Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8895–8899, October 1991
Cell Biology

Inhibitors of DNA topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis

(cytokinesis/concatenation/etoposide/cell cycle controls)

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Communicated by Daniel Mazia, July 15, 1991

ABSTRACT **DNA topoisomerase II (EC 5.99.1.3) is necessary for chromosome condensation and disjunction in yeast but not for other functions. In mammalian cells, it has been reported to be necessary for progression toward mitosis but not for transit through mitosis. We have found, on the contrary, that specific inhibition of topoisomerase II (but not of topoisomerase I) interferes with mammalian mitotic progression. Metaphase is prolonged, and anaphase separation of chromatids is completely inhibited, in cells given high concentrations of topoisomerase II inhibitors; nevertheless these cells attempt cleavage, sometimes generating nucleate and anucleate daughters. Lower concentrations of inhibitors interfere with anaphase and produce abnormalities of segregation. DNA topoisomerase II activity is therefore necessary for mammalian chromatid separation, but it is not tightly coupled to the control of other mitotic events.**

In eukaryotic cells DNA topoisomerase II (EC 5.99.1.3) is a major component of the mitotic chromosome core (1), with sites of action adjacent to the attachment points of supercoiled DNA loops (2). This structural role is conserved in all known cases.

In budding and in fission yeast, temperature-sensitive *top2* mutants show no defect in normal interphase cell functions, including cell cycle progression, at the restrictive temperature; but they are arrested in mitosis (3, 4). In *top2* mutants of the fission yeast *Schizosaccharomyces pombe*, chromosome condensation is partly achieved at the restrictive temperature, but it produces long entangled prophase-like structures, which become fully condensed when the topoisomerase II activity is restored. If topoisomerase II is inactivated after chromosome condensation, the cells remain blocked at metaphase, unable to achieve chromatid separation (4). After release from such arrest, yeast cells show chromosome nondisjunction (5); meiotic nondisjunction can be similarly produced (6). This requirement for topoisomerase II in mitotic condensation and in separation of sister chromatids may be due to the need to separate sister strands of DNA, which inevitably become concatenated during replication but are not decatenated until later (7). Other cellular DNA topoisomerases appear to be adequate for other necessary manipulations of DNA topology but cannot perform this decatenation activity; in *top2* mutants, concatenated plasmids accumulate at the restrictive temperature (3, 8).

Though the topoisomerase II function is necessary for chromatid segregation in fission yeast, other aspects of yeast mitosis continue without it. Temperature-sensitive *top2* cells at the restrictive temperature divide into two portions, joined by chromatid fibers that prevent total cytokinesis; in *top2 cdc11* double mutants, in which septum formation is also blocked, cells reform single active nuclei after an abortive mitosis and enter a new round of replication (8).

In mammalian cells the role of topoisomerase II in cycle progression has been reported to be rather different from its role in yeast. No temperature-sensitive mammalian topoisomerase II mutants are yet available; but in the mouse mutant line tsA159, in which a temperature-sensitive mutation at another locus inhibits topoisomerase II activity (9), cells are arrested at the nonpermissive temperature after a few hours of progression into S phase (10). Mammalian topoisomerase II function has also been studied with the aid of enzyme inhibitors, such as etoposide or its congener teniposide, for lack of temperature-sensitive mutants. Replication of simian virus 40 genomes proceeds normally in the presence of topoisomerase II inhibitors, except for the last 200 or so base pairs, which remain unreplicated in concatenated dimeric genomes (30). The cellular action of topoisomerase II inhibitors is generally to arrest human or rodent cells in G_2 phase (11, 12). Topoisomerase II depletion in a cell-free system has also been shown to prevent chromosome condensation (13).

However, once human cells have begun mitosis, their progress has been reported to be insensitive to topoisomerase II inhibition (14). We have studied the effect of topoisomerase II inhibitors on mitotic cells, and we have observed a profound inhibition of mitotic segregation.

PROCEDURES

Cell Culture. PtK2 *Potorus tridactylis* (rat kangaroo) cells and DM *Muntiacus muntjak* (Indian muntjac) cells (15) were grown in Eagle's minimal essential medium supplemented with nonessential amino acids, glutamine, and 10% fetal calf serum; HeLa cervical tumor-derived human cells were grown in medium with 2.5% fetal calf serum and 2.5% newborn calf serum. Mitotic DM and HeLa cells were synchronized by automatic nitrous oxide arrest (16).

