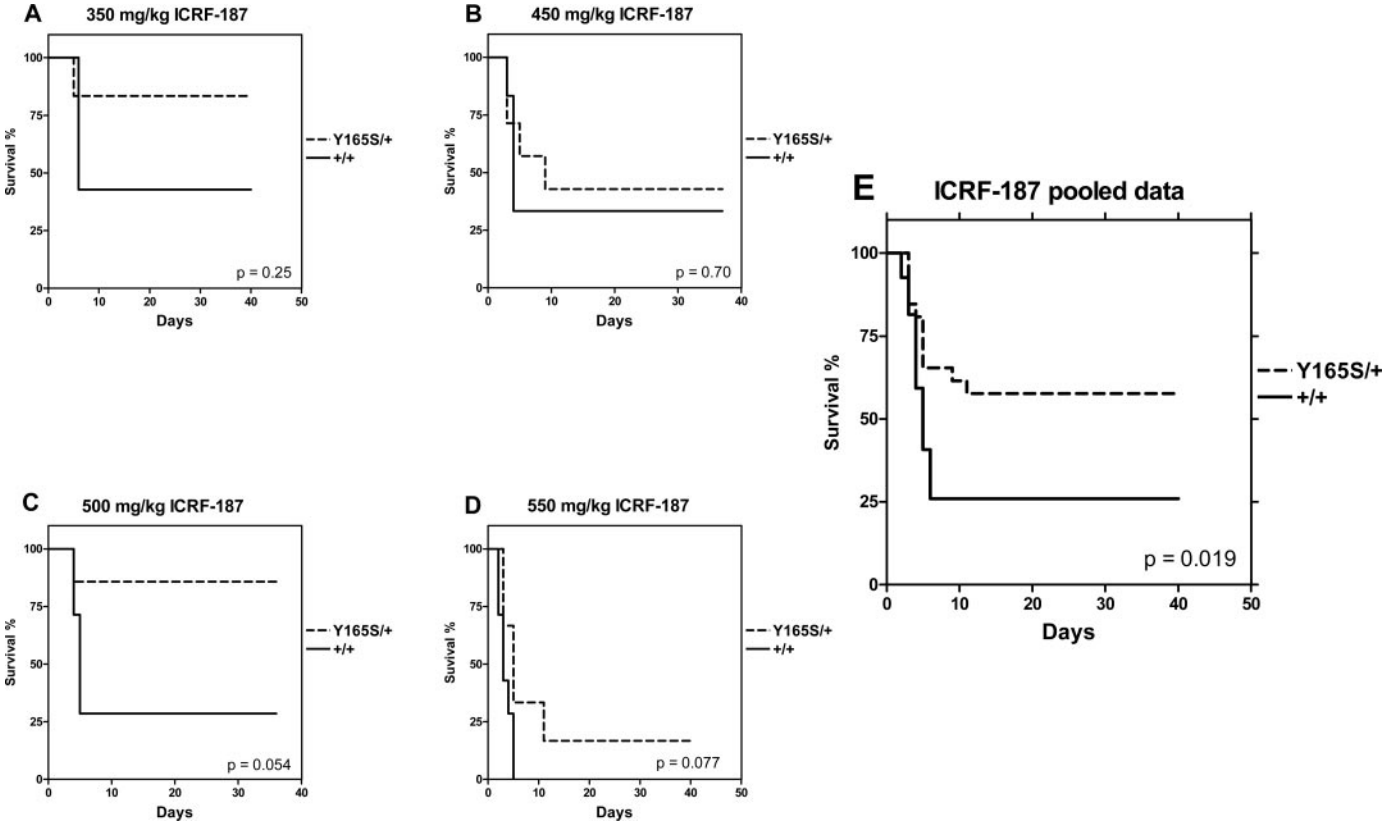

Fig. 4. Kaplan-Meyer survival plots of *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ mice after treatment with different dose levels of ICRF-187 given daily on days 0, 1, and 2 of the study. A, 350 mg/kg ICRF-187; B, 450 mg/kg ICRF-187; C, 500 mg/kg ICRF-187; and D, 550 mg/kg ICRF-187. E, Kaplan-Meyer survival plots of pooled data from A to D. In A to D, the number of animals in each group ranged from six to seven. E, the number of mice in each group was 26. *p* Values from log-rank analysis are shown on the figure for each dose level.

there was little effect of topoisomerase IIα, status because many mice survived treatment and were censored (Fig. 4, A and B). At the 500 and 550 mg/kg dose levels, borderline significance was reached because *TOP2A*$^{Y165S/+}$ mice tended to survive longer with p values of 0.054 and 0.076, respectively. However, when data presented in Fig. 4, A to D, were pooled to obtain larger group sizes, the *TOP2A*$^{Y165S/+}$ allele was found to be a significant determinant for ICRF-187 resistance ($p = 0.019$, $n = 27$ in each group; Fig. 4E). This result demonstrates that *TOP2A*$^{Y165S/+}$ mice are resistant to the general toxicity of ICRF-187.

