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Comparison of effects of fostriecin, novobiocin, and camptothecin, inhibitors of DNA topoisomerases, on DNA replication and repair in human cells

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ABSTRACT

Fostriecin causes a delayed inhibition of replicative DNA synthesis in human cells, consistent with a role for DNA topoisomerase II (its target enzyme) at a late stage in replication. Fostriecin does not inhibit UV-induced excision repair. The less specific inhibitor novobiocin blocks repair in permeabilised cells given a low dose of UV, presumably through a mechanism other than the inhibition of topoisomerase II. Its effect cannot be accounted for by a depletion of the ATP required for incision. Camptothecin, an inhibitor of DNA topoisomerase I, blocks replicative DNA synthesis immediately but incompletely, suggesting a participation of topoisomerase I at the replication fork, but it, too, has no influence on DNA repair. We thus find no evidence for involvement of either topoisomerase I or II in the response of cells to UV damage.

INTRODUCTION

The biochemical pathway for excision repair of UV-damaged DNA in mammalian cells is known only in outline. The rate-limiting step of incision is the earliest defined reaction and is essentially an endonucleolytic breakage of DNA at the site of damage. Although extracts of human cells retain the ability to insert nicks in UV-damaged DNA (1), the enzyme has not been purified. Details of the enzymology of the reaction come from cell-free or permeabilised cell systems, and from indirect studies using inhibitors of later steps in the pathway. Further information is likely to emerge from genetic studies carried out with cells from subjects with the disease xeroderma pigmentosum (associated with defective excision repair), and with mutants derived from rodent cell lines in the laboratory. In both these cases, cell lines are classified as belonging to one of a number of complementation groups, i.e. several distinct proteins are involved in achieving incision. Genes complementing the defect in some of these mutant lines have been isolated (2), but the functions of the gene products remain obscure. They might be involved in recognition of the lesion, or in rendering the DNA more accessible to repair enzymes, rather than in incision itself.

DNA topoisomerases are enzymes with the ability to make transient DNA breaks (single-strand breaks in the case of the class I and double-strand breaks in the case of the class II enzyme) which permit the passage of another strand or double helix before resealing of the break. They thus achieve unwinding of the double helix, reduction of the degree of supercoiling, and release of intertwined DNA molecules; in general, they alter DNA topology. A topoisomerase-mediated change in damaged DNA, rendering it locally less compact, might be a prerequisite for incision. It was found (3, 4) that the topoisomerase II inhibitor novobiocin blocked incision in human cells irradiated with UV, supporting the idea of a pre-incision rearrangement of DNA. However, it later transpired that novobiocin is far from being a specific topoisomerase inhibitor. It disrupts ATP metabolism, leading to a reduced ATP/ADP ratio, and severe changes are seen in mitochondrial structure (5). Thus the inhibition of incision seen with novobiocin might be caused by depletion of ATP, required for incision (6), rather than via topoisomerase.

A role for topoisomerase in a pre-incision step is not excluded by these findings. However, other topoisomerase II inhibitors VP-16 and *m*-AMSA, which act by blocking the enzyme in the intermediate state with a stable enzyme-linked double-strand DNA break, do not inhibit repair of UV damage (7, 8). Here we report experiments with fostriecin, a novel inhibitor of topoisomerase II which, unusually, blocks an early step in the reaction and does not accumulate broken DNA intermediates (9). We have looked for a possible inhibition of incision in HeLa cells. Since other reactions of repair might also be influenced by topoisomerases, we have examined repair synthesis and ligation in the presence of fostriecin. In addition, we have reexamined the effects of novobiocin on repair using a permeabilised cell system, in which the availability of ATP for incision can be artificially controlled.

We also report experiments with camptothecin, an inhibitor of DNA topoisomerase I, which blocks the enzyme at an intermediate stage, leaving it bound to the DNA at the site of the normally transient break (10, 11). We have looked for effects of camptothecin on repair of UV-induced damage. Since it is conceivable that, in the presence of an inhibitor of one topoisomerase, the other topoisomerase takes over an essential role, we have also treated UV-irradiated cells with a combination of camptothecin and fostriecin. Our conclusion is that there is no essential role for either topoisomerase I or II in UV repair.

MATERIALS AND METHODS

Cell culture

HeLa cells (human transformed epithelial cells) were grown in monolayer culture in Eagle's minimal essential medium (MEM: Glasgow modification, from Flow Laboratories), supplemented with 10% horse serum, non-essential amino acids and penicillin and streptomycin. Stocks were maintained in flat glass bottles; for experiments, cells were plated out in plastic Petri dishes (Nunc). Cells were incubated at 37° in a 5% CO_2 atmosphere.

UV irradiation: chemicals

Cells were irradiated as monolayers after removal of medium. A standard dose of 12 Jm^{-2} was delivered at a rate of 1 $Jm^{-2}s^{-1}$ from a germicidal lamp emitting predominantly at 254 nm. Control cells were mock-irradiated.