Analysis of Mitotic Events. Suspensions (30 μl) of mitotic HeLa cells in medium containing topoisomerase inhibitors were pipetted into the wells of Flow Teflon-coated multitest slides, treated with 0.01% polylysine to ensure rapid adhesion. These slides were fixed in methanol at −20°C and stained with toluidine blue for scoring. Mitotic DM cells were treated with topoisomerase inhibitors, washed with medium, and plated out for 18 hr to progress through the next cell cycle. Cells entering the second mitosis were arrested with nocodazole at 0.04 μg/ml, and chromosome preparations from hypotonically swollen cells were spread and stained with crystal violet.

For video analysis, unsynchronized PtK2 cells in 30-mm plastic dishes were incubated in a heated CO_2-equilibrated chamber surrounding the stage of a Nikon Diaphot inverted

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; *m*-AMSA, 4′-(9-acridinyl)aminomethanesulfon-*m*-anisidide.

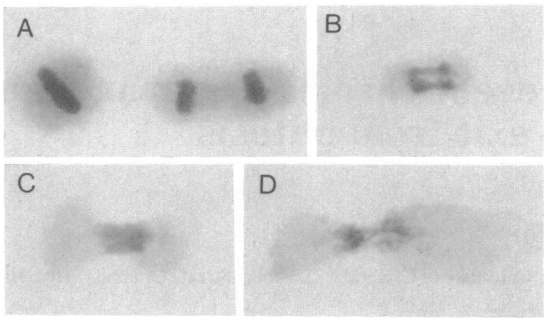

FIG. 1. Light micrographs of HeLa cells 60 min after release from nitrous oxide arrest. (*A*) Control. (*B–D*) Aberrant structures produced by 40 μM etoposide added on release. (×550.)

microscope, so that they could be viewed under phase-contrast conditions while maintained at 37°C in an atmosphere of 5% CO_2. Time-lapse video recordings of selected mitotic cells were made by using a closed circuit TV camera (HV 730 K, Hitachi Denshe) and a Panasonic time-lapse videocassette recorder NV-8051. Single frames were also taken at intervals. At the end of the period of observation, dishes were washed twice in phosphate-buffered saline and fixed in −20°C methanol. After staining in 4′,6-diamidino-2-phenylindole (DAPI; 10 μg/ml), relocated cells were examined by fluorescence microscopy.

Cleavable Complex Formation. Synchronized mitotic HeLa cells, prelabeled in 30-mm dishes for 48 hr with [^{3}H]thymidine at 0.05 μCi/ml (1 Ci = 37 GBq) before nitrous oxide block, were transferred to polylysine-treated 30-mm dishes, given topoisomerase inhibitors, and analyzed in quadruplicate for the formation of cleavable complexes between inhibited topoisomerases and DNA by the potassium/SDS precipitation method of Liu *et al.* (17), as modified for greater sensitivity by lysis of cells in 0.1 M NaOH/0.15 M NaCl before sonication and precipitation of the complex (S. Squires, personal communication).

RESULTS

Inhibition of Anaphase Separation: Population Studies. HeLa cells accumulated in mitosis and treated with the specific topoisomerase II inhibitor etoposide after release from the block form aberrant structures (Fig. 1), which show attempted cleavage in the absence of chromatid segregation. The action of etoposide on mitotic HeLa cells is concentration dependent; population kinetics over 2 hr of treatment are shown in Fig. 2. Progression out of metaphase is delayed, more so at higher etoposide concentrations. Some apparently normal anaphase and telophase structures with separated chromatids are seen after treatment with 20 or even 40 μM etoposide; formation of the latter is delayed. With 60 or 80 μM etoposide, no normal anaphases or telophases are seen, only aberrant structures, and most cells remain arrested in metaphase at the end of treatment. However, a minority of cells produce interphase nuclei despite the absence of normal cytokinesis. It is noteworthy that the concentrations of etoposide needed to prevent segregation are far higher than those needed to arrest the transit from G_2 to prophase, which (as measured by mitotic accumulation) is completely inhibited by 10 μM etoposide (data not shown).

