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Interaction of the DNA Topoisomerase II Catalytic Inhibitor *meso*-2,3-Bis(3,5-dioxopiperazine-1-yl)butane (ICRF-193), a Bisdioxopiperazine Derivative, with the Conserved Region(s) of Eukaryotic But Not Prokaryotic Enzyme

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ABSTRACT. ICRF-193 [*meso*-2,3-bis(3,5-dioxopiperazine-1-yl)butane], a bisdioxopiperazine compound, has been shown to be a catalytic inhibitor of DNA topoisomerase II by stabilizing the enzyme in the form of a closed "protein clamp," an intermediate form in the catalytic cycle (Roca *et al., Proc Natl Acad Sci USA* **91:** 1781–1785, 1994). In view of its usefulness as a probe in the functional analysis of the enzyme, we tried further to define the domain(s) of the enzyme interacting with the drug by examining its inhibitory activity on type II topoisomerases from various species of eukaryotes and prokaryotes. ICRF-193 inhibited the enzyme from yeast, fly, frog, plant, and mammals at IC_{50} values in the range of 1–13 μM. Experiments using fission yeast truncated mutant type II enzyme lacking both amino-terminal 74 amino acids and carboxy-terminal 265 amino acids revealed that ICRF-193 interacts with the 125 kDa "core" polypeptide of the enzyme. In contrast, prokaryotic type II enzymes, *Escherichia coli* DNA gyrase, topo IV, and phage T4 topo, were not affected by the drug. From these results, the domain(s) common to eukaryotic but not to prokaryotic type II enzymes interacting with ICRF-193 was speculated. BIOCHEM PHARMACOL **54**;5:545–550, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. DNA topoisomerase; ICRF-193; cleavable complex; DNA gyrase; catalytic inhibitor; anticancer drug

Type II DNA topoisomerases (topo II**; EC 5.99.1.3) are essential enzymes that play important roles in regulating topological states of DNA by transporting a DNA double helix through a transient double-strand gap created by the enzyme in the same or in another double helix in an ATP-dependent fashion [1, 2]. Topo II is required in many aspects of DNA metabolism including DNA replication, transcription, and recombination, and it plays crucial roles in chromosome structure, condensation/decondensation, and segregation in mitosis [1–3]. These enzymes are known to be the primary cellular targets for some of the most widely prescribed anticancer drugs used in the treatment of human disease [4, 5].

Topo II enzyme can be subdivided into three distinct domains based on its homology to the bacterial type II enzyme, DNA gyrase. When eukaryotic topo II is compared with DNA gyrase at the amino acid level, a colinearity is observed between the GyrB protein and the N-terminal part of topo II, and between the GyrA protein and the central part of topo II. The extreme C-terminal regions—some 300 amino acids—of the eukaryotic enzymes, characterized by a cluster of charged amino acids, however, are unrelated to the bacterial enzyme and are highly divergent among eukaryotes. Whereas eukaryotic type II enzymes are homodimeric, the prokaryotic enzymes gyrase and topo IV are both tetramers consisting of two subunits, GyrA and GyrB, and ParC and ParE, respectively. Topo from phage T4 is a hexamer composed of three distinct subunits encoded by genes 39, 52, and 60 [6]. The functional conservation of these enzymes is remarkable. While they share only about 50% sequence identity, the *Schizosaccharomyces pombe*, *Drosophila*, mouse, and human topo IIα genes can complement a *Saccharomyces cerevisiae* topo II mutation [7–11].

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** *Abbreviations:* ICRF-193, *meso*-2,3-bis(3,5-dioxopiperazine-1-yl)butane; m-AMSA, 4′-(9-acridinylamino)methanesulfon-m-anisidide; MDR, multidrug resistance; topo, topoisomerase; ts, temperature-sensitive; VM-26, 4′-demethylepipodophyllotoxin-9-[4,6-(O-thenylidene)-β-D-glycopyranoside]; and VP-16, 4′-demethylepipodophyllotoxin-9-[4,6-(O-ethylidene)-β-D-glycopyranoside].