[2-^{14}C]dThd and [Me-^{3}H]dThd were from Amersham International. Deoxyribonucleosides, ATP and saponin were from Sigma, and novobiocin and hydroxyapatite from Boehringer Mannheim. Fostriecin, a gift from Parke-Davis, was stored at −80° under desiccation and care was taken to avoid hydration during handling. Camptothecin and aphidicolin (both from Sigma) were dissolved in dimethylsulphoxide at 5 mM and 6 mM respectively; hydroxyurea (Sigma) and fostriecin were dissolved in PBS. These stock solutions were diluted with medium (at 37°) for addition to cells as described in figure legends. When appropriate, the same amounts of dimethylsulphoxide were added to control cultures. We found that the effect of camptothecin on replication, and its ability to break DNA, were very sensitive to pH; if medium was not pre-equilibrated in the 5% CO_2 atmosphere, uptake of the drug was apparently delayed. No such dependence on pH was seen in the case of fostriecin.

Measurement of replicative DNA synthesis

60 mm dishes were set up with 6 × 10^5 cells each, in 2 ml of medium. For the experiments of Figs 1 and 5, cells were prelabelled by incubating for one day with [^{14}C]dThd (57 mCi/mmole) at 0.0025 μCi/ml. They were washed with phosphate-buffered saline (PBS), given fresh medium, and incubated with inhibitors and with [^{3}H]dThd (40 Ci/mmol, 0.2 μCi/ml) as indicated in legends. In the case of the experiment described in Fig. 6, cells were not prelabelled. We followed the cumulative incorporation of [^{3}H]dThd added to the medium at 0.08 μCi/ml (40 Ci/mmole), with or without camptothecin, one day after setting up the cells; sample dishes were taken at intervals.

To measure incorporation of radioactive dThd into DNA, the sample dishes were washed with PBS and lysed with 1 ml 0.5 M NaOH. The lysate was transferred to a test tube, and 1 ml of 20% (w/v) trichloroacetic acid was added to denature macromolecules. After 30 min at 4°, the precipitates were collected on GF/C filters (Whatman), washed with 5% (w/v) trichloroacetic acid and with ethanol, and dried. The filters were immersed in Optiscint T (LKB) and incorporated radioactivity measured by scintillation counting. Double label incorporation was analysed in the standard way.

Measurement of DNA breaks

Cells inoculated at 6 × 10^5 per 60 mm dish were prelabelled by incubating overnight with [^{3}H]dThd (40 Ci/mmole, 0.08 μCi/ml). After removal of radioactive medium and a PBS wash, cells were incubated with or without inhibitors and UV-irradiated as described in figure legends. Cells were lysed in 1.5 ml alkaline solution (0.15 M NaCl, 0.1 M NaOH, 10 mM Na_2EDTA, 5% sucrose) at 4° for 15 min to permit strand unwinding at ends and breaks (12), neutralised by addition of 1 M KH_2PO_4, and the percentage of single-stranded DNA was measured by hydroxyapatite chromatography as described (13). The proportion of single-stranded DNA depends on the extent of DNA unwinding that occurred in alkali, which in turn reflects the number of breaks present. The assay was calibrated in terms of DNA breaks per 10^9 daltons, using cells containing known numbers of DNA breaks introduced by X rays.

Measurement of repair DNA synthesis

Cells inoculated at 2 × 10^5 per 35 mm dish were incubated overnight. The medium was removed for UV irradiation, and replaced with medium containing 5 μCi of [^{3}H]dThd, with or without fostriecin, camptothecin or both, for a 60 min incubation. Control cultures were mock-irradiated. Cells were then washed with PBS, fixed with methanol:glacial acetic acid (3:1), extracted several times with cold 5% (w/v) trichloroacetic acid to remove unincorporated label, and processed for autoradiography as described (14).

Permeabilisation: in vitro repair

HeLa cells inoculated at 1.7 × 10^6 per 60 mm dish were incubated overnight with [^{3}H]dThd (40 Ci/mmole, 0.08 μCi/ml). After washing with PBS and then with Hepes-buffered saline (0.1 M KCl, 20 mM NaCl, 1 mM EGTA, 30 mM Hepes, pH 7.0), the cells were permeabilised by incubation with this buffer containing 0.025% saponin for 2 min at room temperature. The cells were rinsed with Hepes-buffered saline without saponin and then irradiated with UV.

The irradiated, permeabilised cells were incubated for 45 min at 37° in a repair reaction mixture based on that of Kaufmann and Briley (15) containing 0.16 M sucrose, 12 mM KCl, 9 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.6 mM EDTA, 30 mM Tris-HCl, final pH 8.3 at 25°. The repair reaction also contained, for the experiment of Fig. 3, 5 mM ATP and various concentrations of novobiocin. For the experiment of Fig. 4, the concentration of novobiocin was 1 mM, and that of ATP was varied. The concentration of $MgCl_2$ was also varied at the highest concentration of ATP (see legend). DNA breaks were measured as in the incision assay above, except that the alkaline solution comprised 0.3 M NaCl, 0.15 M NaOH, 10 mM Na_2EDTA and 5% sucrose (the higher pH and ionic strength are appropriate for samples where relatively few DNA breaks are expected).