By contrast, camptothecin, a selective inhibitor of topoisomerase I, does not cause the appearance of structures such as are illustrated in Fig. 1, even at concentrations as high as 40 μM. However, an effect similar to that of etoposide has been observed with another agent, 4′-(9-acridinyl)aminomethanesulfon-*m*-anisidide (*m*-AMSA), which selectively inhibits topoisomerase II by a different mechanism from etoposide (18); 40 μM *m*-AMSA entirely inhibits anaphase in HeLa cells (data not shown). Similar effects of etoposide and *m*-AMSA have been observed in several other cell lines (EJ30 human bladder tumor-derived cells, DM Indian muntjac fibroblasts, and WAK *Xenopus laevis* fibroblasts).

Topoisomerase Inhibition. The efficiency of topoisomerase II inhibition in mitotic HeLa cells can be estimated from the concentration dependence of formation, in response to etoposide, of covalently linked alkali-cleavable complexes between inhibited topoisomerase II and cellular DNA (17). These cleavable complexes are formed rapidly on addition of etoposide and dissociate more slowly after its removal (Fig. 3*A*). The concentration dependence of topoisomerase inhibition, as measured by cleavable complex formation, is shown in Fig. 3*B*. Etoposide at 20 μM, which does not block anaphase and only slightly delays progression out of metaphase, does not completely inhibit the cellular topoisomerase II activity; the residual activity is presumably adequate for anaphase segregation. Higher concentrations of etoposide, which produce aberrant anaphase structures, saturate the capacity of mitotic HeLa cells for cleavable complex formation. *m*-AMSA is, in accordance with its greater potency at preventing normal anaphase, a more efficient inducer of cleavable complexes in mitotic HeLa cells (data not shown). Camptothecin-induced cleavable complexes are formed in mitotic HeLa cells (interestingly, in view of the lack of transcription and replication in these cells); complex formation is nearly saturated by 5 μM (Fig. 3*B*), though camptothecin has no effect on anaphase at much higher concentrations.

Time-Lapse Studies with PtK2. In studies of individual PtK2 cells, where the flattened mitotic morphology is convenient for microscopic examination of living cells, it is possible to observe cells that have been treated with topoisomerase inhibitors at defined stages of mitosis without previous synchrony. We have mostly used etoposide, but similar results have again been seen with *m*-AMSA.

In the continuous presence of 60 μM etoposide, added early in prophase before the disappearance of the nucleolus, cells progress to metaphase, with chromosomes assembling

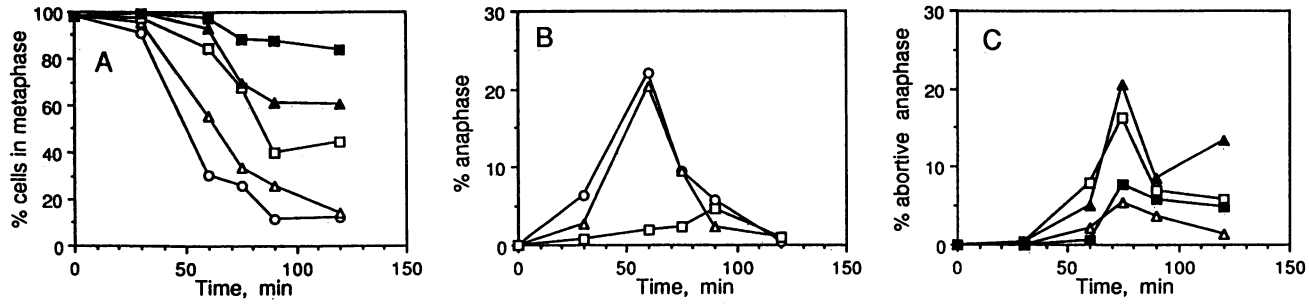

FIG. 2. Effect of etoposide on mitotic progression in HeLa cells after release from metaphase arrest and incubation in medium without etoposide (○), or with etoposide at 20 μM (△), 40 μM (□), 60 μM (▲), or 80 μM (■). Graphs show percentage of cells in metaphase (*A*), in anaphase (*B*), or in aberrant anaphase (*C*) at various times after release.

