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Mechanisms of Cell Killing by Drugs that Trap Covalent Complexes between DNA Topoisomerases and DNA

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SUMMARY

DNA topoisomerases are the molecular targets of a range of anticancer and antimicrobial therapeutics. Many of these drugs act by converting their target enzyme to a DNA-damaging agent through the trapping of the covalent enzyme/DNA intermediate. This drug-mediated trapping of the intermediate is reversible, and the lesion in the DNA disappears on removal of the drug. This reversibility leads to questions concerning how treatment with these drugs effects cell killing. Using drug-permeable yeast strains, we demonstrate that yeast cells arrested in G_1 are refractory to drugs that trap the covalent complex between DNA and either topoisomerase I or topoisomerase II. The cell cycle regulation of topoisomerase II is not responsible for the insensitivity of G_1 cells to drugs targeting this enzyme because ectopic expression of the enzyme in G_1 does not alter drug sensitivity. Commitment to cell killing by anti-topoisomerase II agents predominantly occurs in S phase cells but can also occur as cells progress from G2 through mitosis to G_1. We also demonstrate that yeast cells treated with DNA replication inhibitors such as aphidicolin lose sensitivity to camptothecin but not to topoisomerase II-targeting DNA-damaging agents. Our results suggest that DNA synthesis is a major determinant for cell killing by camptothecin but that other S phase-associated processes can effect cytotoxicity by drugs that convert topoisomerase II to a DNA-damaging agent.

The identification of eukaryotic DNA topoisomerases I and II as the targets of a large number of anticancer drugs has provided a biochemical foundation in the study of the cytotoxicity and clinical applications of these drugs (1–5). There are two distinct classes of topoisomerase-targeting therapeutics. One class, including the eukaryotic DNA topoisomerase I drug camptothecin and the eukaryotic DNA topoisomerase II drugs amsacrine [4′-(9-acridinylamino)-methanesulfon-*m*-anisidide] and etoposide (VP-16), converts their target topoisomerases to DNA-damaging agents (1, 3). *In vitro* and *in vivo*, they prevent the DNA-resealing step normally catalyzed by the topoisomerases; in the drug/enzyme/DNA ternary complexes, the DNA is apparently severed in one or both strands, depending on whether DNA topoisomerase I or II is involved (6, 7). Some recently described agents are capable of preventing resealing by either topoisomerase I or topoisomerase II (8). All of these drugs have sometimes been referred to as "topoisomerase poisons," and they are mechanistically similar to the bactericidal quinolones, which act on bacterial gyrase, the bacterial counterpart of eukaryotic DNA topoisomerase II (9). The other class, which includes aclarubicin, ICRF193 (10), and merbarone (11), seems to act by inhibiting the enzymatic activity of eukaryotic DNA topoisomerase II, an essential enzyme for cell viability (12). Novobiocin is also a member of the latter class of inhibitors, although it is not very active against eukaryotic topoisomerase II or specific for topoisomerases *in vivo* (13).

Intensive studies have been carried out on the mechanism of cell killing by the first class of drugs, or "topoisomerase poisons." It is clear that the formation of the drug/enzyme/DNA ternary complex is, by itself, insufficient for cell killing. The eukaryotic DNA topoisomerase I drug camptothecin, for example, can form the ternary complex in all stages of the cell cycle, yet cytotoxicity of the drug is S phase specific (14, 15). More recent cellular as well as *in vitro* replication studies have provided strong evidence that DNA replication has a major role in converting the camptothecin/topoisomerase I/DNA ternary complex from a latent DNA-damaging state to an irreversible cytotoxic DNA break (16).

Although cell killing by camptothecin clearly involves DNA replication (17), the relation between DNA replication and the cytotoxicity of the first class of drugs that target DNA topoisomerase II is less clear. Studies with mammalian cells showed that these drugs are not entirely S phase specific, and their cytotoxicity persists in other phases of the cell cycle as well (18). Inhibitors of transcription seem to reduce the cytotoxicity of these drugs, which has led to the suggestion that perhaps transcription, like replication, can also convert

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ABBREVIATION: YPDA, yeast extract peptone dextrose adenine medium; DMSO, dimethylsulfoxide.

a latent double-stranded DNA break in a drug/topoisomerase II/DNA ternary complex, but not in a drug/topoisomerase I/DNA ternary complex, to an irreversible break (19). In addition to replication and transcription, drug- and topoisomerase-induced genetic rearrangements have been postulated for the cytotoxic action of drugs that trap the covalent complexes between DNA topoisomerase II and DNA (20). In mammalian cells, actions of antitopoisomerase drugs on replication, transcription, and other processes ultimately activate pathways of programmed cell death (21).

To gain further insight into the mechanism of cell killing by the drugs that trap covalent DNA topoisomerase/DNA complexes, we studied the cell cycle dependence of the cytotoxicity of camptothecin, amsacrine, and etoposide. We show that cell killing by all of these drugs is prevented by arresting the cells in G_1 with α factor mating pheromone; for antitopoisomerase II agents, entry into S phase suffices to commit cells to death. In contrast, active DNA replication is required for cell killing by camptothecin. We also show that the observed pattern of cell killing is independent of the cell cycle regulation of topoisomerase II. These results are interpreted in terms of the replication-driven conversion of the drug/topoisomerase/DNA ternary complexes to irreversible double-stranded DNA breaks. Transcription does not seem to have a significant role in the formation of the unrepairable DNA damage by these drugs.

Materials and Methods

Yeast strains and drug treatment. Yeast strains used in this study are shown in Table 1. All strains were constructed in our laboratories using standard methods. The mutations *ize1* and *ISE2*, which confer permeability to antitopoisomerase drugs, have been described previously (22).