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Numerous potent antitumor drugs have been found to target topo II enzymes by stimulating the formation of a covalently linked enzyme–DNA "cleavable complex" that is presumed to trigger apoptotic processes. These include DNA intercalators such as Adriamycin® and m-AMSA, and non-intercalators such as epipodophyllotoxins, VP-16 and VM-26. Recently, a novel class of topo II inhibitors has been reported; these inhibitors do not stabilize the cleavable complexes, but inhibit catalytic activity of the enzyme [3]. They include bisdioxopiperazines, ICRF-154, -193 [12, 13] and -187 [14], suramin [15], merbarone [16], and aclarubicin [17]. We have shown recently that (1) ICRF-193 inhibits the enzyme by stabilizing the reaction intermediate closed clamp form of the enzyme [18]; (2) ICRF-193 targets topo II in living yeast cells using ts-top 2 mutant [19]; (3) ICRF-193 inhibits SV40 DNA replication *in vitro* [20] and *in vivo* [21] at the final stage of replication, i.e. segregation of the catenated daughter molecules; and (4) ICRF-193, when applied to growing Chinese hamster ovary (CHO) cells, leads to aberrant mitosis and polyploidization of the cells due to the failure of chromosome segregation without affecting other cellular events including DNA replication in the presence of the drug [22]. All of these results support the contention that topo II plays an essential role in chromosome condensation/decondensation and segregation at mitosis [23]. The role of the enzyme in chromosome dynamics has been amply demonstrated in biochemical [24, 25] and pharmacological [26–29] studies in other laboratories.

In view of the unique mode of action of ICRF-193 and its usefulness as a probe in the functional analysis of the enzyme, the present study was undertaken in an attempt to elucidate the domain(s) of the enzyme interacting with ICRF-193 by examining whether this drug inhibits type II topoisomerases from various species of eukaryotes and prokaryotes.

MATERIALS AND METHODS

Drugs and Chemicals

ICRF-193 was obtained from the Zenyaku Kogyo Co., Ltd. (Tokyo, Japan) and was dissolved in DMSO at 10 mM as a stock solution. All other chemicals were of reagent grade.

DNA Substrates

Supercoiled plasmid pBR322 DNA was purified from *Escherichia coli* harboring the plasmid by the conventional method [30]. Relaxed pBR322 plasmid DNA was obtained by relaxation of the supercoiled DNA by purified human topo I. P4 phage DNA was isolated from tail-less phage heads according to the procedure of Liu *et al.* [31].

Enzymes

Purified *Drosophila* topo II and T4 phage topo were gifts from Dr. N. Osheroff, Vanderbilt University, Nashville, TN, U.S.A., and from Dr. K. Kreuzer, Duke University, Durham, NC, U.S.A., respectively. *E. coli* DNA gyrase and topo IV were purified as described [32]. Calf and mouse topo II was partially purified from calf thymus and mouse Ehrlich ascites tumor cells, respectively, by the method of Halligan *et al.* [33]. Human topo II was purchased from TopoGEN, Columbus, OH, U.S.A. Full-length and truncated forms of yeast topo II were obtained from *S. pombe* TM141, a ts mutant of *top2*, harboring pSPTOP2 or pKZ59 encoding full-length topo II and mutant topo II lacking both the N-terminal 74 and the C-terminal 265 amino acids, respectively, as described previously [34]. The host and the plasmids were provided from Dr. M. Yanagida, Kyoto University, Kyoto, Japan. Crude cell extracts were prepared by disrupting the yeast cells with glass beads in 0.4 M NaCl, 1 mM EDTA, and 10 mM Tris–HCl (pH 7.5), and were used for immunoblotting and for the activity assay.

