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RESCUE OF *SCHIZOSACCHAROMYCES POMBE* FROM CAMPTOTHECIN-MEDIATED DEATH BY A DNA TOPOISOMERASE I INHIBITOR, TAN-1518 A

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Abstract—TAN-1518 A is a cytotoxic agent with suppressive activity against Meth A fibrosarcoma *in vivo*. This compound inhibits calf thymus DNA topoisomerase I (Topo I) but does not stimulate cleavable complex formation in the nuclei of Chinese hamster ovary (CHO)-K1 cells, suggesting that it inhibits Topo I in a manner different from that of camptothecin (CPT). To clarify the mode of action of TAN-1518 A, we examined its effects on the eukaryotic microorganism *Schizosaccharomyces pombe (S. pombe)*, which does not require Topo I as an essential factor for growth. TAN-1518 A inhibited purified *S. pombe* Topo I as potently as did CPT. TAN-1518 A, unlike CPT, did not stimulate Topo I-induced DNA cleavage; instead, it inhibited CPT-induced cleavable complex formation. We constructed a *S. pombe* strain, IR9, that produced excess Topo I. IR9 was hypersensitive to CPT, but its growth was not affected by TAN-1518 A. The CPT-mediated death of IR9 cells was reduced dramatically in the presence of TAN-1518 A. These findings clearly demonstrate that TAN-1518 A is a specific inhibitor of Topo I in eukaryotic cells and also suggest that this agent inhibits some earlier step(s) that occurs before the formation of cleavable complex on DNA strands in the catalytic cycle of this enzyme.

Key words: TAN-1518 A; camptothecin; DNA topoisomerase I; cleavable complex; rescue; *Schizosaccharomyces pombe*

DNA topoisomerases are the nuclear enzymes responsible for DNA topology, and they are essential for DNA metabolism in mammalian cells, being involved in replication, transcription, and recombination [1]. Recently, these enzymes in cancer cells have attracted considerable attention as intracellular targets for cancer therapeutics, since many drugs with antitumor properties interact with them [2]. While there are many potent antitumor agents that inhibit Topo II†, few agents that inhibit Topo I have been identified [3–7]. The only Topo I inhibitor that has been characterized thus far is a plant alkaloid, CPT. CPT interrupts the breakage–reunion cycle of mammalian Topo I by trapping a reversible Topo I–DNA cleavable complex [8, 9]. The antitumor activity of CPT is most likely due to the processing of these cleavable complexes by the replication machinery in drug-treated cells, which induces the breakdown of chromosomal DNA, cell cycle arrest in the G_2 phase, and subsequent apoptosis [10–13].

TAN-1518 A is a cytotoxic agent with suppressive activity against Meth A fibrosarcoma *in vivo* (Fig. 1). This compound inhibits the relaxation activity of calf thymus Topo I as potently as CPT and suppresses the growth of various tumor cells, inducing apoptosis. Unlike CPT, TAN-1518 A does not stimulate the formation of cleavable complex in the nuclei of Chinese hamster ovary (CHO)-K1 cells and has weak activity in intercalating into DNA strands. This compound arrests the growth of human tumor cells in the G_1 phase of the cell cycle [14]. These findings indicate that TAN-1518 A inhibits Topo I in a manner different from that of CPT, but its precise mechanism of inhibition is still unknown. Mammalian

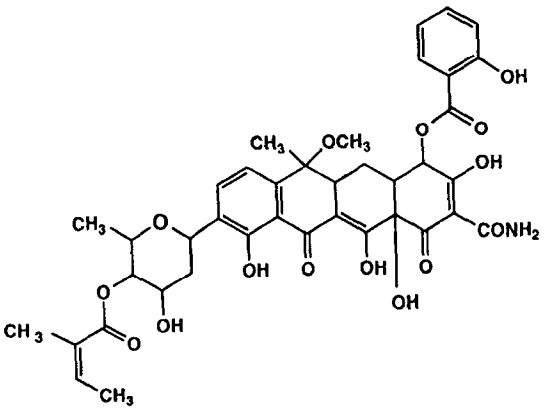

Fig. 1. Structure of TAN-1518 A.