Growth and drug treatments of cells were done in YPDA medium (22). Viability of yeast cells was determined by plating appropriate dilutions of cultures to YPDA medium containing 1.5% agar. Plates were incubated for 3–4 days at 30° before counting. All experiments were performed at least twice with similar results; representative data are shown.

Camptothecin, amsacrine, teniposide, and etoposide were the kind gifts of Dr. Leroy Liu (Robert Wood Johnson Medical School, Rutgers University, New Brunswick, New Jersey). Drugs were dissolved in 100% DMSO at a concentration of 2–10 mg/ml. Unless otherwise indicated, camptothecin was used at a concentration of 50 μg/ml, and the other antitopoisomerase drugs were used at a concentration of 100 μg/ml.

Cell cycle arrest. Cells were arrested with α factor by diluting logarithmically growing cultures to 3–5 $\times$ 10^6 cells/ml and adding α factor (Sigma) from a 1 mg/ml solution in 100% methanol containing 3 mM sodium acetate, pH 5.2, to a final concentration of 10–20 μg/ml. After shaking at 30° for 3–4 hr in the presence of α factor, the cells were examined microscopically to determine the percentage of cells with a morphology of α factor-arrested cells. Under the conditions used, typically >90% of the cells seemed to be unbudded or showed the "schmooing" characteristic of α factor treatment. Cells were collected by centrifugation, washed twice with prewarmed YPDA, and resuspended in YPDA at a concentration of 2 $\times$ 10^6 cells/ml. Appropriate drugs or fresh α factor was added at this time, the zero time for the experiments described below. For cultures held in α factor for long periods after t = 0, an additional aliquot of α factor was added at t = 4 hr.

Cells were also arrested at other points in the cell cycle using the DNA synthesis inhibitors hydroxyurea and aphidicolin and the microtubule-destabilizing agent nocodazole. Stock solutions of hydroxyurea (Sigma Chemical, St. Louis, MO) were dissolved in H_2O at 50 mg/ml, and aphidicolin was dissolved in DMSO at a concentration of 10 mg/ml. Nocodazole (Sigma) was dissolved in DMSO at 4 mg/ml and heated to 60° briefly to ensure complete solution. The nocodazole solution was freshly prepared before use.

Determination of topoisomerase II activity in α factor-arrested cells. Topoisomerase II activity was determined using the yeast strain a bar1 carrying the plasmid pDED1TOP2 (23). Cells were grown to an absorbance at 600 nm of 0.4 in YPDA. The culture was then divided in two equal portions, and α factor was added to one half of the culture at a final concentration of 0.5 μg/ml. This lower concentration of α factor is sufficient to arrest cells carrying the *bar1* mutation in G_1. The other half of the culture was incubated without α factor. Aliquots (50 ml) were removed at hourly intervals and quick frozen in dry ice/ethanol. After all of the samples were collected, extracts were prepared from the frozen samples as described previously (24). Topoisomerase II activity was determined by decatenation of kinetoplast DNA (25), and protein concentrations in the cell extracts were determined according to the Bradford method (26).

TABLE 1
Yeast strains

JN362a	*MATa ISE2 ura3-52 leu2 trp1 his7 ade1-2*
JN392	*MATa ise1 rad52::LEU2 leu2-3,112 his7*
JN394	*MATa ISE2 ura3-52 leu2-3,112 trp1 his7 ade1-2 rad52::LEU2*
JN394t1	as JN362a but *top1::LEU2 rad52::TRP1*
JN394t2-4	as JN394 but *top2-4*
a bar1	*MATa bar1-1 ura3-52*

Results

Effect of arresting cells in G_1 on the cytotoxicity of camptothecin, amsacrine, and etoposide. To test the relation between DNA replication and cell killing by topoisomerase drugs that induce latent DNA breaks, we used *MATa* yeast strains bearing a *rad52* mutation and an *ise1* or *ISE2* mutation. As was shown previously, the *rad52* mutation prevents the repair of double-stranded DNA breaks in yeast and sensitizes yeast cells to drugs that trap covalent DNA topoisomerase/DNA complexes (22, 27), and the *ise1* or *ISE2* mutation apparently makes the cells more permeable to drugs. It is well established that *MATa* cells are arrested by the α factor mating pheromone at the Start point in G_1; on removal of the mating pheromone, the arrested cells resume the normal cell cycle progression and proceed rapidly into S.

JN392 (*MATa rad52 ise1 TOP*$^+$) cells were first treated with α factor for 3 hr to synchronize the cells in G_1. Cells were then washed thoroughly to remove the pheromone and resuspended in media with or without α factor and in the presence or absence of camptothecin. The results of this experiment are shown in Fig. 1. Cells resuspended in medium containing α factor continue to be blocked in division but show no significant decrease in viable titer. Removal of the α factor allows the cells to resume growth after a short lag. The addition of camptothecin to cells released from α factor arrest induces lethality within a short time. However, if camptothecin is added to α factor-arrested cells resuspended again in the presence of α factor to prevent cell-cycle progression, no loss of viability is seen. Similar results were obtained with strain JN394 (*MATa rad52 ISE2 TOP*$^+$) (data not shown). Thus, yeast cells arrested in G_1 are resistant to camptothecin, as would be expected on the basis of the S phase specificity of the drug.