Topo II Activity Assay

Topo II activity was assayed by the ATP-dependent unknotting of knotted DNA or relaxation of supercoiled DNA. The unknotting activity was measured using knotted P4 phage DNA as a substrate as described by Liu *et al.* [31]. The relaxation activity of supercoiled Col E1 plasmid DNA was determined by the method of Uemura and Yanagida [35]. One unit of activity is defined as the activity of the enzyme that converts 200 ng of the substrate DNA to the reaction product under the conditions used. When inhibitors were tested, serially diluted drugs dissolved in DMSO were added to the reaction mixtures as specified in the figure legends. The DMSO concentration was kept at 5% in the reactions. Reaction products were subjected to electrophoresis in 0.8% agarose gels in TAE buffer (0.04 M Tris-acetate, pH 8.0, 0.001 M EDTA) at 50 V. The gels were stained with ethidium bromide and photographed under UV light.

RESULTS

Inhibition by ICRF-193 of Type II Topos from Eukaryotes but not those from Prokaryotes

We measured the ATP-dependent strand passing activities of topo II enzymes from various eukaryotes, i.e. unknotting activity using knotted phage P4 DNA as a substrate and relaxing activity using a supercoiled plasmid DNA. One unit equivalent of the activity was used to measure the inhibition by ICRF-193. Human topo II was inhibited by the drug with an IC_{50} value of 5 μM (data not shown; see Refs. 12 and 13 for details). Enzymes from other eukaryotes, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Xenopus laevis*, cauliflower, and calf, were all inhibited by ICRF-193

TABLE 1. IC_{50} Values of ICRF-193 on topo IIs derived from various species

Species	IC_{50} (μM)
Human placenta	5.5
Calf thymus	2.0
Xenopus laevis	12.6*
Drosophila melanogaster	0.9
Schizosaccharomyces pombe	1.9
Cauliflower	1.3

The IC_{50} values were calculated by densitometric scanning of the photographs of ethidium bromide stained gels followed by analysis on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, U.S.A., part number PB95-500195GEI). Independent experiments with some of the enzymes, performed at different times, gave values in the same range of concentrations.

* Assay was performed with crude extract.

to a variety of extents depending on the species from which they were derived (Table 1), thus demonstrating that ICRF-193 interacts with domains shared by all eukaryotic enzymes.

Next we examined whether the drug interacts with prokaryotic type II enzymes, *E. coli* DNA gyrase, topo IV, and phage T4 topo, as assayed by supercoiling of relaxed plasmid DNA, relaxation of supercoiled DNA, and unknotting of knotted phage P4 DNA, respectively. As shown in Fig. 1A, no effect, whatsoever, of ICRF-193 was observed on the activities of the bacterial enzymes, gyrase and topo IV, even at the highest concentration (500 μM). Although topo IV but not gyrase did unknot the knotted phage P4 DNA, ICRF-193 did not affect the enzyme in the unknotting assay (data not shown). Another prokaryotic type II enzyme, T4 phage topo, is known to be inhibited by typical eukaryotic topo II inhibitors such as m-AMSA, ellipticine derivatives, mitoxantrone, and the epipodophyllotoxins

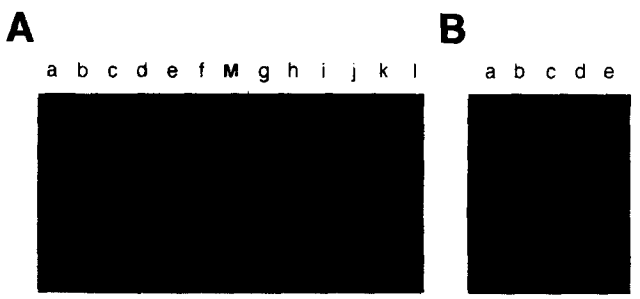

FIG. 1. Inability of ICRF-193 to inhibit *E. coli* DNA gyrase (panel A, lanes g–l), topo IV (panel A, lanes a–f), and T4 phage topo (panel B). (A) Supercoiled plasmid pBR322 DNA (lane a) or relaxed pBR322 DNA (lane g) was reacted with 1 unit each of topo IV or DNA gyrase, respectively, in the absence (lanes b and h) or presence of 500 (lanes c and i), 50 (lanes d and j), 5 (lanes e and k), and 0.5 (lanes f and l) μM ICRF-193. M, marker *Hin*dIII digest of λ phage DNA. (B) Knotted phage P4 DNA (lane a) was reacted with 1 unit of T4 phage topo in the absence (lane a) or presence of 10, 50, 100, and 500 μM ICRF-193 (lanes b–e, respectively).