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† Abbreviations: CPT, camptothecin; MIC, minimum inhibitory concentration; *S. pombe*, *Schizosaccharomyces pombe*; Topo I, DNA topoisomerase I, and Topo II, DNA topoisomerase II.

cells need both Topo I and Topo II for their survival. In contrast, eukaryotic microorganisms, such as the fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae*, do not require Topo I as an essential factor for their growth [15–17]. Mutants that lack the Topo I gene are resistant to CPT, and a strain that carries a plasmid that produces excess Topo I is hypersensitive to CPT [18–20]. These findings indicate that Topo I is the unique molecular target of CPT in yeast cells. If TAN-1518 A specifically inhibits Topo I in some step(s) before the formation of cleavable complex in yeast cells, it is possible that TAN-1518 A would interfere with the stabilization of cleavable complex induced by CPT and would suppress the induction of chromosomal DNA breakdown, resulting in the prevention of the CPT-mediated death of yeast cells. To confirm this speculation, we constructed a strain of *S. pombe* that produced excess Topo I.

In this study, we show evidence that TAN-1518 A is a specific inhibitor of Topo I in eukaryotic cells, and that its action differs from that of CPT.

MATERIALS AND METHODS

Yeast strains. S. pombe strains NCYC1914 (*leu1-32*, h^+) and NCYC1962 (*rad1*, h^-), were purchased from the National Collection of Yeast Cultures (NCYC). Topo I and chromosomal DNA were purified from NCYC1914. A PN501 strain (*rad1*, *leu1-32*, h^+) was obtained by a cross of NCYC1914 with NCYC1962.

Enzymes, nucleic acids, and chemicals. S. pombe Topo I was purified to homogeneity following the method of Goto *et al.* [21], except that the cells were disrupted with glass beads in a buffer [50 mM Tris–HCl (pH 7.4), 0.1 M KCl, 1 mM Na_3EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM 2-mercaptoethanol]. TAN-1518 A was prepared in our laboratories as described previously by Horiguchi *et al.* [14]. Supercoiled pBR322 DNA, ethidium bromide (EtBr), dithiothreitol (DTT), and proteinase K were purchased from the Wako Pure Chemical Co. (Osaka, Japan). CPT and BSA were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Amplitaq DNA polymerase was purchased from Perkin Elmer Cetus (Norwalk, CT, U.S.A.).

DNA relaxation and DNA cleavage reaction with Topo I. The reaction mixture contained 50 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 30 μg/mg BSA, 0.25 μg pBR322 DNA, 2 μL sample solution, and 1 μL Topo I in a total volume of 20 μL. The mixture was incubated at 37° for 40 min. The reaction was terminated by the addition of 6× loading buffer (0.25% bromophenol blue, 40% glycerol, and 2.5% SDS), and analysis was carried out by agarose gel electrophoresis, as described below (DNA relaxation assay). For DNA cleavage, the reaction was terminated by the addition of a solution containing 5% SDS and 2.5 mg/mL proteinase K, and incubation was performed for an additional 30 min at 37° (DNA cleavage assay). After the addition of loading buffer, samples were subjected to agarose gel electrophoresis in TBE buffer [89 mM Tris-borate (pH 8.3), 2 mM EDTA] containing 0.1% SDS. Gels were stained with EtBr and washed thoroughly with distilled water. The DNA band was visualized over UV light and photographed with Polaroid type 667 positive/negative films. One unit of Topo I activity was defined as the amount of the enzyme that converted 0.25 μg of supercoiled pBR322 DNA to its relaxed form in 40 min at 37°.

Construction of IR9. Standard genetic procedures for *S. pombe* were carried out as described by Gutz *et al.* [22]. The construction of the plasmid pIATOP1 is summarized in Fig. 2. We cloned the *S. pombe* $top1^+$ gene by the polymerase chain reaction method. Two oligonucleotides with *Sac*I linker, [5′-GCGAGCTCGTCATGACAACGAGTG-3′] and [5′-GCGAGCTCTTCACAATTTGTCTGCCG-3′] were designed for the 5′ and 3′ primers, respectively. One hundred microliters of the reaction mixture contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 units of Amplitaq DNA polymerase, 0.1 ng of each primer, and 1 μg of *S. pombe* chromosomal DNA. Thirty cycles of reactions at 94° for 1 min, 55° for 2 min, and 72° for 3 min were performed in a DNA thermal cycler (Perkin Elmer Cetus). The reaction products were digested with *Sac*I and separated on an agarose gel. The 2.7-kbp fragment was subcloned into pART1, an expression vector encoding alcohol dehydrogenase promoter (*adh*). The resultant pATOP1 was digested with *Eco*RI and *Sph*I, and a 3.4-kbp fragment was recovered. This fragment was ligated with linearized pUC119-LEU2 after blunt-ending, giving pIATOP1. pIATOP1 was linearized with *Bgl*II and transformed to pN501 by the lithium acetate method. The stable transformant, IR9, was screened on minimal essential medium, and its hypersensitivity to CPT was confirmed.