Similarly, when the same experiment was carried with

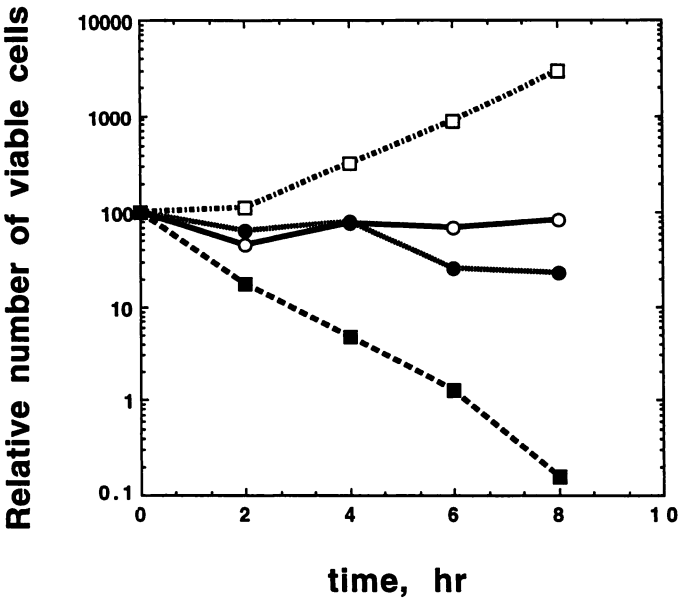

Fig. 1. Yeast cells arrested with α factor are resistant to camptothecin. JN392 cells were treated with 10 μg/ml α factor for 3 hr as described in Materials and Methods. The α factor was washed out, and cells were resuspended in YPDA. Drugs and fresh α factor were added to the appropriate samples. Aliquots were removed at various times, diluted, and plated to YPDA plates to determine cell viability. All survivals are shown relative to t = 0, the time at which camptothecin was added. Cells were treated with DMSO (□) or 50 μg/ml camptothecin (■) without additional α factor or treated with DMSO (○) or 50 μg/ml camptothecin (●) with fresh α factor at a final concentration of 10 μg/ml.

amsacrine instead of camptothecin, it was found that JN394 (*MATa rad52 ISE2 TOP*$^+$) cells synchronized in G_1 show little loss of viability when treated with amsacrine in the presence of α factor. In contrast, cells released from G_1 arrest in the presence of amsacrine begin to lose viability within 30 min (Fig. 2). The time of commitment to cell death approximately coincides with the appearance of budded cells, a landmark of entrance into S phase. Similar results were obtained with the epipodophyllotoxins etoposide and teniposide (data not shown).

The lethality that is seen in Figs. 1 and 2 at longer incubation times in α factor-arrested cells in the presence of a topoisomerase drug is consistent with a fraction of cells escaping the α factor block: the reduction in viable cells correlated well with the fraction of cells that were seen with small or large buds under the microscope.

Effect of ectopic expression of topoisomerase II on cell killing by amsacrine in G_1 arrested cells. A trivial explanation for the lack of sensitivity of α factor-treated cells to amsacrine is in the cell cycle regulation of topoisomerase II. The insensitivity of G_1 cells may reflect a lack of topoisomerase II protein. To examine whether a lack of topoisomerase II activity affected drug sensitivity, we transformed JN394 cells with pDED1TOP2, a plasmid that constitutively expresses TOP2 from the yeast *DED1* promoter. These cells were arrested with α factor as before, and sensitivity to amsacrine was determined. As shown in Fig. 3, pDED1TOP2-bearing JN394 cells released from α factor arrest show a rapid loss of viability when treated with 100 μg/ml amsacrine, similar to that seen in Fig. 2. Although some loss of viability is seen in α factor-arrested cells on exposure to amsacrine, the survival in arrested cells is nearly 10-fold higher than that in cells released from α factor arrest.

The arrest of cells carrying pDED1TOP2 with α factor has no effect on topoisomerase II activity. To measure topoisomerase II activity in cells arrested with α factor, we took advantage of a yeast strain carrying a mutation in the *bar1* gene. *BAR1* encodes a protease that inactivates α factor; thus, cells lacking BAR1 activity are hypersensitive to α factor. Cell extracts were made from the *a bar1* strain, which also carried pDED1TOP2. Topoisomerase II activity

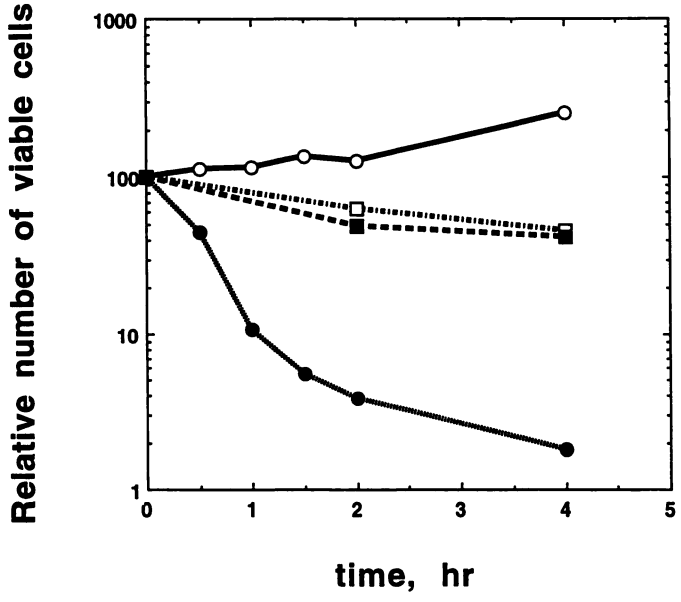

Fig. 2. Yeast cells arrested with α factor are resistant to amsacrine. JN394 cells were pretreated with 20 μg/ml α factor for 3 hr as described in the legend to Fig. 1. Cells were treated with DMSO (○) or 100 μg/ml amsacrine (●) without additional α factor or treated with DMSO (□) or 100 μg/ml amsacrine (■) with fresh α factor at a final concentration of 20 μg/ml.