RESULTS

Effects of fostriecin and novobiocin

Fostriecin is an inhibitor of topoisomerase II in mammalian cells (9). Since topoisomerase II is thought to participate in DNA replication, we looked first at the effect of fostriecin on incorporation of [^{3}H]dThd into DNA in proliferating HeLa cells. [^{3}H]dThd was added for 30 min pulses up to 150 min after adding fostriecin. It is clear from Fig. 1 that the inhibition of replication by fostriecin increases progressively with time, from zero inhibition in the first pulse interval to 68% inhibition in the last interval. Unpublished experiments showed over 90% inhibition of [^{3}H]dThd incorporation measured 20 hours after

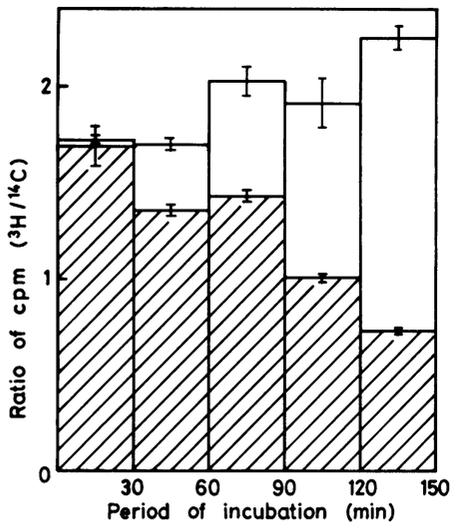

Figure 1. Replicative DNA synthesis; effect of fostriecin. HeLa cells prelabelled with [^{14}C]dThd were pulse labelled with [^{3}H]dThd for 30 min intervals after addition of fostriecin at 0.22 mM (hatched columns) or the corresponding volume of PBS (open columns). Incorporation into acid-insoluble material, expressed in terms of the ratio of ^{3}H to ^{14}C cpm, reflects synthesis of new DNA. Bars represent standard errors of means.

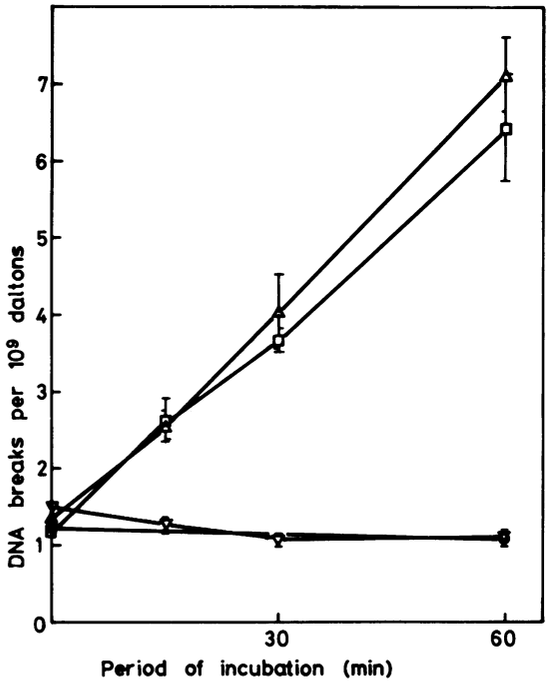

Figure 2. Incision after UV irradiation. HeLa cells, prelabelled with [^{3}H]dThd, were incubated for 30 min with fostriecin (0.22 mM), ▽; with hydroxyurea (10 mM) and aphidicolin (15 μM), △; with fostriecin, hydroxyurea and aphidicolin, □; or with no inhibitors, ○. They were then irradiated with UV and incubated for various times, as indicated, with the same inhibitors. DNA breaks were assayed by alkaline unwinding and hydroxyapatite chromatography. Bars indicate range of duplicates.

adding 0.22 mM fostriecin; this concentration is therefore sufficient to produce a maximal effect. The strong but delayed inhibition of DNA synthesis by fostriecin is in marked contrast

Table 1. Repair DNA synthesis in UV-irradiated HeLa cells

	Grains per nucleus (± S.E. of mean)	
	No UV	Plus UV
Experiment 1		
No inhibitor	7.8 (±1.3)	55.9 (±4.5)
Plus fostriecin	8.6 (±1.4)	54.4 (±2.8)
Experiment 2		
No inhibitor	9.5 (±1.2)	55.6 (±6.5)
Plus camptothecin (1 μM)		55.6 (±7.7)
Plus camptothecin (10 μM)	11.5 (±1.1)	49.3 (±4.5)
Experiment 3		
No inhibitors	13.4 (±1.8)	41.1 (±1.7)
Plus camptothecin, fostriecin	12.6 (±1.2)	42.5 (±2.6)

The concentration of camptothecin was 10 μM, and of fostriecin, 0.22 mM, except where stated otherwise. Data represent the mean number of grains from up to 20 non-S phase nuclei scored in autoradiographs.

with the rapid inhibition that would be seen with a drug acting directly at the DNA polymerisation step.

We then investigated whether fostriecin has any effect on DNA repair after UV irradiation, looking at different aspects; the initial event of incision at sites of damage in the DNA, the subsequent synthesis of a repair patch by polymerisation of deoxyribonucleotides, and the final ligation or sealing of the patch into the pre-existing DNA.