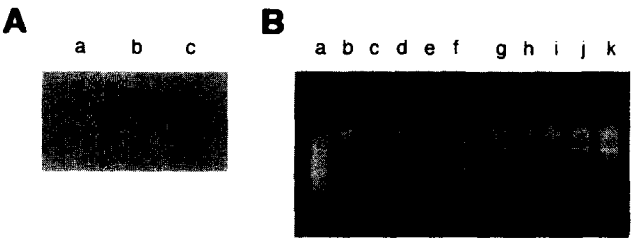

FIG. 2. Inhibition of unknotting activity of truncated 125 kDa core polypeptide of fission yeast topo II. Extracts were prepared from a ts-*top2* mutant of *S. pombe* harboring plasmids encoding the wild-type or truncated mutant form of topo II. Extracts from both wild-type and mutant-type topo II expressing cells were immunoblotted with monoclonal antibody 2H5 raised against N-terminal 75 kDa polypeptide of human topo IIα (panel A), and assayed for unknotting activity (panel B). (A) Extracts from host ts-*top2* mutant cells (lane a), wild-type topo II expressing cells (lane b), and truncated mutant topo II expressing cells (lane c) were immunoblotted. (B) One unit each of the wild-type topo II (lanes a–f) and truncated mutant topo II (lanes g–k) were assayed in the absence (lanes b and g) and presence of 1 (lanes c and h), 5 (lanes d and i), 10 (lanes e and j), and 50 (lanes f and k) μM ICRF-193. Lane a, substrate P4 phage DNA.

VP-16 and VM-26 [36]. However, ICRF-193 did not inhibit the enzyme at the highest concentration used (Fig. 1B). This is a very interesting result, which suggests that ICRF-193 interacts with the site(s) or domain(s) commonly shared by eukaryotic but not prokaryotic enzymes, and those distinct from the sites interacting with the classical eukaryotic topo II inhibitors mentioned above.

Inhibition by ICRF-193 of 125 kDa Core Polypeptide of Fission Yeast Topo II

Further experiments to define the binding site(s) of ICRF-193 were performed using a mutant yeast enzyme. Two kinds of plasmids harboring genes for *S. pombe* wild-type topo II and truncated mutant-type topo II at both N- (74 amino acids) and C- (265 amino acids) termini were transfected into TM141 (ts-*top2* mutant of *S. pombe*). The truncated enzyme was reported to be as active as the wild-type enzyme but unable to complement the null allele of the gene [34]. Cell extracts were prepared from the transformants, and the expression of the enzymes was examined by western blot analysis. As shown in Fig. 2A, no positive signal was detected in the extract from the host cell TM141 by anti-topo II monoclonal antibody 2H5, raised against *N*-terminal 75 kDa human topo II polypeptide (manuscript in preparation). However, 165 kDa wild-type and 125 kDa truncated enzymes were found in the extracts of the transformants. Enzymatic activities in both extracts were almost the same. One-unit equivalents of the two enzymes were assayed for sensitivity to ICRF-193. As shown in Fig. 2B, activities of both wild-type and truncated enzymes were inhibited by the drug, although the latter apparently was a little less sensitive, with IC_{50} values of 12.5

and 23.6 μM, respectively. The result demonstrates that ICRF-193 interacts with domains within the 125 kDa core polypeptide with amino acid residues 75–1219.