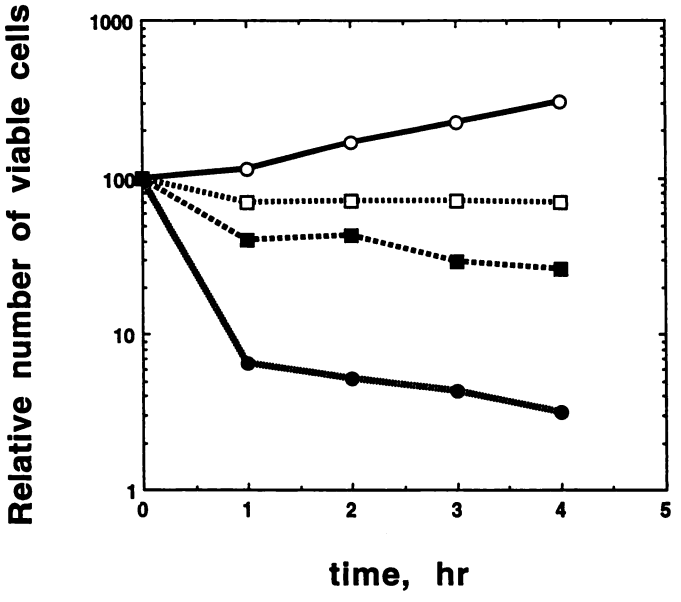

Fig. 3. Amsacrine sensitivity of JN394 cells carrying the plasmid pDED1TOP2. JN394 cells carrying pDED1TOP2 were pretreated with 20 μg/ml α factor for 3 hr as described in the legend to Fig. 2. Cells were treated with DMSO (○) or 100 μg/ml amsacrine (●) without additional α factor or treated with DMSO (□) or 100 μg/ml amsacrine (■) with fresh α factor at a final concentration of 20 μg/ml.

was compared for α factor-arrested cells and cells that were not exposed to α factor. A titration of topoisomerase II activity is shown in Fig. 4. Cells treated with or without α factor for 1–3 hr showed no detectable differences in topoisomerase II activity (Fig. 4, compare *top* with *bottom*). Therefore, the difference in sensitivity in α factor-arrested cells cannot be solely accounted for by differences in the level of topoisomerase II activity.

Cytotoxicity of the topoisomerase II-targeting drug amsacrine is not limited to S phase. Two experiments were carried out to assess the cytotoxicity of amsacrine at other points in the cell cycle. In one, cells were treated with the microtubule-destabilizing agent nocodazole. Three hours after the addition of nocodazole, at which time cells were arrested in mitosis, the culture was spun in a centrifuge to pellet the cells. Cells were washed to remove nocodazole and then resuspended in fresh media: one containing α factor and the other containing both amsacrine and α factor.

The presence of α factor after the removal of nocodazole prevents reentry of the cells into S phase. Cells treated with nocodazole alone do not lose viability, and in the presence of α factor, they proceed efficiently through mitosis to the next G_1. Four hours after release from nocodazole, >80% of the cells in medium containing α factor and no amsacrine exhibited the mononucleated "schmoo" morphology characteristic of α factor-arrested cells in G_1. When cells were released from nocodazole into medium containing both α factor and amsacrine, however, loss of viability is apparent (Fig. 5). It was also observed that in the presence of both amsacrine and α factor, only 60% of cells assumed the "schmoo" shapes 4 hr after their release from nocodazole arrest. The latter observation suggests that in the presence of amsacrine, the exit of cells from mitosis is delayed.

In the second experiment, cells were synchronized in G_1 with α factor and released into medium containing both amsacrine and nocodazole to block cell-cycle progression at mitosis. Fig. 6 shows that the killing of cells by amsacrine in the presence of nocodazole is not significantly different than the killing of cells with amsacrine alone. This result indicates that progression through mitosis is not necessary to kill yeast cells with amsacrine. Taken together, these two experiments indicate that the topoisomerase-targeting drug amsacrine can kill cells at multiple stages in the cell cycle.

Arrest in G_1 by α factor also prevents the killing of *top1* cells by etoposide and amsacrine. Previous studies with *top2* temperature-sensitive mutants of both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* showed that although inactivation of DNA topoisomerase II in *TOP1*$^+$ cell led to cell killing only if the cells traversed mitosis, in *top1* cells devoid of DNA topoisomerase I, thermal inactivation of DNA topoisomerase II was cytotoxic in all stages of the cell cycle (28). Therefore, it seemed plausible that the arrest of *top1* cell in G_1 by α factor might not prevent cell killing by amsacrine or etoposide because of the inhibition of DNA topoisomerase II by the drug. The isogenic pair of *S. cerevisiae* strains JN394 and JN394t1, a Δ*top1::LEU2* derivative of JN394 in which a segment of the *TOP1* gene was replaced by the *LEU2* gene, were used to test this possibility.