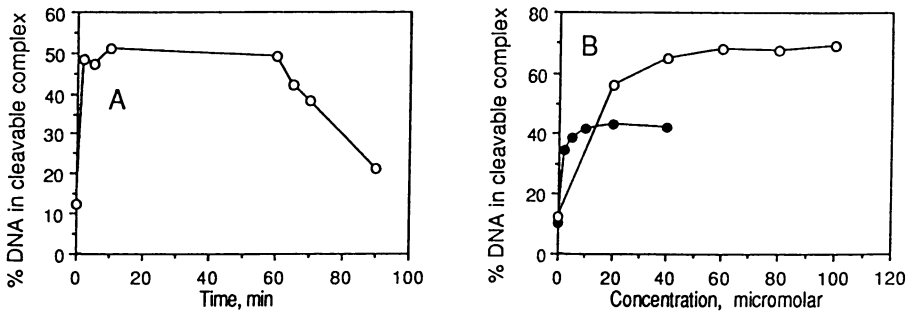

FIG. 3. (*A*) Formation and disappearance of cleavable complexes, as determined by mean percentage of DNA precipitated by SDS/KCl treatment, in mitotic HeLa cells given 60 μM etoposide and washed free of etoposide after 60 min. (*B*) Concentration dependence of cleavable complex formation over 30 min, in mitotic HeLa cells given etoposide (○) or camptothecin (●).

onto a metaphase plate normally. However, metaphase continues for longer than in control cells (Table 1) and is not followed by anaphase chromatid separation. Instead, at the end of metaphase, chromosomes begin to aggregate, and an asymmetric cleavage furrow forms, separating the cells into two unequally sized daughters, typically with chromosomes only in the larger cell (Fig. 4*A*). Cleavage may be completed within the normal time scale, but in some cases it is protracted and accompanied by vigorous apoptotic blebbing, as is often seen in other kinds of perturbed mitoses (19). After cleavage, the two daughter cells spread out, chromosomes decondense, and a single nucleus with nucleolus reforms. Apoptotic blebbing may then continue, particularly in the cell without DNA. Separation between daughters is not always complete, and occasionally the two cells fuse to form a single mononucleate cell. It is notable that 60 μM etoposide has no effect on the morphology of adjacent interphase PtK2 cells within the time scale of these observations.

With 30 μM etoposide added early in prophase, this abolition of segregation is sometimes also seen. More typically, however, the mass of partially aggregated metaphase chromosomes is partitioned during cleavage (Fig. 4 *B* and *C-1–4*), giving rise to daughter cells, both with nuclei; often these are of unequal size, and/or remain connected by a narrow thread of DNA (Fig. 4 *B-8* and *E-4*). Some cells contain a large and a small satellite nucleus (Fig. 4*E-5*). Metaphase is again of longer duration, as with higher drug concentrations (Table 1).

A 15-min pulse of 60 μM etoposide, added in prophase before the disappearance of the nucleolus, leads to a delay in metaphase similar in duration to that seen in the continuous presence of the drug (Table 1). However, subsequent mitotic stages are less severely affected. Anaphase segregation is not prevented, though it is somewhat disordered, and segregation may not be completed before cleavage starts.

When incubation with 60 μM etoposide starts later in mitosis, but before the start of anaphase, segregation is also affected. The behavior of different cells varies, but two patterns are common. In the first, anaphase separation starts on schedule but aborts before chromatids have completely separated (Fig. 4*C-1–4*); subsequent cleavage separates two chromosome masses. In the second, anaphase progresses to completion, but separation of sister chromatids is slow compared with control cells, where chromatids separate within 2–3 min from the start of anaphase and well before the appearance of the cleavage furrow (Fig. 4*E-6–8*). Pairs of chromatids in treated cells may still be joined at the telomeres 6 min after the start of anaphase, and segregation may not be complete until the cleavage furrow forms (Fig. 4 *D* and *E-1–3*). However, cleavage occurs at the normal time and symmetrically, giving rise to two daughter cells, each containing a separate nucleus with nucleolus. Chromosome segregation in PtK2, even if not arrested, is abnormal when 60 μM etoposide is added at any time during metaphase, even when treatment is delayed until the last few minutes before the start of anaphase. Once anaphase has begun, however, the drug is without effect (data not shown).