Incision is normally hard to detect, because the DNA breaks are transient. However, if the synthetic steps – repair synthesis and ligation – are blocked with DNA synthesis inhibitors, these repair sites remain unsealed, and DNA breaks accumulate with time. This is seen in Fig. 2, on incubation of UV-irradiated cells with the combination of hydroxyurea, an inhibitor of ribonucleotide reductase, which provides DNA precursors (16), and aphidicolin, blocking DNA polymerase (17). If fostriecin were able to inhibit polymerisation directly, it would emulate hydroxyurea and aphidicolin and cause incomplete repair sites to accumulate. We have shown that fostriecin is not a direct inhibitor of replication, and Fig. 2 shows that, accordingly, breaks do not accumulate with fostriecin alone after UV irradiation. Fig. 2 also gives the result of incubating cells with fostriecin in conjunction with hydroxyurea and aphidicolin. In this case, fostriecin has no effect on the accumulation of breaks; i.e. incision is unimpaired by the presence of the topoisomerase inhibitor. Even when cells were preincubated for 3 hours with fostriecin before UV irradiation, and then incubated with fostriecin, hydroxyurea and aphidicolin, breaks due to incision accumulated, though to a slightly lower final level than with just hydroxyurea and aphidicolin (results not shown). Overall, there is no evidence for a necessary involvement of topoisomerase II at the incision stage of repair. Furthermore, the fact that breaks do not appear with fostriecin alone after UV irradiation implies that ligation, as well as repair synthesis, takes place as normal.

We confirmed the normal occurrence of repair DNA synthesis by autoradiography, after incubation of HeLa cells for 60 min with [^{3}H]dThd and fostriecin. Unscheduled DNA synthesis was estimated by counting grains over nuclei of non-S phase cells (i.e. excluding those heavily labelled). Fostriecin had no effect on the level of UDS after UV irradiation, nor on the background level of nuclear silver grains in unirradiated cells (Table 1).

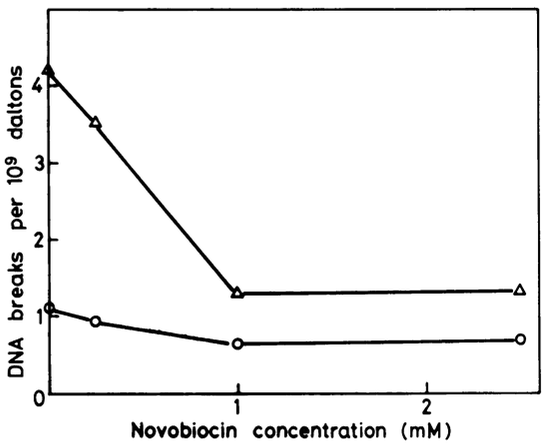

Figure 3. Repair in permeabilised cells; effect of novobiocin on incision. Permeabilised cells were UV-irradiated (△) and incubated for 45 min with novobiocin at the concentrations shown, in the presence of 5 mM ATP. Unirradiated controls: ○. DNA breaks, representing incision, were assayed by alkaline unwinding and hydroxyapatite chromatography. Means of duplicate values are shown.

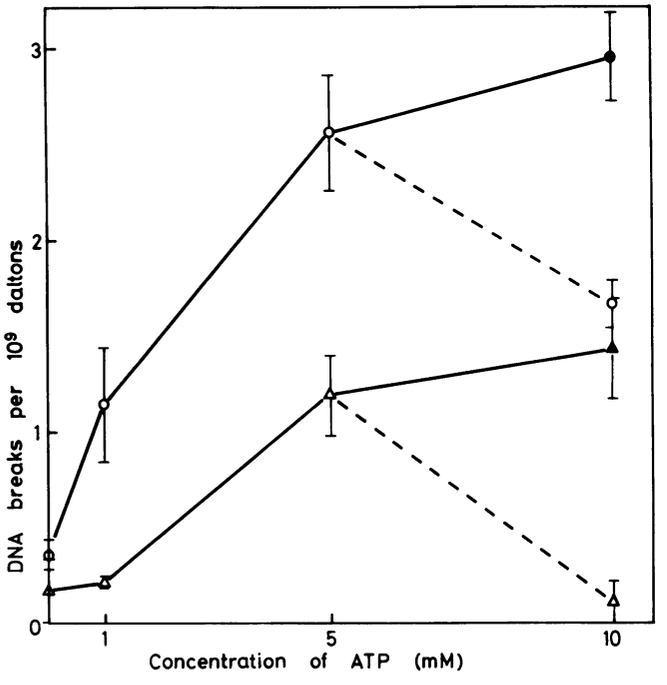

Figure 4. Repair in permeabilised cells; dependence of novobiocin effect on concentration of ATP. Permeabilised cells were UV-irradiated and incubated for 45 min with (△) or without (○) novobiocin (1 mM), in the presence of different concentrations of ATP. Solid symbols; $MgCl_2$ present at 13 mM (otherwise at 9 mM). DNA breaks, representing incision, were assayed by alkaline unwinding and hydroxyapatite chromatography. DNA breaks accumulated in control incubations (i.e. without UV, with or without novobiocin) have been subtracted. Bars indicate standard errors of means.