Rescue experiment. Freshly prepared IR9 cells (1×10^4) were inoculated into 5 mL of liquid YPD (yeast extract 1%, polypeptone 2%, and glucose 2%) medium in a test tube and shaken for 20 hr at 28°. Four hundred microliters of the culture was transferred to 40 mL of fresh medium in a 200-mL flask and shaken for 6 hr at 28°. When the cell density reached 1×10^6/mL, CPT and TAN-1518 A were added simultaneously, and the culture was further incubated for 6 hr. The cells were then harvested and washed with distilled water. Appropriately diluted culture was spread on a YPD agar plate and incubated for 2 days at 28°. Colonies were then counted, and relative survival was estimated. In the study on the effect of the timing of the addition of TAN-1518 A, TAN 1518 A was added 1 hr before or 1 hr after the addition of CPT, and the cells were incubated for 6 hr.

Measurement of MIC. The MIC value was determined by the agar dilution method [23]. Briefly, 1×10^6 cells in 5 μL of distilled water were inoculated on YPD agar plates each including 2-fold-diluted drug and incubated for 2 days at 28°. The minimum concentration of the drug at which the cells could not grow was defined as MIC.

RESULTS

Inhibition of relaxation activity of S. pombe *Topo*

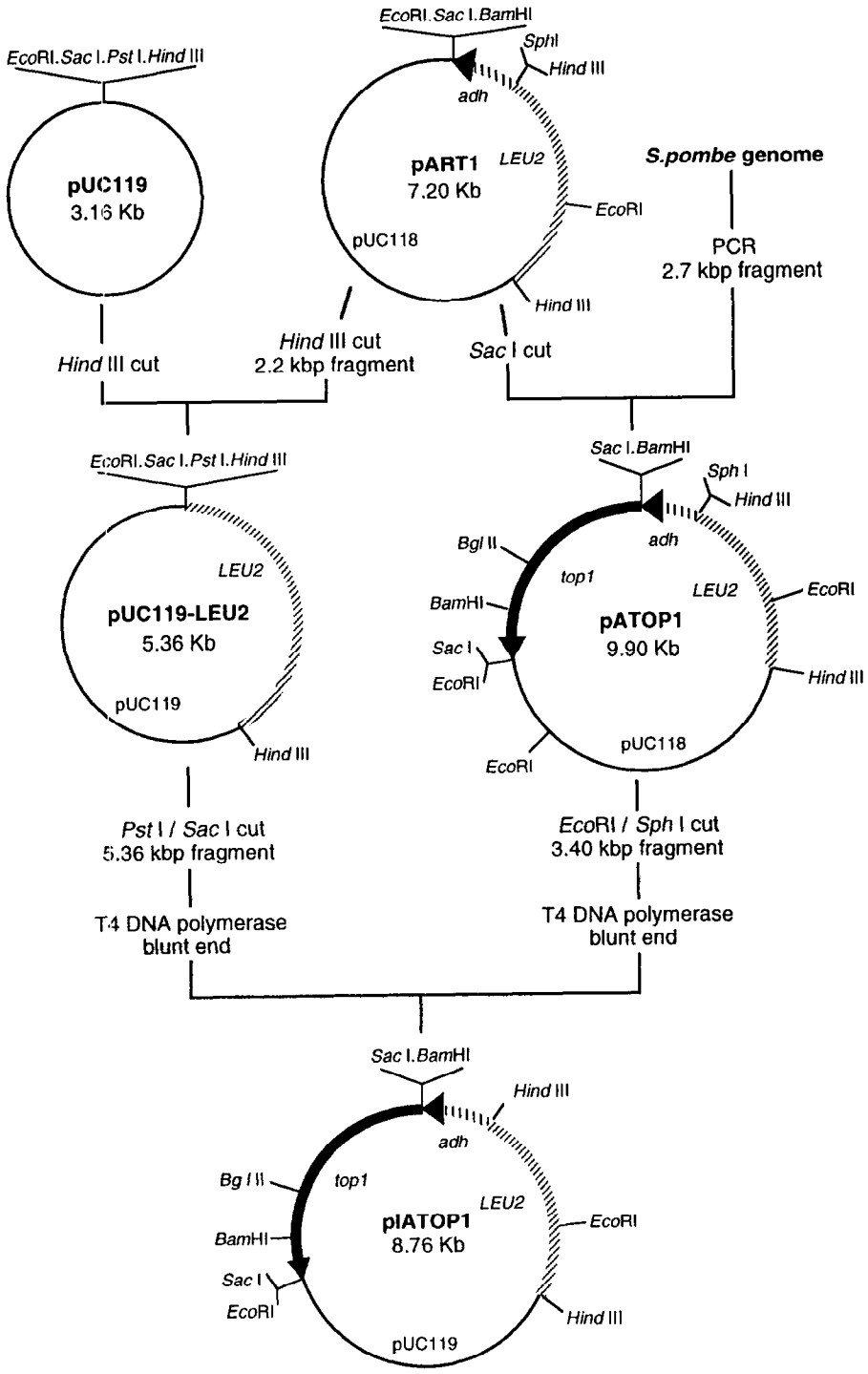

Fig. 2. Construction of pIATOP1.