Table 1. Topoisomerase II inhibition of PtK2 mitotic progression

Treatment			Timing of individual stages of mitosis, min						Time from nucleolar breakdown to cleavage, min
Etoposide, μM	Time added								
	Stage	min	A	B	C	D	E	F	
60	Prophase	18	14	9	—	—	32	—	55
		(1–44)	(10–22)	(5–21)			(22–54)		(38–83)
30	Prophase	5	18	7	—	—	34	—	59
		(1–10)	(16–21)	(7–9)			(19–55)		(48–80)
60	Metaphase	6	11	9	11	7	—	26	38
		(2–15)	(8–14)	(8–10)	(6–15)	(6–7)		(22–31)	(37–43)
30	Metaphase	10	12	8	11	7	—	—	38
		(0–17)	(8–13)	(6–9)	(7–15)	(7–8)			(35–45)
60 for	Prophase	10	15	7	—	—	32	—	54
15-min pulse		(5–16)	(11–18)	(5–8)			(23–50)		(45–75)
0	Prophase		14	7	13	7	—	28	41
			(11–18)	(5–10)	(5–27)	(5–9)		(17–33)	(32–58)

The effect of adding etoposide to PtK2 cells either in prophase or in metaphase is shown. Dimethyl sulfoxide alone was added for the control. Mean timings of addition, and range of values in parentheses, are given for prophase addition in terms of minutes before breakdown of nucleolus, and for metaphase addition in terms of minutes after beginning of metaphase. Durations of subsequent mitotic stages as analyzed by time-lapse videomicroscopy are given for the following mitotic stages: A, nucleolar disappearance to early metaphase; B, early metaphase to full metaphase; C, full metaphase to anaphase; D, anaphase to cleavage; E, full metaphase to cleavage (in cases where anaphase was prevented); F, cleavage to appearance of nucleolus. The number of cells observed for each set of conditions was in the range 3–8.