These results with fostriecin seem to contradict the earlier findings of an inhibition of incision by novobiocin (3, 4). They do not support the idea that topoisomerase II – the presumed target of novobiocin – is required for incision to take place. The action of novobiocin on incision, an ATP-dependent reaction (6), could be explained by its disruption of the cellular ATP supply

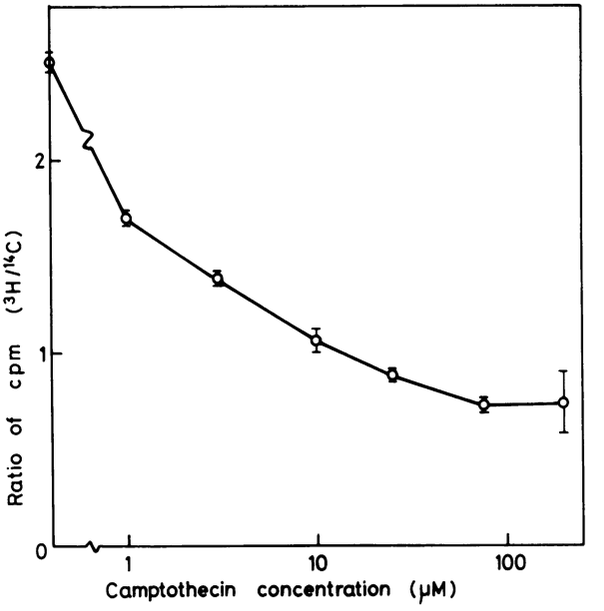

Figure 5. Replicative DNA synthesis; effect of increasing concentrations of camptothecin. Cells prelabelled with [^{14}C]dThd were incubated in fresh medium for one hour before addition of camptothecin and [^{3}H]dThd. Synthesis of DNA during the subsequent hour is indicated by the ratio of ^{3}H to ^{14}C cpm in acid-insoluble material. Bars represent standard errors of means.

(5). We therefore examined the ability of novobiocin to block incision in a permeabilised HeLa cell system in which the supply of ATP is independent of cellular metabolism. Cells were permeabilised with saponin, irradiated with UV, and incubated under conditions (without dNTPs) which allow incision but not repair synthesis or ligation. ATP was provided at 5 mM. Over a period of 45 min, a substantial number of breaks accumulate (Fig. 3). Control experiments using dye exclusion confirmed that more than 99% of the cells remained permeable during this period. Fig. 3 clearly shows the inhibitory effect of novobiocin; at 1–2.5 mM, DNA breaks are reduced by about three quarters. In another experiment, the concentration of novobiocin was constant (Fig. 4); there was no indication that increasing the concentration of ATP caused any decrease in the effectiveness of novobiocin as an inhibitor. Incision was depressed by about half, whether ATP was present at 5 mM or 10 mM. (In the latter case, a higher concentration of $MgCl_2$ was optimal for incision; see ref. 6.) Thus novobiocin acts on repair independently of the supply of ATP.

Effects of camptothecin

To establish the likely range of concentration of camptothecin over which a possible effect on repair might be seen, we first measured the inhibition of replicative DNA synthesis in terms of incorporation of [^{3}H] thymidine into DNA in a randomly proliferating culture of HeLa cells incubated for one hour with the drug (Fig. 5). A concentration of 1 μM had a substantial inhibitory effect, and at 10 μM incorporation was reduced to about 40% of the control level. Even at 20 × this concentration, there was still a residual 29% of control incorporation, so it seems that inhibition of replication is only partial. We decided to use up to 10 μM camptothecin in further experiments, since this concentration has maximal effect in terms of inducing DNA breaks (18). Camptothecin is strikingly rapid in taking effect;

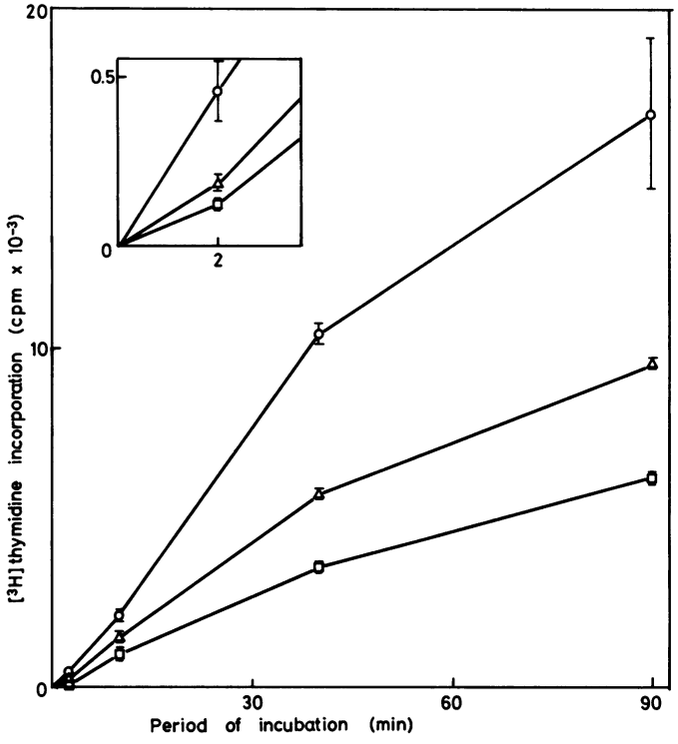

Figure 6. Time course of inhibition of DNA replication by camptothecin. The cumulative incorporation of [^{3}H]dThd into DNA was measured in the absence (○) or presence of camptothecin at 1 μM (△) and 10 μM (□). Bars indicate standard errors of means.