I by TAN-1518 *A*. To test whether TAN-1518 A inhibited the catalytic activity of *S. pombe* Topo I, we performed a relaxation assay with supercoiled pBR322 DNA and purified *S. pombe* Topo I. TAN-1518 A inhibited the Topo I activity in a concentration-dependent manner, and partial inhibition was observed, even at a concentration of 5.0 μM. The inhibitory activity was comparable to that of CPT (Fig. 3). These findings demonstrate that TAN-1518 A inhibits yeast Topo I as potently as does CPT, as was the case with calf thymus Topo I, described previously [14].

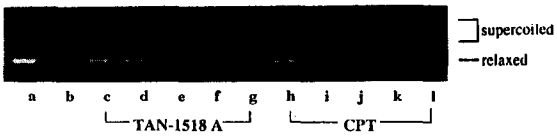

Fig. 3. Inhibitory effects of TAN-1518 A on the relaxation activity of *S. pombe* Topo I. Electrophoresis was carried out in a 0.7% agarose gel with 0.1% SDS at 100 V for 40 min. *Lane a*, ccc DNA control; *lane b*, relaxed DNA control; *lanes c* to *g*, TAN-1518 A; *lanes h* to *l*, CPT. Drug concentrations: *lanes c* and *h*, 100 μM; *lanes d* and *i*, 50 μM; *lanes e* and *j*, 20 μM; *lanes f* and *k*, 10 μM; *lanes g* and *l*, 5 μM.

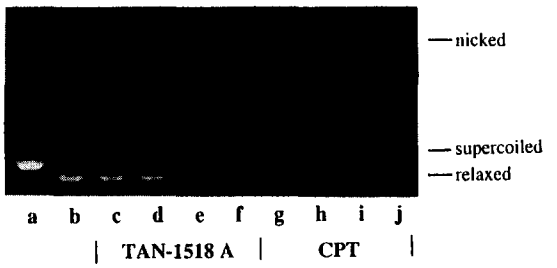

Fig. 4. Effects of TAN-1518 A on Topo I-mediated DNA cleavage. One hundred units of enzyme was used. Electrophoresis was carried out in a 1.2% agarose gel with 0.1% SDS and 0.5 μg/mL ethidium bromide at 100 V for 2 hr. *Lane a*, ccc DNA control; *lane b*, relaxed DNA control; *lanes c* to *f*, TAN-1518 A; *lanes g* to *j*, CPT. Drug concentrations: *lanes c* and *g*, 5 μM; *lanes d* and *h*, 10 μM; *lanes e* and *i*, 20 μM; *lanes f* and *j*, 50 μM.