We also performed survival studies with the bisdioxopiperazine analog ICRF-193 that is approximately 10 times more potent against topoisomerase II than ICRF-187 (Hasinoff et al., 1995). In these experiments, doses of 10, 20, and 30 mg/kg/day were used. *TOP2A*$^{Y165S/+}$ mice were highly resistant toward the toxicity of ICRF-193 compared with normal mice at two different dose levels, namely 20 and 30 mg/kg/day for 3 days (Fig. 5, B and C; $p = 0.009$). At the lowest dose level, 10 mg/kg, there was a tendency of the *TOP2A*$^{Y165S/+}$ mice to have a better survival than wild-type mice (Fig. 5 A, $p = 0.14$). When these results (A–C) were pooled, *TOP2A*$^{Y165S/+}$ mice were resistant to ICRF-193 toxicity ($p < 0.0001$, $n = 18$ in each group; Fig. 5D). This result shows that *TOP2A*$^{Y165S/+}$ mice are extremely resistant toward ICRF-193 general toxicity.

***TOP2A*$^{Y165S/+}$ Mice Are Resistant toward Bisdioxopiperazine-Induced Bone Marrow Cell and Peripheral White Blood Cell Depression.** Bisdioxopiperazines like ICRF-187 are known to cause myelosuppression at dose levels required for clinical activity (Bakowski et al., 1979; Von Hoff et al., 1981). Although bisdioxopiperazines are known to target topoisomerase IIα in vitro and in cell lines (Hasinoff et al., 1995; Ishida et al., 1995; Sehested et al., 1998; Wessel et al., 1999, 2002; Kobayashi et al., 2001), a direct role of the topoisomerase IIα isoform in bisdioxopiperazine-induced myelosuppression has never been established. We therefore assessed whether there was any difference in the effect of ICRF-187 on bone marrow cell counts and peripheral blood cell counts (WBCs, RBCs, and PLTs) between *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ mice. Daily doses of 250 or 450 mg/kg ICRF-187 were given i.p. on days 0, 1, and 2 to groups of six to seven mice. The hematological measurements were performed on day 3. Although there was no significant difference in the values for bone marrow cell counts obtained from *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ mice in the absence of drug (Fig. 6A, $p = 0.68$), there was significantly less suppression of bone marrow cell counts in the *TOP2A*$^{Y165S/+}$ mice compared with the *TOP2A*$^{+/+}$ mice at the 250 mg/kg dose level (Fig. 6A; $p = 0.0001$) and a tendency to less suppression in mutant mice at the 450 mg/kg dose level (Fig. 6A; $p = 0.06$). We also looked at suppression of peripheral white blood cell counts (Fig. 6B). Here, the *TOP2A*$^{Y165S/+}$ genetic background was found to significantly reduce the effect of ICRF-187 on depressing the white blood cell numbers (Fig. 6B; $p = 0.04$ in comparisons at both dose levels).

The effect of ICRF-193 on hematology values was also examined. Daily doses of 10, 20, and 30 mg/kg were given in