The Δ*top1::LEU2* mutation does not affect the time course

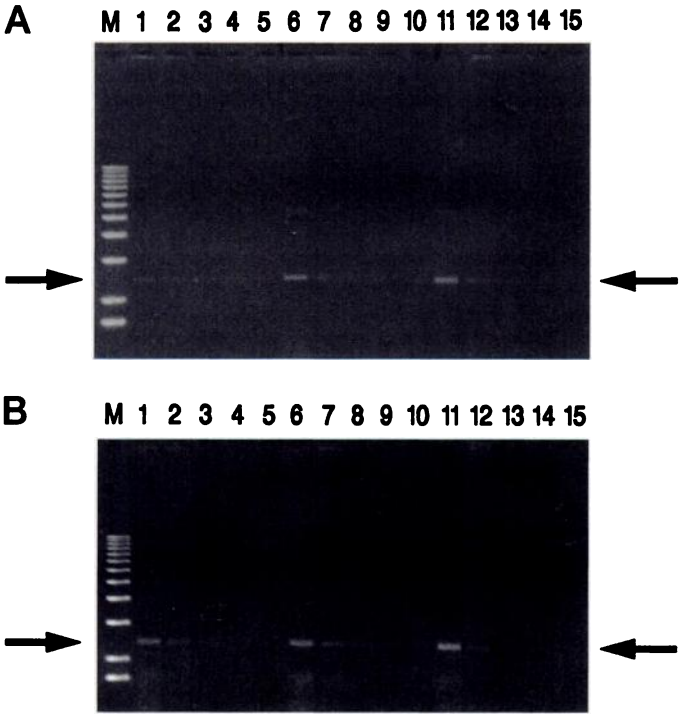

Fig. 4. Topoisomerase II activity of α factor arrested cells carrying the plasmid pDED1TOP2. a bar1 cells were grown to an absorbance at 600 nm of 0.4 in YPDA, and half of the culture was then exposed to α factor. Aliquots (50 ml) were removed at 1, 2, or 3 hr from cultures with or without α factor, and extracts were prepared. *Top*, samples from the culture without α factor. *Lanes 1–5*, 1 hr after α factor was added (to the other half or the culture); *lanes 6–10*, 2 hr after α factor was added; *lanes 11–15*, 3 hr after α factor was added. *Lanes 1, 6, and 11*, 1 μg of protein; *lanes 2, 7, and 12*, 0.5 μg of protein; *lanes 3, 8, and 13*, 0.2 μg of protein; *lanes 4, 9, and 14*, 0.1 μg of protein; *lanes 5, 10, and 15*, 0.05 μg of protein. *Lane M*, molecular mass markers. *Arrow*, mobility of the decatenated product. *Bottom*, identical to those shown on *top* except the culture contained 0.5 μg/ml α factor.

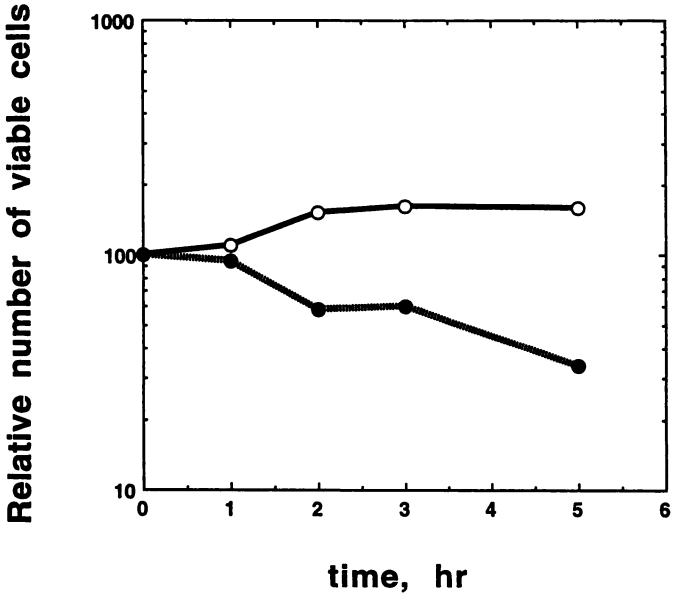

Fig. 5. Amsacrine sensitivity of nocodazole-treated cells. JN394 cells were pretreated with nocodazole as described in the text. The nocodazole was washed out, and cells were resuspended in fresh YPDA containing 20 μg/ml α factor. Incubation was continued at 30° with shaking. Cells were treated after nocodazole synchronization with DMSO (○) or 100 μg/ml amsacrine (●).

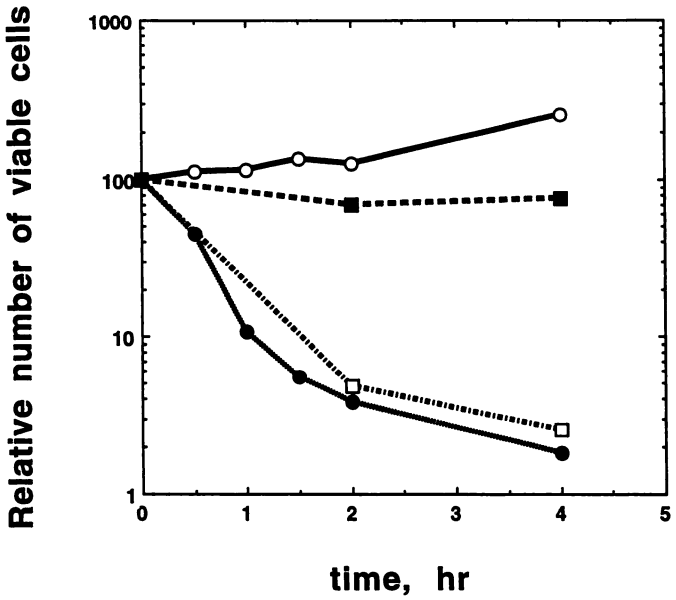

Fig. 6. Nocodazole does not affect sensitivity of JN394 cells to amsacrine. Cells were synchronized with 20 μg/ml α factor for 3 hr as described in the legend to Fig. 2. The α factor was removed, and cells were treated with DMSO (○) or 100 μg/ml amsacrine (●) without nocodazole or treated with DMSO (■) or 100 μg/ml amsacrine (□) with nocodazole.

of α factor arrest. Four hours after the addition of α factor, >80% of the JN394t1 or JN394 cells show the "schmoo" morphology characteristic of α factor-arrested cells, and no cells with small buds were observed. As shown in Fig. 7, JN394t1 cells released from α factor arrest in the presence of amsacrine or etoposide showed reduced viability within 1 hr, similar to that observed for JN394 cells (Fig. 2); 4 hr after release, <1% of the JN394t1 cells were viable in the presence of 100 μg/ml amsacrine and ~8% were viable in the presence of 100 μg/ml etoposide. In contrast, treatment with 100 μg/ml amsacrine in the continued presence of α factor resulted in little killing of JN394t1 cells: the viability was >70% after 4 hr. Therefore, α factor-arrested cells are nearly refractory to drugs that trap covalent complexes of DNA and DNA topoisomerase II regardless of whether DNA topoisomerase I activity is present.