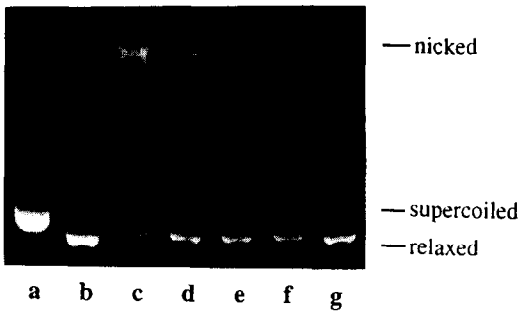

Fig. 5. Inhibition of camptothecin-induced DNA cleavage by TAN-1518 A. Drugs were added simultaneously to the reaction system. Electrophoresis was carried out under the same conditions as those described in Fig. 4. *Lane a*, ccc DNA control; *lane b*, relaxed DNA control; *lane c*, CPT 20 μM; *lanes d* to *g*, same as *lane c* except that the concentration of TAN 1518 A was increased to 10, 30, 100, and 200 μM, respectively.

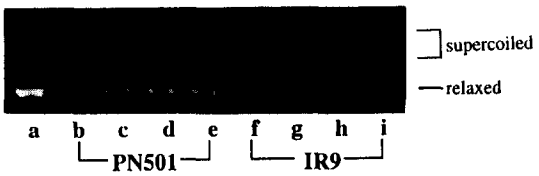

Fig. 6. Relaxation activity of Topo I in lysates from parent and IR9 cells. Electrophoresis was carried out in a 0.7% agarose gel with 0.1% SDS at 100 V for 40 min. *Lane a*, ccc DNA control; *lanes b* to *e*, lysate from parent cells; *lanes f* to *i*, lysate from IR9 cells. *Lanes b* and *f*, 1200 ng; *lanes c* and *g*, 600 ng; *lanes d* and *h*, 300 ng; *lanes e* and *i*, 150 ng.

Effect of TAN-1518 A on the induction of Topo I-mediated DNA cleavage. To determine whether TAN-1518 A inhibited *S. pombe* Topo I in the same manner as does CPT, we investigated the effect of TAN-1518 A on the stimulation of cleavable complex formation. The amount of cleavable complex was measured by detecting nicked open circular DNA. An increase in the concentration of CPT resulted in the conversion of closed circular DNA to nicked open circular DNA, indicating that CPT inhibits yeast Topo I by stabilizing the cleavable complex, as has been shown in mammalian cells. In contrast, no nicked open circular DNA was produced, even in the presence of 50 μM TAN-1518 A (Fig. 4). These findings clearly indicate that TAN-1518 A inhibits yeast Topo I without stabilizing the cleavable complex, a result consistent with the findings in labeled CHO-K1 cells [14].

Inhibition of CPT-induced DNA cleavage by TAN-1518 A. If TAN-1518 A were to inhibit Topo I before cleavable complex formation, then CPT-induced DNA cleavage could be suppressed by TAN-1518 A. In fact, the amount of nicked open circular DNA formed in the presence of 20 μM CPT was decreased markedly when TAN-1518 A was added at concentrations of 30 μM or greater (Fig. 5). Also, when 75 μM TAN-1518 A was added to the reaction system prior to the addition of CPT, DNA cleavage was inhibited as potently as when TAN-1518 A (200 μM) and CPT were added simultaneously (data not shown).

Character of IR9. We constructed a *S. pombe* strain hypersensitive to CPT. A plasmid, pIATOP1, that overexpresses yeast Topo I was constructed, as shown in Fig. 2. This plasmid was linearized and transformed to PN501, which has the *rad1* mutation, since the sensitivity of the *rad1* mutant to CPT is about 4 times higher than that of wild type cells. A stable transformant, IR9, was thus obtained and was shown by genomic Southern hybridization to have one copy of pIATOP1 in its *top1*$^+$ locus (data not shown). Figure 6 shows the relaxation activity of Topo I in lysates from parent and IR9 cells. The activity in 0.15 μg of the lysate from IR9 cells was greater than that in 1.2 μg of the lysate from parent cells. These results suggest that the amount of Topo I in IR9 cells was more than 8 times higher than that in parent cells.

Table 1. Susceptibility of parent and integrant to various drugs

Drug	MIC (μM) PN501 (a)	MIC (μM) IR9 (b)	Index a/b
CPT	6.25	0.1	62.5
TAN-1518 A	>100	>100	
Distamycin	25	25	1.0
Bleomycin A_1	6.25	3.13	2.0
Dnacin B_1	12.5	6.25	2.0
Mitomycin C	>100	100	
Actinomycin D	3.13	3.13	1.0
Adriamycin	>100	100	
m-AMSA	>100	>100	
Etoposide	>100	>100	
Cycloheximide	6.25	6.25	1.0
Nystatin	0.8	3.13	0.25
Amphotericin B	0.2	0.4	0.5

Minimum inhibitory concentration (MIC) values were determined by an agar dilution method, using YPD medium.