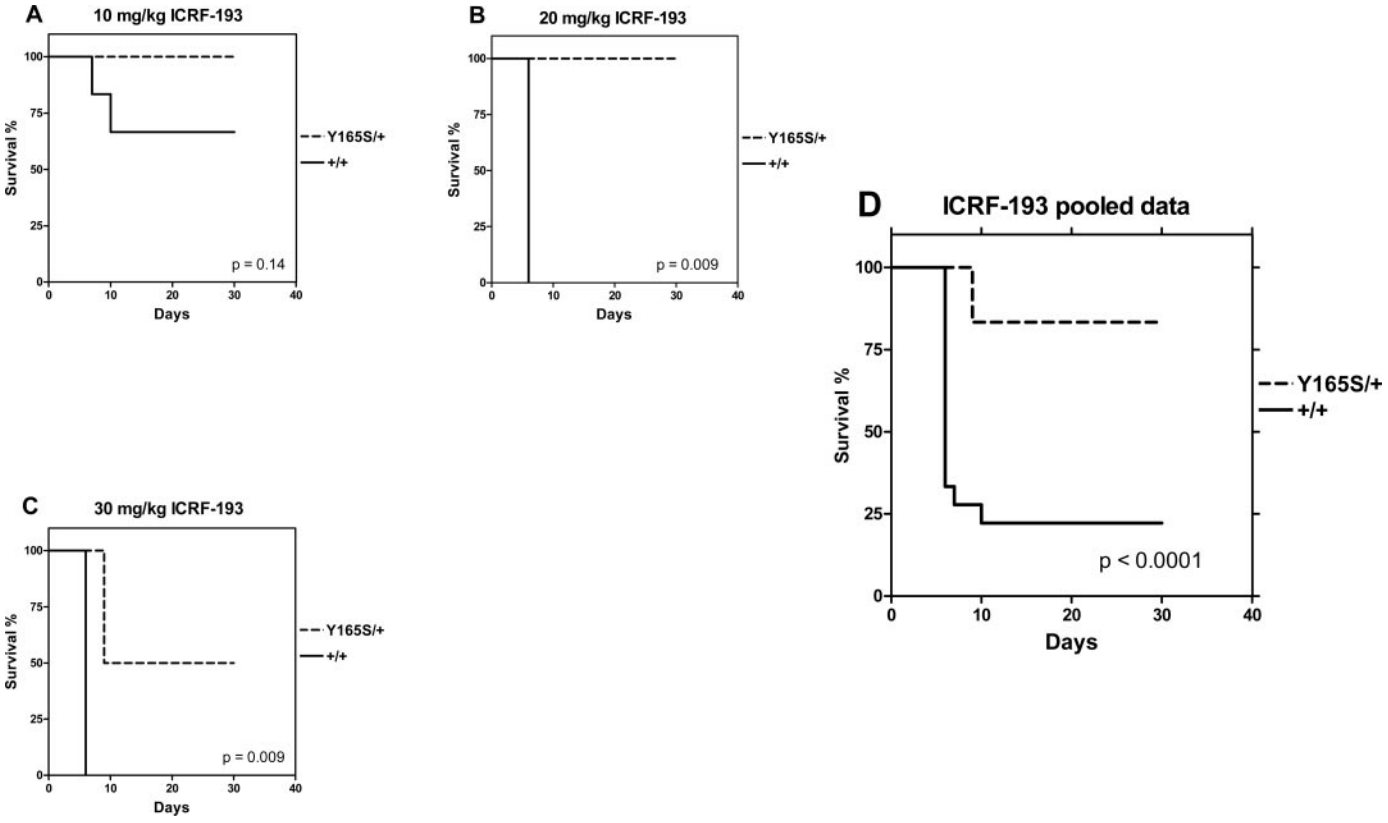

Fig. 5. Kaplan-Meyer survival plots of *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ mice after treatment with different dose levels of ICRF-193 given daily on days 0, 1, and 2 of the study. A, 10 mg/kg ICRF-193; B, 20 mg/kg ICRF-193; C, 30 mg/kg ICRF-193. D, Kaplan-Meyer survival plots including all mice treated with ICRF-193 in A to C. In A to C, the number of animals in each group was six. In D, the number of mice in each group was 18. p Values from log-rank analysis are shown on the figure for each dose-level.

the same 3-day scheme, and blood/bone marrow samples were taken on day 3. As seen for ICRF-187, the *TOP2A*$^{Y165S/+}$ genetic background reduced the effect of ICRF-193 on bone marrow cell depression at all three doses (Fig. 7A, $p \leq 0.02$). The peripheral white blood cell counts were also lower in *TOP2A*$^{+/+}$ mice compared with *TOP2A*$^{Y165S/+}$ mice after daily doses of 20 or 30 mg/kg for 3 days (Fig. 7B; $p = 0.01$ in both comparisons).

Intestinal Alterations Induced by ICRF-187 and ICRF-193 Concentrations Resulting in Selective Toxicity Toward wt Mice Are Insufficient to Explain Death. To examine the possibility that the mice died as a result of intestinal toxicity, we exposed groups of wt and *TOP2A*$^{Y165S/+}$ mice to three daily doses of 30 mg/kg ICRF-193 or 550 mg/kg ICRF-187 and performed histopathology on longitudinal sections of the duodenum, jejunum, and ileum by using hematoxylin and eosin straining. We used these concentrations because they had been found previously to confer selective toxicity to wt mice in the survival assay. Furthermore, as a control experiment, we performed hemotoxicity measurements and could demonstrate that the expected differential bone marrow and WBC depression did actually occur. The sections were then evaluated in a blinded fashion. Although an increase in apoptotic basal crypt cells and enlarged nuclei in parabasal cells was observed in both treated wt and *TOP2A*$^{Y165S/+}$ animals, no severe changes such as ulceration or shedding were found.

Thus, the extent of the cellular alterations was judged to be insufficient to cause severe intestinal toxicity. This result is in line with the fact that the mice (wt and *TOP2A*$^{Y165S/+}$) showed no signs of diarrhea during the toxicity assays. Together, these results indicate that intestinal toxicity was not the cause of the selective killing of wt mice by ICRF-193 and ICRF-187.

Discussion

The bisdioxopiperazines were originally developed as agents showing moderate anticancer activity as single agents, with their main side effect being myelosuppression, which was seen at pharmacologically active concentrations (Bakowski et al., 1979; Von Hoff et al., 1981). Besides interacting with topoisomerase II, ICRF-187 is also a strong iron chelator (Hasinoff et al., 1998). It is the ring-opened hydrolysis product ADR-925 that has activity as iron chelator. ICRF-187 is approved with the indication of reducing anthracycline-induced cardiomyopathy [dexrazoxane (Cardioxane, Zinecard)]. It is believed that the iron chelating activity of its ring-opened hydrolysis product, ADR-925, is responsible for its cardioprotective effect through the removal of iron from its highly toxic complexes with anthracyclines (Hasinoff et al., 2003b), in turn protecting the cardiac myocytes against mitochondrial damage caused by redox-cycling of the anthracycline-iron complex (Hasinoff et al., 2003a). However, an effect through topoisomerase II inhibition has never been