Arrest of cells in G_1 by α factor blocks killing by a combination of both camptothecin and amsacrine. Unlike what has been frequently observed in mammalian cells (29, 30), the combination of camptothecin and a topoisomerase II "poison" results in synergistic cell killing (31). In cells released from α factor, the combination of amsacrine and camptothecin results in greater cytotoxicity than does either agent alone; survival at 4 hr after α factor release is ~0.1% (Fig. 8). However, treatment with both drugs resulted in no cytotoxicity in cells maintained under continued α factor arrest. These results suggest that the combination of drugs trapping the cleavage complexes of both topoisomerase I and topoisomerase II has the potential to produce a high level of cell killing provided that both drugs are present during S phase.

Effects of DNA replication inhibitors on cell killing by DNA topoisomerase-targeting drugs that trap covalent DNA/enzyme intermediates. In addition to the use of α factor to prevent yeast cells from entering S phase, we used inhibitors of DNA replication to gain information on the effects on the cytotoxicity of camptothecin and amsacrine of blocking DNA replication.

Fig. 9, top, depicts the effect of aphidicolin, an inhibitor of several DNA polymerases involved in replication, on the viability of yeast JN394 cells in the presence of camptothecin. As expected, the addition of aphidicolin alone to a final con-

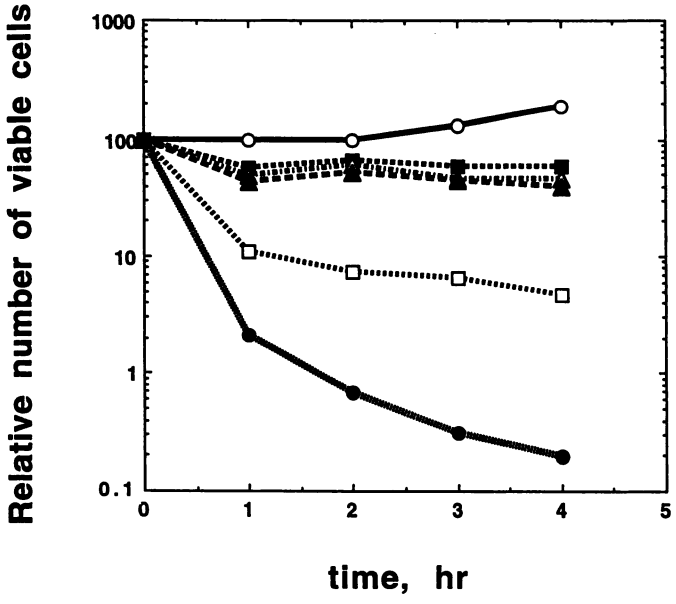

Fig. 7. Sensitivity of JN394t1 cells treated with α factor to amsacrine and etoposide. JN394t1 cells were pretreated with 20 μg/ml α factor for 3 hr as described in the legend to Fig. 2. Cells were treated with DMSO (○), 100 μg/ml amsacrine (●), or 100 μg/ml etoposide (□) without additional α factor or treated with DMSO (■), 100 μg/ml amsacrine (△), or 100 μg/ml etoposide (▲) with fresh α factor at a final concentration of 20 μg/ml.

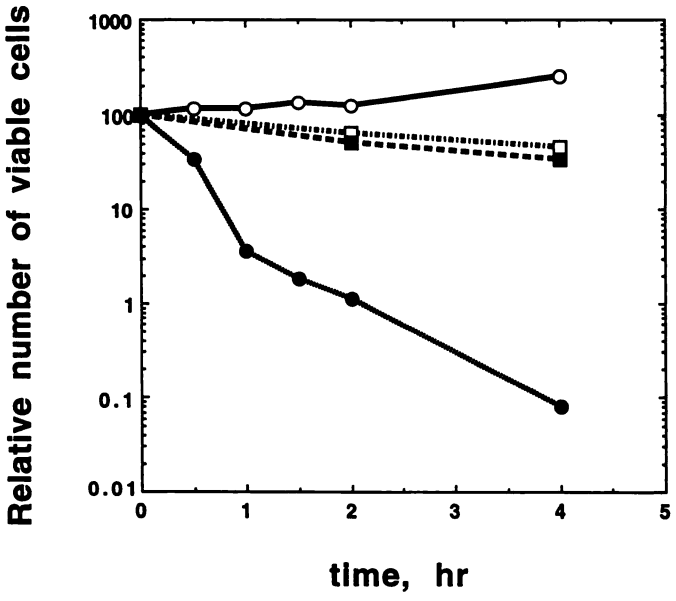

Fig. 8. Effect of simultaneous treatment of α factor-arrested cells with camptothecin and amsacrine. JN394 cells were pretreated with 20 μg/ml α factor for 3 hr as described in legend to Fig. 2. Cells were treated with DMSO (○) or 100 μg/ml amsacrine and 50 μg/ml camptothecin (●) without additional α factor or treated with DMSO (□) or 100 μg/ml amsacrine and 50 μg/ml camptothecin (■) with fresh α factor at a final concentration of 20 μg/ml.