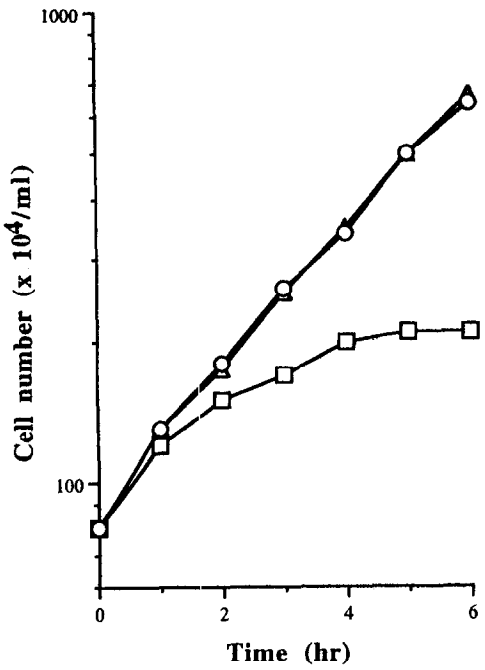

Fig. 7. Effects of TAN-1518 A on the growth of IR9 cells. Exponentially growing cultures of IR9 cells (8×10^5/mL) were exposed to each drug. Cell numbers represent the average of triplicate experiments. Key: (○) drug-free control; (△) 10 mM TAN-1518 A; and (□) 0.1 mM CPT.

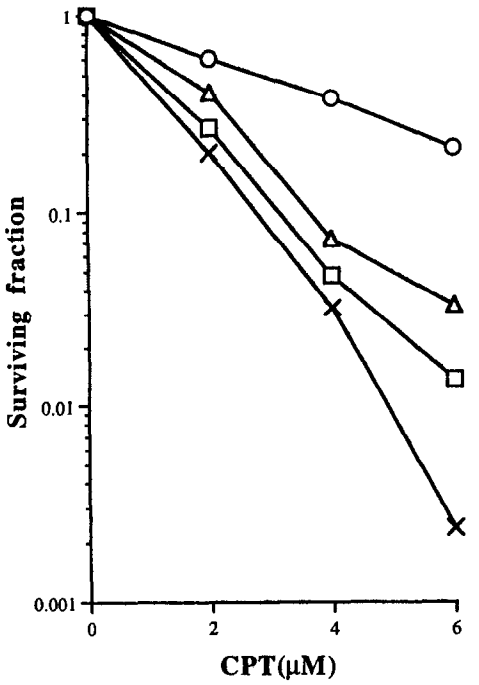

Fig. 8. Rescue of IR9 cells from CPT-mediated death by TAN-1518 A. Various concentrations of TAN-1518 A and CPT were added simultaneously to exponentially growing cultures of IR9 cells (1×10^6/mL). After a 6-hr incubation, the viability of IR9 was evaluated as described in Materials and Methods. Data represent the average of triplicate experiments. Key: (○) 1 mM TAN-1518 A; (△) 0.1 mM TAN-1518 A; (□) 0.01 mM TAN-1518 A; and (X) control.

The susceptibility of the parent cells and IR9 to various drugs is shown in Table 1. IR9 was 62.5 times more sensitive than the parent strain to CPT, this correlating with the increase of Topo I expression. CPT-treated IR9 cells died and were extremely small (data not shown), suggesting the induction of a mitotic catastrophe [24]. In contrast, no increase in the susceptibility of IR9 to TAN-1518 A was observed. A 10 mM concentration of TAN-1518 A did not affect the exponential growth of IR9 at all, whereas 0.1 mM CPT completely inhibited this growth (Fig. 7).