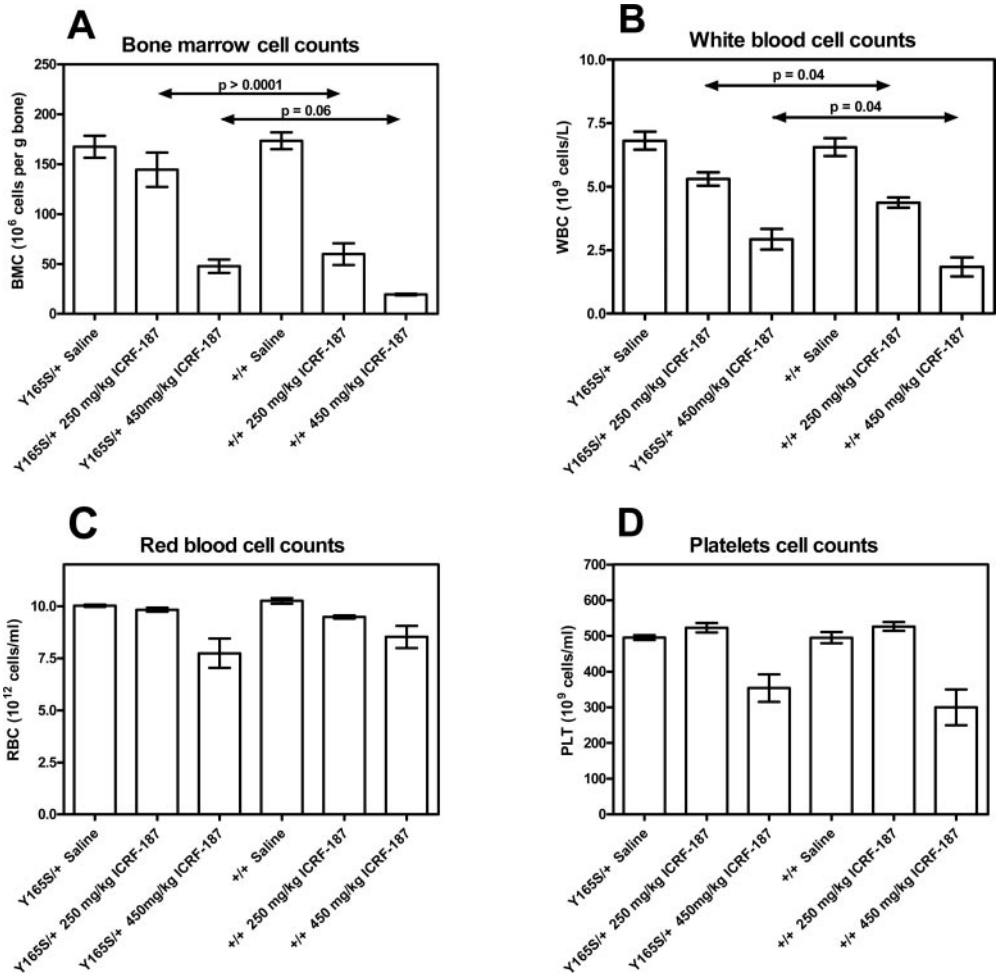

Fig. 6. Hematological toxicity induced by different dose levels of ICRF-187 in *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ mice given the shown doses daily for 3 consecutive days. Bone marrow and blood samples were taken the day after the last dose. A, effect of ICRF-187 on bone marrow cell counts. B, effect of ICRF-187 on peripheral white blood cell counts. C, effect of ICRF-187 on red blood cell counts. D, effect of ICRF-187 on platelet counts. Error bars represent S.E.M. of data from 6 to 12 individual animals.

excluded. ICRF-187 is also approved as an antidote for accidental anthracycline extravasation (dexrazoxane; Mouridsen et al., 2007), and the role of iron chelation versus topoisomerase II inhibition in this process still remains to be fully elucidated. Finally, ICRF-187 has proven effective in modulating the pharmacological effects of topoisomerase II poisons in preclinical models (Holm et al., 1998; Hofland et al., 2005b), and the role for topoisomerase IIα versus topoisomerase IIβ in these processes also needs to be fully understood.

We here present an animal model developed with the aim of elucidating the role of topoisomerase IIα in the pharmacological effects of bisdioxopiperazines in vivo. In these studies, we also include the more potent (toward topoisomerase II) analog ICRF-193 (Hasinoff et al., 1995). We chose to look at the topoisomerase IIα isoform because we have described previously several mutations in this enzyme rendering it highly resistant to the actions of bisdioxopiperazines (Sehested et al., 1998; Wessel et al., 1999, 2002; Jensen et al., 2000b). In our present work, we take advantage of a Y165S mutation in human topoisomerase IIα that is dominant and specific with regard to bisdioxopiperazine resistance (Wessel et al., 2002).