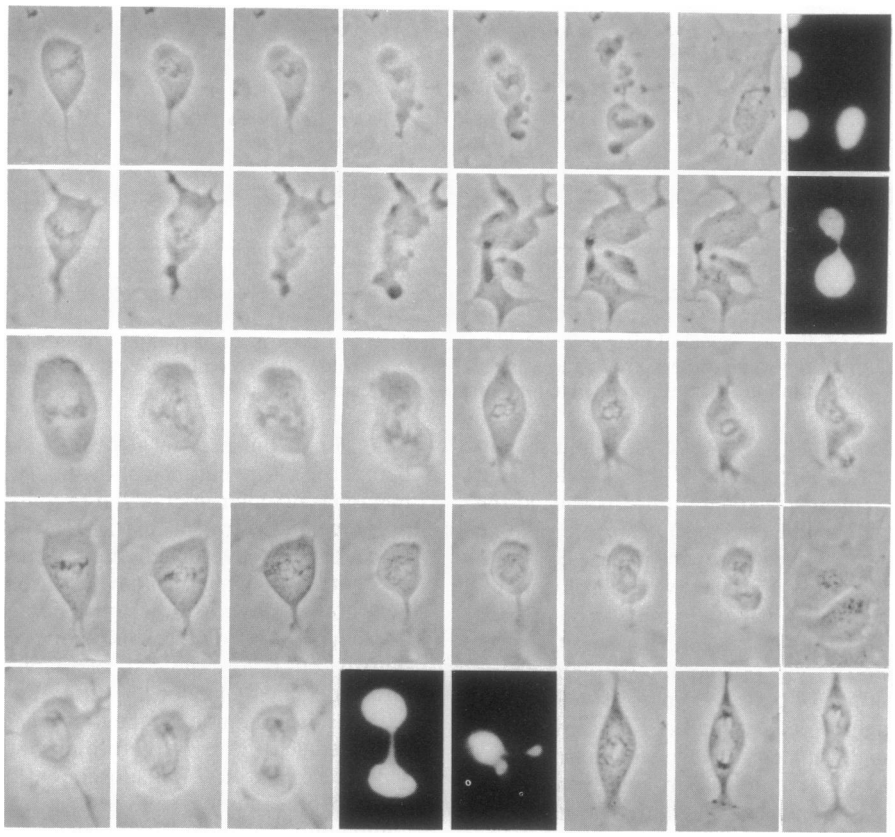

FIG. 4. Time series of phase-contrast micrographs of living PtK2 cells (except *A-8*, *B-8*, *E-4*, and *E-5*, which are fluorescence micrographs of fixed, DAPI-stained cells) incubated with 30–60 μM etoposide, added in prophase (*A*, *B*, *C-1–4*) or metaphase (*C-5–8*, *D*, *E-1–3*) or not at all (*E-6–8*). Times to the nearest minute are, relative to the first picture in each series, as follows: *A-1–8*, 0, 9, 10, 12, 13, 20, 59, 59; *B-1–8*, 0, 3, 7, 12, 19, 22, 25, 107; *C-1–4*, 0, 22, 22, 23; *C-5–8*, 0, 1, 4, 5; *D-1–8*, 0, 3, 4, 18, 19, 20, 21, 55; *E-1–3*, 0, 1, 2; and *E-6–8*, 0, 5, 8. *E-4* and *5* are isolated frames of cells not represented in phase-contrast series. Note the considerable time interval between *B-7* and *B-8*, during which the relative orientation of the two nuclei has changed. (All ×340 except *B-8*, ×740; *C-1–4* and *E-1–3*, ×500; *A-8*, ×270; *E-4* and *5*, ×410.)

These effects observed with metaphase PtK2 cells differ in detail from those seen with HeLa cells. The differences may well be due to the differing protocols, for the PtK2 cells were given etoposide during prophase or metaphase of previously unaffected mitoses, while the HeLa cells were exposed to the drug during the period of recovery from nitrous oxide arrest.

Nondisjunction in Muntjac Cells. These effects of extensive inhibition of chromatid separation are perceptible only at concentrations of etoposide that, for mitotic cells, are ultimately lethal; the relative plating efficiency of mitotic HeLa cells after 1 hr in 40 μM etoposide is only 2%. However, it is possible to perceive etoposide-induced chromosome nondisjunction in mammalian cells given low concentrations of etoposide in mitosis. With the Indian muntjac line DM, which is convenient because it has a small number of large chromosomes (generally nine), treatment in mitosis with low micromolar concentrations of etoposide allows cells to proceed through mitosis with reasonable survival. Mitotic cells given 1 μM etoposide for 60 min, when arrested in the next mitosis, show a high frequency of chromosome abnormalities; and 15% have two to four supernumary chromosomes, indicative of nondisjunction.

DISCUSSION

These experiments show that DNA topoisomerase II inactivation prevents mitotic chromatid separation in mammalian cells, as it does in yeast. Earlier reports to the contrary (14) were based on work with less specific topoisomerase inhibitors, novobiocin and nalidixic acid, which have a variety of nontopoisomerase targets; this can produce considerable confusion (20, 21). We have shown that the metaphase–anaphase transition is sensitive to specific inhibitors of DNA topoisomerase II, but not of topoisomerase I, both in cells accumulated in mitosis and in unsynchronized PtK2 cells, in a largely reversible manner analogous to the effects of *top2* but not *top1* mutations in yeast (3–7). The presumptive role of topoisomerase II in mitosis is to separate intercalated DNA loops on adjacent chromatids—a fundamental requirement for segregation at cell division, effected by type 2 topoisomerases even in *Escherichia coli* (22). We have not formally excluded the possibility that both etoposide and *m*-AMSA prevent chromatid separation, but not cytokinesis, by inhibiting activities other than topoisomerases—for example, the engagement of kinetochores with the spindle (23). However, in view of the entirely different molecular structures and mechanisms of action of these two topoisomerase inhibitors (18), and the parallel with yeast *top2* mutants, we consider this possibility unlikely.

The recent demonstration by Roberge *et al.* (24) that histone H1 dephosphorylation occurs in mitotic BHK (baby hamster kidney) cells treated with the topoisomerase II inhibitor teniposide may provide evidence for the molecular events corresponding to the cellular activities we observe. A partial chromosome decondensation is reported by Roberge *et al.* (24) in cells treated with teniposide while arrested in mitosis by microtubule inhibitors, and the decondensation is interpreted by them as a relaxation of the chromatin to allow for the repair of teniposide-induced DNA strand breaks; this is probably distinct from the decondensation we observe after abortive cytokinesis. An earlier report, that chromosome aberrations induced in grasshopper neuroblasts by treatment in interphase with *m*-AMSA may be due to "chromosome stickiness" (25), is consistent with the effects described here.