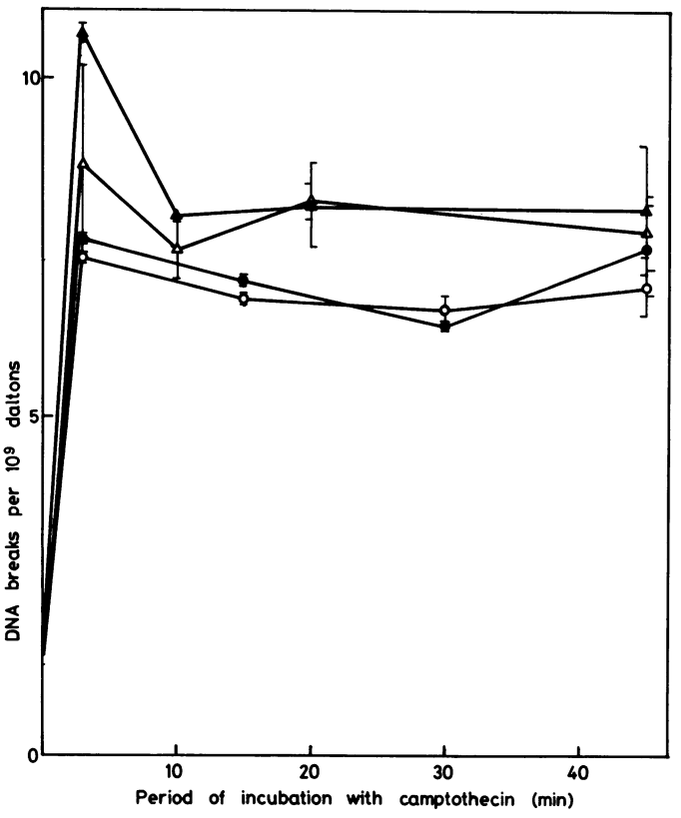

Figure 7. Induction of DNA breaks by camptothecin. HeLa cells were prelabelled with [^{3}H]dThd and incubated for 1 hour in fresh medium before addition of camptothecin at 1 μM (○, ●) or 10 μM (△, ▲). Open symbols indicate that the cells were UV-irradiated just before adding inhibitor. The data point at t_0 represents cells neither irradiated nor incubated with camptothecin. DNA breaks were measured by alkaline unwinding/hydroxyapatite chromatography. Bars indicate standard errors of means.

the inhibition is established to its maximum extent within the first two minutes of incubation (Fig. 6).

We next looked for an effect of camptothecin on DNA repair, studying the incorporation of [^{3}H]thymidine into DNA in the nuclei of non S-phase cells, i.e. UDS. Table 1 shows, first, that 10 μM camptothecin does not change the background level of grains in unirradiated cells. Nor does it have a significant effect on the UDS occurring as a result of UV irradiation of cells.

Camptothecin is known to produce DNA breaks by interfering with the reaction of topoisomerase I, blocking the DNA rejoining step. We confirmed that, in HeLa cells, camptothecin induces DNA breaks at both 1 μM and 10 μM (Fig. 7). These topoisomerase-dependent breaks make it difficult to investigate the possible effect of camptothecin on the early and rate-limiting step of nucleotide excision repair, incision, since incision itself is measured by the production of DNA breaks. Fig. 7 does at least show clearly that there is no significant difference between the number of breaks accumulating in unirradiated and in UV-irradiated cells, whether 1 or 10 μM camptothecin is present.

Because of the difficulty of distinguishing repair-related DNA breaks and topoisomerase-dependent breaks induced by camptothecin itself, it was not possible to look for an effect of this inhibitor on the final step of DNA repair, the ligation of repair sites.

A substitution of topoisomerase II for topoisomerase I when the latter is inhibited could explain the lack of effect of camptothecin on repair. Similarly, the lack of effect of the topoisomerase II inhibitor, fostriecin, could be explained by a substitution of topoisomerase I for II. To test these possibilities, we applied camptothecin and fostriecin in combination. Table 1 demonstrates that repair occurs normally even when both topoisomerases are inhibited, since UDS after UV irradiation is not affected by the presence of camptothecin and fostriecin together.