Crosses between heterozygous *TOP2A*$^{Y165S/+}$ animals failed to produce any homozygous *TOP2A*$^{Y165S/Y165S}$ mice. Instead, only *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ animals were born at the ratio of 1:2, suggesting that the homozygous *TOP2A*$^{Y165S/Y165S}$ embryos die in utero. The mechanistic basis for this is not clear, but the simplest explanation would be that the level of topoisomerase IIα catalytic activity in *TOP2A*$^{Y165S/Y165S}$ embryos is insufficient to meet the requirements during development. Another possibility would be that the embryonic lethality of the homozygous *TOP2A*$^{Y165S/Y165S}$ mutation is related to the decreased levels of DNA-stimulated ATP hydrolysis seen with the Y165S protein (Sorensen et al., 2005). The DNA transport mechanism of eukaryotic topoisomerase II is directly coupled to ATP hydrolysis (Baird et al., 1999). Consequently, the reduced ATP hydrolysis of the Y165S protein could be associated with subtle alterations in its catalytic DNA strand passage reaction that could be lethal in the absence of wt topoisomerase IIα. The phenotype of the homozygous mutant embryos will be described elsewhere.

As described above, we assumed that the heterozygous *TOP2A*$^{Y165S/+}$ animals would show an increased resistance to treatment with bisdioxopiperazines. To test this hypothesis, we conducted toxicity experiments with groups of wt and mutant mice (Figs. 4 and 5). When the survival data for ICRF-187, as presented in Fig. 4, A to D, are pooled, it is evident that the mutant mice are resistant to the general toxicity of ICRF-187 (Fig. 4E). In experiments in which the more topoisomerase II-potent analog ICRF-193 was used, topoisomerase IIα mutant status was clearly a highly significant determinant for toxicity even in small experiments where groups of six mice were used (Fig. 5, B and C). When pooled, these ICRF-193 experiments demonstrate that the

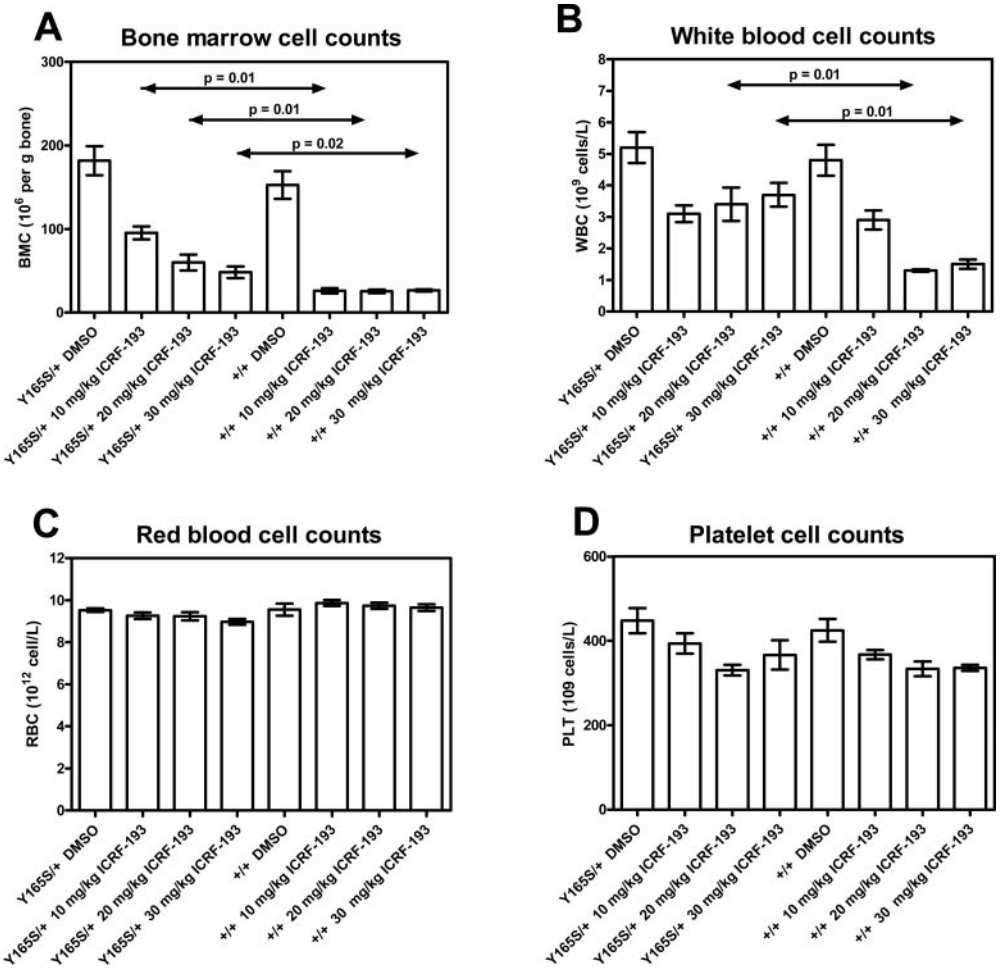

Fig. 7. Hematological toxicity induced by different dose levels of ICRF-193 in *TOP2A*$^{+/+}$ and *TOP2A*$^{Y165S/+}$ mice given the shown doses daily for 3 consecutive days. Bone marrow and blood samples were taken the day after the last dose. A, effect of ICRF-193 on bone marrow cell counts. B, effect of ICRF-193 on peripheral white blood cell counts. C, effect of ICRF-193 on red blood cell counts. D, effect of ICRF-193 on platelet counts. Error bars represents S.E.M. of data from seven individual animals.

mutant mice are highly resistant toward the general toxicity of ICRF-193 (Fig. 5D).