Rescue of IR9 cells from CPT-mediated death by TAN-1518 A. If TAN-1518 A were to inhibit CPT-mediated cleavage in *S. pombe* cells, the breakdown of chromosomal DNA could be suppressed and, consequently, the death mediated by CPT could be prevented. To examine this possibility, we performed a rescue experiment following the protocols described in Materials and Methods. As shown in Fig. 8, decreased viability of IR9 cells was observed in proportion to increased CPT concentration, and only 0.2% of the cells survived in the presence of 6 μM CPT. In contrast, the simultaneous addition of TAN-1518 A and CPT dramatically increased the viability in a concentration-dependent manner; 22% of the cells survived in the presence of 6 μM CPT and 1 mM TAN-1518 A. When TAN-1518 A (1 mM or 0.1 mM) was added simultaneously with CPT or 1 hr before CPT, the CPT-mediated death of IR9 cells was reduced dramatically. In contrast, when TAN-1518 A was added 1 hr after CPT, the viability of IR9 was almost the same as that in the absence

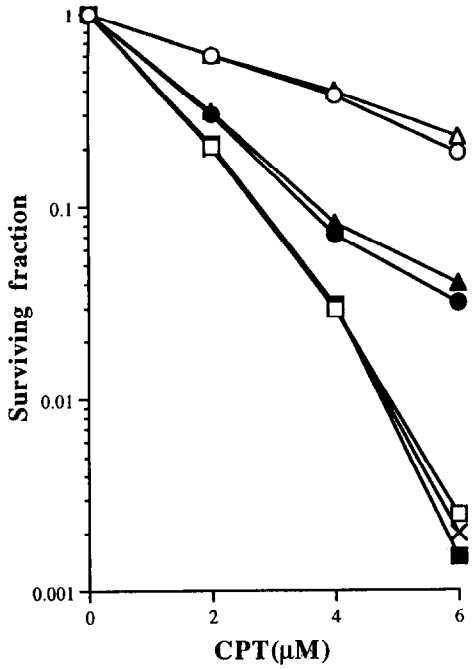

Fig. 9. Effects of timing of the addition of TAN-1518 A. Data represent the average of triplicate experiments. Key: (●,○) both drugs were added simultaneously; (▲,△) TAN-1518 A was added 1 hr before CPT and incubation was performed for 6 hr in the presence of both drugs; (■,□) same as triangles, except that TAN-1518 A was added 1 hr after CPT; and (X) control. Open symbols, 1 mM TAN-1518; closed symbols, 0.1 mM TAN-1518 A.

of TAN-1518 A (Fig. 9). These findings clearly indicate that TAN-1518 A rescues IR9 cells from the death mediated by CPT.

DISCUSSION

Using the fission yeast *S. pombe*, we clarified that TAN-1518 A specifically inhibited Topo I in eukaryotic cells, and our findings suggested that this compound inhibited some earlier step(s) prior to cleavable complex formation in the catalytic cycle of Topo I.

In the rescue experiment, TAN-1518 A dramatically rescued the Topo I-overproducing yeast from CPT-mediated death. Moreover, the growth of the yeast was not affected by TAN-1518 A itself. Thus, it appears that TAN-1518 A may directly and specifically interact with Topo I in eukaryotic cells. Preincubation of TAN-1518 A with Topo I enhanced the inhibition of CPT-mediated DNA cleavage, indicating a direct interaction between the two agents. TAN-1518 A weakly inhibits the decatenation activity of calf thymus Topo II *in vitro* [14], but the compound may have little effect on Topo II function in eukaryotic cells.

The catalytic cycle of Topo I can be divided into five functional steps: (i) noncovalent binding of Topo I to DNA, (ii) strand cleavage and simultaneous covalent attachment of enzyme to DNA, (iii) swiveling or strand passage, (iv) religation of the nick(s), and (v) readoption of an active enzyme conformation [25]. Kjeldsen *et al.* [26] suggested that CPT inhibited not only step (iv) but also step (ii); in fact, the inhibition of step (iv), which is equivalent to the stabilization of cleavable complex, is critical for the chromosomal DNA breakdown and for the subsequent death of eukaryotic cells. In the rescue experiment, the rescue effect of TAN-1518 A was abolished completely when it was added 1 hr after CPT. This clearly indicates that TAN-1518 A inhibits some earlier step(s) before step (iv) in yeast cells. Furthermore, unlike CPT, TAN-1518 A did not induce Topo I-mediated DNA cleavage. Therefore, it is presumed that TAN-1518 A inhibits step (i) or step (ii), or both, in the catalytic cycle of Topo I [7].

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