DISCUSSION

The use of novobiocin as a topoisomerase inhibitor is problematical, since it has various other actions on cells. One of its principal effects is on mitochondrial function; the ATP/ADP ratio is grossly disturbed, and it was suggested (5) that this is the primary cause of its effect on incision. Dresler and Robinson-Hill (19) studied novobiocin in permeabilised cells, where it is possible to provide ATP and thus by-pass the cellular ATP supply system; they found that there was still an inhibitor effect of novobiocin on incision. However, as Downes *et al.* commented (7), the UV dose used was high, and different modes of repair of high and 'normal' levels of UV-induced damage (15) might account for this result. We have therefore repeated the experiment in permeabilised cells using a low UV dose, and we find, still, a strong inhibitory effect. Furthermore, the novobiocin inhibition of incision is not reduced at all by doubling the concentration of ATP available. So novobiocin, after all, must inhibit UV repair by some means other than the disruption of mitochondrial function.

As to whether this inhibition is due to a blockage of topoisomerase II, the answer is probably no, on the evidence of

experiments with other topoisomerase II inhibitors. Fostriecin is a more specific inhibitor than novobiocin; it does not decrease ribonucleotide or deoxyribonucleotide pools, nor does it directly inhibit RNA or DNA polymerase (20). Fostriecin inhibits the overall catalytic activity of the enzyme, unlike the inhibitors such as *m*-AMSA, VP-16 and VM-26 that block topoisomerase II after it has made DNA breaks (see review, 21). The absence of inhibitor-induced breaks makes it much simpler to analyse effects of fostriecin on repair, where the most sensitive assays depend on the detection of repair-related DNA breaks. We find no sign of any inhibitory effect of fostriecin on incision, repair synthesis or ligation – at a concentration that strongly inhibits replicative DNA synthesis. The ability of novobiocin to inhibit incision in mammalian cells remains unexplained, but it is reported (8) to be a potent inhibitor of the *Micrococcus luteus* UV endonuclease *in vitro*.

The effect of fostriecin on replication follows an interesting time course, with no inhibition at first and then a steadily increasing degree of inhibition. This pattern fits very well with the commonly held idea, based on work with yeast (22) and with an *in vitro* SV40 replication system (23), that the role of topoisomerase II in eukaryotic DNA replication is to separate daughter double helices which inevitably become intertwined during the process of replication. DNA replication in mammalian cells is organised temporally into clusters of replication units, each undergoing replication for only a fraction of the total S-phase length of 7–8 hours (24–26). The order of replication of these units of DNA is fixed, and initiation of one set of replication units depends on the completion of replication of preceding sets (27). So fostriecin, by preventing the final stage of replication, perhaps blocks subsequent initiation in other units – accounting for the delayed effect we observe. Although a similar time course was previously recorded (20), the possible significance was not appreciated; at that time the target of the inhibitor was not known. A delayed action of the drug could, alternatively, be due to inefficient entry into the cells. It is thought (28) that fostriecin is taken up via the reduced folate carrier system. Although methods do not exist to detect fostriecin within cells, the uptake of methotrexate via the same carrier has been measured, and the $t_{1/2}$ for entry is about 5 minutes (28), so we consider it unlikely that fostriecin is inactive because of slow uptake. This conclusion is supported by our experiments with prolonged preincubation, which did not significantly alter the effectiveness of the inhibitor.

There is an apparent inconsistency between our claim, on the basis of a 1 hour incubation, that there is no effect of fostriecin on repair DNA synthesis, and the recognition of an inhibition of replicative synthesis which becomes pronounced only after 1 hour. However, DNA repair and replication are very different processes. In the case of repair, each individual event lasts only a few minutes and the incorporation during an hour is the summation of many such events; if the total incorporation is not affected by fostriecin, then the individual repair events are evidently immune to this inhibitor. The inhibition of replication, by contrast, is an effect on a continuous, long-term process.

Camptothecin has been known for many years as an antitumour agent with inhibitory effects on DNA and RNA synthesis, and with a capacity for causing a reversible fragmentation of DNA in mammalian cells, but only recently was its target enzyme identified as topoisomerase I (10).

Camptothecin has an immediate effect on incorporation of [^{3}H]dThd into DNA (in unirradiated cells), and yet the inhibition is only partial; replication is depressed by about 70% (Fig. 5). The rapidity of action implies an effect at the replication fork, i.e. the point at which incorporation occurs, and is in marked contrast to the delayed effect of fostriecin. The only known target of camptothecin is DNA topoisomerase I; the role of this enzyme in replication is most likely to be in unwinding the double helix (by passing one strand through a transient gap in the other) in advance of the replication fork.

The DNA breaks caused by camptothecin appear rapidly, in line with the immediate effect on replication. Camptothecin-induced breaks have been detected close to the replication fork in SV40 minichromosomes (29). A recent report (30) that cotreatment of cells with aphidicolin (to inhibit replication fork movement) protects them from the lethal effect of camptothecin, gives support to the idea that the cytotoxicity of camptothecin is due to a cessation of replication when the fork encounters the DNA breaks complexed with topoisomerase I.

The incomplete nature of the inhibition of replicative DNA synthesis by camptothecin (see also refs 29 and 31) remains to be explained. It might be that topoisomerase II is responsible for unwinding the parental DNA at a proportion of replication forks. At least in an *in vitro* SV40 replication system (23), either topoisomerase I or II can perform this role.