To identify a biological mechanism underlying the bisdioxopiperazine resistance conferred by the *TOP2A*$^{Y165S/+}$ allele, we looked at a well known effect of bisdioxopiperazines, namely myelosuppression (Bakowski et al., 1979; Von Hoff et al., 1981). Bisdioxopiperazines, including ICRF-187 and ICRF-193, trap eukaryotic topoisomerase II as a closed protein clamp on DNA, which is toxic to cells (Ishida et al., 1995; van Hille and Hill, 1998; Jensen et al., 2000a; Xiao et al., 2003; Oestergaard et al., 2004). This configuration of topoisomerase II on DNA has furthermore been associated with increased levels of DNA strand breaks in some reports (Huang et al., 2001; Hajji et al., 2003). Because bone marrow stem cells are rapidly proliferating and contain high levels of topoisomerase IIα, we hypothesized that topoisomerase IIα would be involved in this effect and that the heterozygous *TOP2A*$^{Y165S/+}$ allele might abrogate or reduce this effect in *TOP2A*$^{Y165S/+}$ animals (Wessel et al., 2002). This turned out to be the case. The *TOP2A*$^{Y165S/+}$ allele had a significant impact on the ability of both ICRF-187 and ICRF-193 to cause depression of bone marrow cell counts (Figs. 6A and 7A). The same picture was seen for peripheral white blood cell counts, which were also significantly less depressed in *TOP2A*$^{Y165S/+}$ animals (Figs. 6B and 7B). These results for the first time demonstrate directly that topoisomerase IIα is playing an important and significant role in the myelosuppressive effect of bisdioxopiperazines. Furthermore, our finding that intestinal toxicity was not related to the selective toxicity of ICRF-187 and ICRF-193 toward the wt mice points toward hemotoxicity being responsible for this.

We found the RBC and PLT counts to be decreased after ICRF-187 treatment ($p < 0.0001$ for both RBC and PLT), regardless of genotype ($p = 0.48$ and 0.60, respectively; see Fig. 6, C and D). This suggests that the acute toxicity of ICRF-187 on RBC and platelets is caused by a mechanism independent of topoisomerase IIα, which also is in concordance with the fact that the time point for blood sampling (day 3 after start of treatment) is too early to reveal an effect on the erythropoietic precursor cells in the bone marrow/spleen, because erythrocytes and platelets have a longer transit time in the blood than white blood cells. As seen for ICRF-187, the red blood cells and platelet counts also tended to decrease with increasing ICRF-193 dose (Fig. 7, C and D; $p < 0.0001$ in both cell types), and in the case of platelets, there was no effect of genotype, whereas the *TOP2A*$^{Y165S/+}$ mice had lower RBC counts than *TOP2A*$^{+/+}$ mice ($p = 0.0003$). This difference in RBC counts is probably caused by the dehydration and consequent hemoconcentration in the *TOP2A*$^{+/+}$ mice after treatment with ICRF-193, because these mice were much less tolerant to the general toxicity of ICRF-193. The short-term effects observed on RBC and platelets after bisdioxopiperazine treatments are not associated with effects depending on topoisomerase IIα but possibly connected to the compound's iron chelating properties.

In this article, we present a model for assessing the role for topoisomerase IIα in bisdioxopiperazine action in the mouse. We found this enzyme to be associated with resistance to the general toxicity of both ICRF-187 and ICRF-193, and we used this model to demonstrate directly that topoisomerase IIα is involved in mediating bisdioxopiperazine-induced myelosuppression. Experiments aimed at clarifying the role of the *TOP2A*$^{Y165S/+}$ allele on the ability of ICRF-187 to antagonize the effects of topoisomerase II poisons in vivo are under way. We also aim at using this model to investigate the role of topoisomerase IIα in ICRF-187-mediated protection against anthracycline-induced cardiomyopathy and to assess whether topoisomerase II α might also be involved in ICRF-187-mediated protection against anthracycline-mediated extravasation necrosis.

Acknowledgments

The technical assistance of Annette Nielsen, Sanne Christiansen, and Mette Frandsen is highly appreciated. The expertise of Anita, Lillian, and Jannie in animal care is appreciated. Support from the Danish Biotechnology Instrument Center is also highly appreciated.

References

Akimitsu N, Adachi N, Hirai H, Hossain MS, Hamamoto H, Kobayashi M, Aratani Y, Koyama H, and Sekimizu K (2003) Enforced cytokinesis without complete nuclear division in embryonic cells depleting the activity of DNA topoisomerase IIalpha. *Genes Cells* **8:**393–402.