DISCUSSION

The present study was undertaken to elucidate the domains of topo II interacting with the specific inhibitor ICRF-193, a bisdioxopiperazine derivative, by examining its inhibitory activities on type II enzymes from various species of eukaryotes and prokaryotes. The drug is a very important agent in that (a) it is a catalytic inhibitor of topo II [12, 13], and (b) its sole *in vivo* target is topo II [19]; thus, it is a useful probe in the functional analysis of the enzyme. We demonstrated that the drug inhibited all eukaryotic topo IIs examined, which were derived from yeast, fly, frog, plant, and mammals, whereas it did not affect prokaryotic type II enzymes such as E. *coli* DNA gyrase, topo IV, and T4 phage topo II. Thus, the drug appears to interact with domains shared by eukaryotic enzymes but presumably not by prokaryotic enzymes. It is also relevant to refer to the results reported by Huff and Kreuzer [36] that T4 phage topo is inhibited by conventional eukaryotic topo II inhibitors such as m-AMSA, ellipticines, mitoxantrone and the epipodophyllotoxins VP-16 and VM-26. We have also demonstrated that the drug inhibited N- and C-terminally truncated polypeptides of yeast topo II, suggesting that the domains interactive with ICRF-193 are located within this region called "core" polypeptide possessing amino acid residues from 75 to 1219.

When the sequences of various eukaryotic type II topos are compared, three discrete domains can be discerned: the N-terminal region homologous to the B subunit of bacterial gyrase (GyrB), a central region containing active site tyrosine and homologous to the GyrA subunit, and a C-terminal region characterized by clusters of charged amino acids [37–42]. Within the central region three common motifs, EGDSA, PLRGK, and IMTD(Q/A)D, conserved among all type II enzymes are found.

Susceptibility to protease digestion also defines at least three regions of the topo II polypeptide [11, 34, 43]. Thus, yeast topo II was cleaved by *Staphylococcus aureus* V8 protease at Glu-410 and Glu-1200, defining the three domains described above. A new cleavage site, Glu-680, appears upon binding of the ATP analogue AMPPNP. These regions may reflect distinct folded domains, since the cleavage sites are well conserved among enzymes from different species [11, 34, 43]. It is relevant to refer to the study of Roca *et al.* [18] showing that ICRF-193 holds topo II in a "closed clamp" conformation once it is bound to DNA, much like the form induced by the nonhydrolyzable ATP analogue AMPPNP. This may be taken to imply that one of the domains interacting with ICRF-193 is the N-terminal ATPase domain.

Many clinically relevant antitumor drugs act by inhibiting the topo IIs by trapping the enzyme on DNA in a covalently bound state, often referred to as "cleavable complex" [4]. The occurrence of MDR in tumor cells is frequent, and poses major obstacles to successful cancer chemotherapy. One form of MDR, frequently called atypical (at) MDR, is often correlated with the expression of mutated enzyme. These alterations render the tumor cells resistant to a number of topo II-targeting antitumor drugs of the cleavable complex-stabilizing type such as m-AMSA, VP-16, and VM-26. Many topo II mutations have been determined and mapped in two restricted regions, one in the vicinity of common motifs, EGDSA, PLRGK and IMTD(Q/A)D, around amino acid residues 430–490, and the other close to the active site tyrosine around residues 740–860 [44, 45]. These sites are presumed to be the sites interacting with the conventional topo II inhibitors. One of the VP-16-resistant cell lines, human epidermoid carcinoma cells, KB/VP-2 [46], harboring a point mutation in the topo IIα gene at residue 861 changing serine to phenylalanine was tested for sensitivity to ICRF-193. The cells were partially cross-resistant to ICRF-193*, the result suggesting that ICRF-193 interacts with at least some domain(s) overlapping active site Tyr-804. However, the identification of the enzyme site(s) interactive with the drug awaits further investigation including ICRF-193-resistant mutation of the enzyme.

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*Personal communication from Dr. K. Kohno, Department of Molecular Biology, University of Occupational and Environmental Health, Fukuoka, Japan. Cited with permission.

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