The parallel between yeast and larger eukaryote mitotic topoisomerase functions is not, however, complete. We have not observed anything analogous to the long, entangled prophase-like structures seen in *top2* yeast entering mitosis at the restrictive temperature (4), even in cells given etoposide early in prophase. This may reflect a difference in the scheduling of topoisomerase II activities in the yeast and mammalian cycles; alternatively, it may be a consequence of our mode of investigation. Even specific inhibitors of topoisomerase II have a secondary radiomimetic effect on cell cycle controls, probably exerted through the formation of DNA strand breaks: they indirectly inhibit the mitotic $p34^{cdc2}$ kinase (26). This effect, which prevents progression through G_2 phase into prophase, is exerted by etoposide up to 30 min before prophase (14).

But topoisomerase II inhibitors are believed to affect decatenation at a late stage in mammalian DNA replication (27). Likewise, in yeast, it is known that much decatenation may be performed in interphase; cell-cycle mutants of *Saccharomyces cerevisiae* carrying circular minichromosomes, arrested after replication but before mitosis, had most minichromosomes decatenated (28). So if a topoisomerase II action some time before prophase is necessary for full mitotic chromosome condensation in mammalian cells as in yeast, such an action would not be detectable in our system, since a primary action of etoposide in blocking such condensation at the prophase stage would be masked by the secondary action in preventing prophase from starting. The most we can say is that if topoisomerase II action is required in PtK2 cells for progression through prophase to metaphase, this action occurs well before prophase starts.

Continued topoisomerase II action in mammalian prophase may also be necessary for the metaphase–anaphase transition, for the cycle progression of PtK2 cells is more sensitive to etoposide given in prophase than in metaphase (Table 1). Again, we cannot be sure that the effects of etoposide in prophase are not due to an indirect inhibition of the $p34^{cdc2}$ kinase. Later effects, however, are more clearly attributable. Studies on the mitotic delay imposed by ionizing radiation in CHO (Chinese hamster ovary) cells (29), which also involves $p34^{cdc2}$ inhibition (26), indicate that after a transition point in midprophase no delay can be induced. By analogy, we would suppose that the effects seen with higher concentrations of etoposide later in mitosis reflect an action on topoisomerase II rather than on $p34^{cdc2}$. We have not, incidentally, observed an etoposide-induced reversal of early prophase, such as can be seen in irradiated prophase CHO cells (29).

The resemblance between the action of topoisomerase II inhibitors on mammalian cells and of *top2* mutations on yeast extends to the uncoupling of cell division from chromatid separation; mitotic cleavage furrows can be seen over the metaphase chromosomes, instead of between the telophase chromosomal sets (Figs. 1 and 4). Thus, as in yeast, cytokinesis is attempted even when a lack of topoisomerase function prevents chromatid separation. Again, the parallel is not exact; fission yeast, with their architecture rigidly constrained by the cell wall, attempt an accurate cleavage when disjunction is inhibited. Mammalian cells may attempt something similar and produce figures analogous to the *top2* aberrant mitosis; we do not know by what mechanism, or how accurately, chromatin is thus separated in the absence of complete chromatid separation. Alternatively, if topoisomerase inhibition is severe and prolonged, mammalian cells may eventually achieve a distorted cleavage without any separation (Fig. 4).

We conclude, though, that the later events of mammalian mitosis occur even when chromatid separation is inhibited. The prolongation of metaphase in etoposide-treated PtK2 (Table 1) may indicate that cytokinesis is partly coupled to chromatid separation, but after a time, the cells proceed through the metaphase–anaphase transition even though they still have inseparable chromatids. This action of topoisomerase II inhibitors on mitotic mammalian cells has considerable potential as a system for studying the regulation of late mitotic events.

We thank Roger Northfield, Hugh Buckner, and Martin Mamo for skilled assistance, Paul Smith for topoisomerase inhibitors, Charles ffrench-Constant for the loan of the viewing chamber, Richard Warn for PtK2 cells, and James Fawcett for the loan of the video equipment. We are grateful to the Cancer Research Campaign, of which R.T.J. is a Fellow, for continued support, and to Newnham College, Cambridge, for support to A.M.M.

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