Austin CA and Marsh KL (1998) Eukaryotic DNA topoisomerase II beta. *Bioessays* **20:**215–226.

Baird CL, Gordon MS, Andrenyak DM, Marecek JF, and Lindsley JE (2001) The ATPase reaction cycle of yeast DNA topoisomerase II. Slow rates of ATP resynthesis and P_i release. *J Biol Chem* **276:**27893–27898.

Baird CL, Harkins TT, Morris SK, and Lindsley JE (1999) Topoisomerase II drives DNA transport by hydrolyzing one ATP. *Proc Natl Acad Sci U S A* **96:**13685–13690.

Bakowski MT, Prentice HG, Lister TA, Malpas JS, McElwain TJ, and Powles RL (1979) Limited activity of ICRF-159 in advanced acute leukemia. *Cancer Treat Rep* **63:**127–129.

Classen S, Olland S, and Berger JM (2003) Structure of the topoisomerase II ATPase region and its mechanism of inhibition by the chemotherapeutic agent ICRF-187. *Proc Natl Acad Sci U S A* **100:**10629–10634.

Cvetković RS and Scott LJ (2005) Dexrazoxane: a review of its use for cardioprotection during anthracycline chemotherapy. *Drugs* **65:**1005–1024.

Falck J, Jensen PB, and Sehested M (1999) Evidence for repressional role of an inverted CCAAT box in cell cycle-dependent transcription of the human DNA topoisomerase IIα gene. *J Biol Chem* **274:**18753–18758.

Hajji N, Pastor N, Mateos S, Dominguez I, and Cortes F (2003) DNA strand breaks induced by the anti-topoisomerase II bis-dioxopiperazine ICRF-193. *Mutat Res* **530:**35–46.

Hasinoff BB, Hellmann K, Herman EH, and Ferrans VJ (1998) Chemical, biological and clinical aspects of dexrazoxane and other bisdioxopiperazines. *Curr Med Chem* **5:**1–28.

Hasinoff BB, Kuschak TI, Yalowich JC, and Creighton AM (1995) A QSAR study comparing the cytotoxicity and DNA topoisomerase II inhibitory effects of bisdioxopiperazine analogs of ICRF-187 (dexrazoxane). *Biochem Pharmacol* **50:**953–958.

Hasinoff BB, Schnabl KL, Marusak RA, Patel D, and Huebner E (2003a) Dexrazoxane (ICRF-187) protects cardiac myocytes against doxorubicin by preventing damage to mitochondria. *Cardiovasc Toxicol* **3:**89–99.

Hasinoff BB, Schroeder PE, and Patel D (2003b) The metabolites of the cardioprotective drug dexrazoxane do not protect myocytes from doxorubicin-induced cytotoxicity. *Mol Pharmacol* **64:**670–678.

Hofland KF, Thougaard AV, Dejligbjerg M, Jensen LH, Kristjansen PE, Rengtved P, Sehested M, and Jensen PB (2005a) Combining etoposide and dexrazoxane synergizes with radiotherapy and improves survival in mice with central nervous system tumors. *Clin Cancer Res* **11:**6722–6729.

Hofland KF, Thougaard AV, Sehested M, and Jensen PB (2005b) Dexrazoxane protects against myelosuppression from the DNA cleavage-enhancing drugs etoposide and daunorubicin but not doxorubicin. *Clin Cancer Res* **11:**3915–3924.

Holm B, Sehested M, and Jensen PB (1998) Improved targeting of brain tumors using dexrazoxane rescue of topoisomerase II combined with supralethal doses of etoposide and teniposide. *Clin Cancer Res* **4:**1367–1373.

Huang KC, Gao H, Yamasaki EF, Grabowski DR, Liu S, Shen LL, Chan KK, Ganapathi R, and Snapka RM (2001) Topoisomerase II poisoning by ICRF-193. *J Biol Chem* **276:**44488–44494.

Ishida R, Hamatake M, Wasserman RA, Nitiss JL, Wang JC, and Andoh T (1995) DNA Topoisomerase II is the molecular target of bisdioxopiperazine derivatives ICRF-159 and ICRF-193 in *Saccharomyces cerevisiae*. *Cancer Res* **55:**2299–2303.

Jensen LH, Nitiss KC, Rose A, Dong J, Zhou J, Hu T, Osheroff N, Jensen PB, Sehested M, and Nitiss JL (2000a) A novel mechanism of cell killing by anti-topoisomerase II bisdioxopiperazines. *J Biol Chem* **275:**2137–2146.

Jensen LH, Renodon-Cornière A, Wessel I, Langer SW, Sokilde B, Carstensen EV, Sehested M, and Jensen PB (2002) Maleimide is a potent inhibitor of topoisomerase II in vitro and in vivo: a new mode of catalytic inhibition. *Mol Pharmacol* **61:**1235–1243.