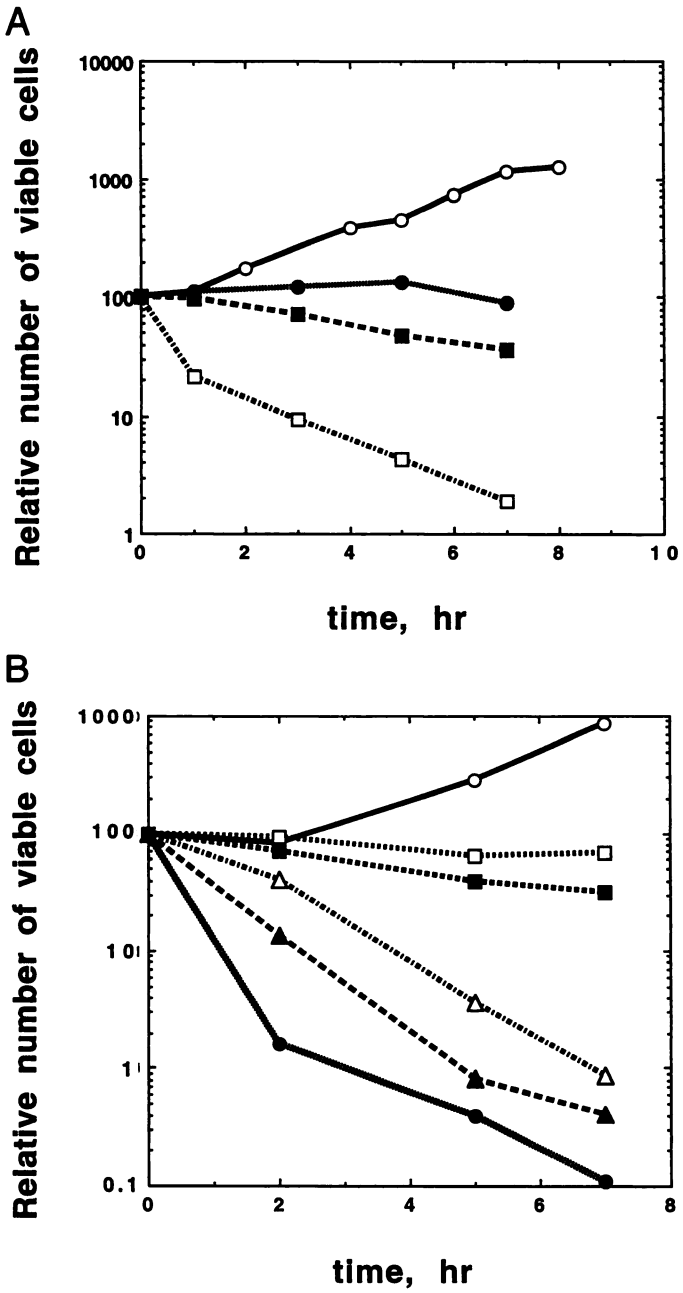

Fig. 9. Effects of DNA synthesis inhibitors on yeast cell sensitivity to camptothecin or amsacrine. *Top*, JN394 cells were synchronized with α factor as described in legend to Fig. 2 and then treated with DMSO (○) or 50 μg/ml camptothecin (□) without aphidicolin or treated with DMSO (●) or 50 μg/ml camptothecin (■) with aphidicolin. *Bottom*, JN394 cells synchronized with α factor were treated with DMSO (○), 100 μg/ml amsacrine (●), 100 μg/ml aphidicolin alone (□), 50 mg/ml hydroxyurea alone (■), 100 μg/ml amsacrine and 100 μg/ml aphidicolin (△), or 100 μg/ml amsacrine and 50 mg/ml hydroxyurea (▲).

centration of 100 μg/ml rapidly stops cell division, but the DNA polymerase inhibitor is not cytotoxic, and cell division resumes after its removal (data not shown). In agreement with results with mammalian cells, the killing of yeast *rad52 ISE2* cells by camptothecin was significantly reduced when aphidicolin was added at the same time as camptothecin.

For the DNA topoisomerase II drug amsacrine, the killing of logarithmically growing unsynchronized *rad52 ISE2* cells by this drug is reduced by several-fold when aphidicolin is added together with the drug (data not shown). To examine whether cell killing in the presence of both aphidicolin and amsacrine might be due to events outside the S phase, synchronized *rad52 ISE2* cells released from α factor arrest were used. Unlike the situation with camptothecin, it was found that aphidicolin affords only partial protection from cell killing after release from α factor arrest (Fig. 9, *bottom*).

We also examined the effects of hydroxyurea, a ribonucleotide reductase inhibitor known to block DNA replication, on the cytotoxicity of amsacrine in synchronized *rad52 ISE2* cells. As shown in Fig. 9, *bottom*, hydroxyurea at 50 mM stopped cell growth, and there was a low but measurable level of cell killing by this inhibitor. The addition of hydroxyurea at the same time as amsacrine showed only a marginal inhibition of cell killing compared with cell killing by amsacrine alone. These results indicate that high levels of cell killing can be obtained with anti-topoisomerase II agents in the absence of DNA synthesis.

Discussion

In yeast (31), and probably in mammalian cells as well, DNA topoisomerase I is the only cellular target of camptothecin; yeast cells devoid of the enzyme are refractive to the drug. The known S phase specificity of camptothecin cytotoxicity (15, 16) and the present finding that α factor-arrested yeast cells are not killed by camptothecin, when combined with the observation that *rad52 TOP+* mutants of yeast are hypersensitive to the drug, strongly support the hypothesis that the mechanism of cell killing by camptothecin is the conversion of the drug/DNA topoisomerase I/DNA ternary complex to an irreversible double-stranded DNA break. The formation of the ternary complex by itself is not cytotoxic; as long as the pair of broken DNA ends are properly juxtaposed in the ternary complex, they are readily rejoined by the topoisomerase after the dissociation of the drug. The latent DNA break in the ternary complex is converted to an irreversible double-stranded break, however, when a ternary complex encounters a replication fork. This mechanism is further supported by the reduction of camptothecin cytotoxicity by aphidicolin in mammalian cells (21) and yeast (current study). Previous studies on the effect of camptothecin on SV40 DNA replication *in vitro* and *in vivo* have also provided more direct evidence that a camptothecin/DNA topoisomerase I/DNA ternary complex can be converted to a double-stranded break by replication.