Jensen LH, Wessel I, Moller M, Nitiss JL, Sehested M, and Jensen PB (2000b) N-terminal and core-domain random mutations in human topoisomerase II alpha conferring bisdioxopiperazine resistance. *FEBS Lett* **480:**201–207.

Kobayashi M, Adachi N, Aratani Y, Kikuchi A, and Koyama H (2001) Decreased

topoisomerase IIalpha expression confers increased resistance to ICRF-193 as well as VP-16 in mouse embryonic stem cells. *Cancer Lett* **166:**71–77.
Langer SW, Sehested M, and Jensen PB (2000) Treatment of anthracycline extravasation with dexrazoxane. *Clin Cancer Res* **6:**3680–3686.
Morris SK, Baird CL, and Lindsley JE (2000) Steady-state and rapid kinetic analysis of topoisomerase II trapped as the closed-clamp intermediate by ICRF-193. *J Biol Chem* **275:**2613–2618.
Mouridsen H, Langer S, Buter J, Eidtmann H, Rosti G, de WM, Knoblauch P, Rasmussen A, Dahlstrom K, Jensen P, et al. (2007) Treatment of anthracycline extravasation with Savene (dexrazoxane): results from two prospective clinical multicentre studies. *Ann Oncol* **18:**546–550.
Oestergaard VH, Knudsen BR, and Andersen AH (2004) Dissecting the cell-killing mechanism of the topoisomerase II-targeting drug ICRF-193. *J Biol Chem* **279:**28100–28105.
Ramírez-Solis R, Davis AC, and Bradley A (1993) Gene targeting in embryonic stem cells. *Methods Enzymol* **225:**855–878.
Renodon-Cornière A, Jensen LH, Nitiss JL, Jensen PB, and Sehested M (2003) Analysis of bisdioxopiperazine dexrazoxane binding to human DNA topoisomerase II alpha: decreased binding as a mechanism of drug resistance. *Biochemistry* **42:**9749–9754.
Roca J, Ishida R, Berger JM, Andoh T, and Wang JC (1994) Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp. *Proc Natl Acad Sci U S A* **91:**1781–1785.
Roca J and Wang JC (1994) DNA transport by a type II DNA topoisomerase: evidence in favor of a two-gate mechanism. *Cell* **77:**609–616.
Sehested M, Wessel I, Jensen LH, Holm B, Oliveri RS, Kenwrick S, Creighton AM, Nitiss JL, and Jensen PB (1998) Chinese hamster ovary cells resistant to the topoisomerase ii catalytic inhibitor ICRF-159: a Tyr49Phe mutation confers high-level resistance to bisdioxopiperazines. *Cancer Res* **58:**1460–1468.
Sorensen TK, Grauslund M, Jensen PB, Sehested M, and Jensen LH (2005) Separation of bisdioxopiperazine- and vanadate resistance in topoisomerase II. *Biochem Biophys Res Commun* **334:**853–860.
Swiatek PJ and Gridley T (1993) Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. *Genes Dev* **7:**2071–2084.
van Hille B and Hill BT (1998) Yeast cells expressing differential levels of human or yeast DNA topoisomerase II: a potent tool for identification and characterization of topoisomerase II-targeting antitumour agents. *Cancer Chemother Pharmacol* **42:**345–356.
Von Hoff DD, Howser D, Lewis BJ, Holcenberg J, Weiss RB, and Young RC (1981) Phase I study of ICRF-187 using a daily for 3 days schedule. *Cancer Treat Rep* **65:**249–252.
Wang JC (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* **3:**430–440.
Wessel I, Jensen LH, Jensen PB, Falck J, Rose A, Roerth M, Nitiss JL, and Sehested M (1999) Human small cell lung cancer NYH cells selected for resistance to the bisdioxopiperazine topoisomerase II catalytic inhibitor ICRF-187 demonstrate a functional R162Q mutation in the Walker A consensus ATP binding domain of the alpha isoform. *Cancer Res* **59:**3442–3450.
Wessel I, Jensen LH, Renodon-Cornière A, Sorensen TK, Nitiss JL, Jensen PB, and Sehested M (2002) Human small cell lung cancer NYH cells resistant to the bisdioxopiperazine ICRF-187 exhibit a functional dominant Tyr165Ser mutation in the Walker A ATP binding site of topoisomerase II alpha. *FEBS Lett* **520:**161–166.
Xiao H, Mao Y, Desai SD, Zhou N, Ting CY, Hwang J, and Liu LF (2003) The topoisomerase IIβ circular clamp arrests transcription and signals a 26S proteasome pathway. *Proc Natl Acad Sci U S A* **100:**3239–3244.
Yang X, Li W, Prescott ED, Burden SJ, and Wang JC (2000) DNA topoisomerase IIbeta and neural development. *Science* **287:**131–134.

Address correspondence to: Dr. Lars Høllund Jensen, Experimental Pathology Unit, Department of Pathology, Rigshospitalet afs. 3731, Biocenter, Bygning 2, 3 sal., Ole Maaløes vej 5, DK-2100 Copenhagen O, Denmark. E-mail: lhj@topotarget.com