When a camptothecin/DNA topoisomerase I/DNA complex is converted by the replication fork into a double-stranded break, two types of double-stranded DNA ends are expected: one with a 5′ OH terminus and the other with a DNA topoisomerase I covalently linked to a 3′ terminal phosphoryl group. Because the covalently linked enzyme is capable of serving as a DNA strand-transferase and can transfer the DNA strands attached to it to a 5′ OH terminus (32), it is not clear why the yeast *RAD52* function is necessary to repair such a break. Perhaps the broken ends are subsequently processed by cellular entities: a repair system might, for example, remove the protein-linked terminus or change the 5′ OH to 5′ phosphoryl ends.

The camptothecin/DNA topoisomerase I/DNA ternary complex is known to block transcription (33). Data presented in Results indicate that in α factor-arrested yeast cells, inhibi-

tion of transcription by camptothecin does not lead to cell killing. Our results with α factor are based on the assumption that the major effect of the mating pheromone on antitopoisomerase drug sensitivity are due to the arrest of cells in G_1. Pheromone-treated cells assimilate mass and carry on transcription and protein synthesis, and it is known that the cell cycle arrest is due to a signal transduction pathway that terminates with inhibition of G_1-specific cyclins.

The mechanism of cell killing by drugs that trap the covalent intermediate between DNA and DNA topoisomerase II seems to be more complicated than the mechanism of camptothecin. The S phase cytotoxicity of drugs such as amsacrine and etoposide can be interpreted in terms of the same mechanism described for camptothecin (i.e., the conversion of the ternary complex to irreversible double-stranded DNA breaks by replication). In this case, the 5′ end of each broken duplex end is presumably associated with a single polypeptide of the dimeric DNA topoisomerase II. Unlike the covalent complexes formed with camptothecin, DNA, and topoisomerase I, enzymes that track along DNA such as RNA polymerase or DNA helicases should be capable of generating an (irreversible) double-stranded break. Recent evidence suggests that DNA helicases may be capable of generating such secondary double-stranded breaks (34).

The DNA ends produced by the breaking of the covalent DNA/DNA topoisomerase II complex might be highly recombinogenic because they might reassociate through dimerization of the attached polypeptides. As postulated earlier for the broken ends generated in the presence of camptothecin, cellular processing of the ends attached to DNA topoisomerase II polypeptides might also occur to yield protein-free ends, which are again expected to be recombinogenic. In yeast as well as in mammalian cells (20), topoisomerase drugs that trap enzyme/DNA complexes are known to induce a high level of recombination events. Although it has been suggested that genetic rearrangements might contribute significantly to the cytotoxicity of amsacrine and etoposide (35), the yeast *RAD52* dependence of cell killing by these drugs argues in favor of a more direct relation between cytotoxicity of these drugs and the generation of double-stranded DNA breaks.

The experiment in which yeast cells were first arrested in M phase by nocodazole and then released from the arrest in the presence of both amsacrine and α factor demonstrates clearly that cell killing by amsacrine is not limited to S phase. From the biological roles of eukaryotic DNA topoisomerase II, its inactivation by any mechanism is expected to lead to cell killing when the cells progress through M phase (12). The result of the nocodazole experiment is therefore in concordance with the known essential nature of eukaryotic DNA topoisomerase II in chromosome segregation.

The results of cytotoxicity of amsacrine and etoposide in yeast seem to differ from those in mammalian cells in one major respect: yeast cells arrested in G_1 by α factor are resistant to killing by these drugs, whereas previous studies indicate that mammalian cells in G_1 can be killed by the same drugs (36). In yeast, there is clear evidence that DNA topoisomerase II is the only significant target of amsacrine and etoposide (37, 38). It has been suggested that transcription, similar to replication, might also convert a drug-trapped DNA/DNA topoisomerase II covalent complex, but not a camptothecin-trapped DNA/DNA topoisomerase I complex, to an irreversible double-stranded break. In yeast cells arrested in G_1 by α factor, transcription is known to occur normally. Thus, the finding that α factor-arrested yeast cells are resistant to amsacrine and etoposide is inconsistent with the hypothesis of transcription-mediated conversion of drug/DNA topoisomerase II/DNA ternary complexes to irreparable double-stranded DNA breaks.

For both *S. cerevisiae* (28) and *S. pombe* (39), it is known from studies of *top1 top2 ts* double mutants that inactivation of DNA topoisomerases I and II leads to cell killing at all stages of the cell cycle. The arrest of *S. cerevisiae* cells at Start by α factor, for example, does not completely prevent cell killing resulting from the simultaneous inactivation of DNA topoisomerases I and II (28). Therefore, the findings that α factor protects *ISE2 rad52 top1* cells from killing by amsacrine and *ISE2 rad52 TOP*$^+$ cells from killing by a combination of camptothecin and amsacrine are unexpected. One trivial explanation is that there might be sufficient residual cellular DNA topoisomerase I or II activity at the levels of the drugs used in these experiments. More likely, DNA topoisomerases are not completely inhibited by either amsacrine or camptothecin, and the cell killing is solely effected by the generation of irreversible double-stranded breaks. In any case, the results presented here clearly support the importance of active cellular processes and cell proliferation in killing by antitopoisomerase agents.

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