Topoisomerase I is implicated not just in DNA replication but also in transcription (32), so camptothecin, acting on topoisomerase I, introduces breaks at sites of RNA as well as DNA synthesis (33). DNA synthesis involves only a small fraction of the DNA at any one time. But sites of RNA synthesis are more widespread and occur at intervals of 10^8 daltons on average (34), so the frequency of DNA breaks detected by the alkaline unwinding assay could be fully explained by DNA topoisomerase acting at transcription and replication sites. It has been suggested (11) that some breaks induced by camptothecin are not associated with a covalently bound DNA topoisomerase; but a recent report (18) claims that this finding resulted from an underestimation of protein-DNA complexes through the use of a detergent which allows some reversal of topoisomerase-DNA binding, and that in fact topoisomerase I inhibition can quantitatively account for the camptothecin induced DNA breaks.

Although camptothecin causes large numbers of DNA breaks, it does not resemble a typical DNA-damaging agent, as it does not induce a repair response in the cells (Table 1). This is consistent with the fact that the enzyme-linked DNA breaks are held in a stable state as long as the camptothecin is present; on removal of the inhibitor, the breaks are rapidly rejoined (11).

Camptothecin does not reduce the UDS induced by UV irradiation. As evidence for lack of an inhibitory effect on repair, this may be thought inconclusive, for established repair synthesis inhibitors such as hydroxyurea are well known not to affect UDS. Hydroxyurea inhibits repair synthesis and thus holds repair sites open, which allows polymerisation – although very slow – to proceed for much longer than normal and achieve substantial incorporation of [^{3}H]dThd (35). However, camptothecin is unlikely to have this effect, in view of its quite different target enzyme and mode of action. The results of the experiment of Fig. 7, looking at DNA breaks accumulating with and without UV in the presence of camptothecin, support this conclusion. If camptothecin blocked a topoisomerase required for incision, the resulting topoisomerase-linked DNA breaks would show as an increase in breaks in UV-irradiated compared with unirradiated cells. Similarly, an inhibition of repair synthesis or ligation by camptothecin would also give an increase in DNA breaks, as

incomplete repair sites would accumulate. Neither effect is seen.

In yeast, the topoisomerase activities are to a certain extent interchangeable. Temperature-sensitive (ts) mutants defective in topoisomerase II are blocked at nuclear division after one round of replication at the restrictive temperature and the DNA is found to be intertwined (22, 36). Topoisomerase I mutants are viable; but ts double mutants, defective in both topoisomerases, arrest (at the restrictive temperature) at various stages of the cell cycle (36). So, while topoisomerase II is essential for segregation of chromosomes, the role of topoisomerase I in maintaining chromatin organisation throughout the cycle can be taken over (in the topoisomerase I mutants) by topoisomerase II. In mammalian cells, however, topoisomerase I seems essential. A mutant human cell line has been isolated which is not killed by camptothecin; but rather than lacking topoisomerase I activity, its enzyme shows a 125-fold resistance to inhibition by the drug (37). To check for the possible substitution of one topoisomerase for the other in the case of repair, we incubated cells with both camptothecin and fostriecin, and detected no effect on repair DNA synthesis.

Repair of UV-induced DNA damage does not, then, depend on topoisomerase I or II – at least up to the stage of ligation. Ligation is the final step in restoring the continuity of the DNA, but repair involves also changes in the chromatin. In interphase cells, chromatin structure can be visualised by fusing them with mitotic cells; factors in the latter induce premature condensation of interphase chromosomes (PCC). It was found that UV irradiation prevented this induced condensation and instead the PCC appeared attenuated, the degree of attenuation correlating with the amount of repair DNA synthesis occurring (38). It seems that a topological rearrangement of DNA is associated with incision at UV lesions. Although the attenuation is prevented if novobiocin is present (39), from the present work it seems that the chromatin change may be a simple consequence of incision, allowing unwinding of supercoiling, rather than depending on active topoisomerase involvement. It remains to be seen whether a topoisomerase is involved in repacking the chromatin when DNA repair and ligation are complete.

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REFERENCES

1. Wood,R.D., Robins,P. and Lindahl,T. (1988) *Cell* **53**, 97–106.
2. Thompson,L.H. (1989) *Environ. Molec. Mutagenesis*, in press.
3. Collins,A. and Johnson,R.T. (1979) *Nucl. Acids Res.* **7**, 1311–1320.
4. Mattern,M.R., Paone,R.F. and Day,R.S. (1982) *Biochim. Biophys. Acta* **697**, 6–13.
5. Downes,C.S., Ord,M.J., Mullinger,A.M., Collins,A.R.S. and Johnson,R.T. (1985) *Carcinogenesis* **6**, 1343–1352.
6. Dresler,S.L. and Lieberman,M.W. (1983) *J. Biol. Chem.* **258**, 12269–12273.
7. Downes,C.S., Mullinger,A.M. and Johnson,R.T. (1987) *Carcinogenesis* **8**, 1613–1618.
8. Snyder,R.D. (1987) *Photochem. Photobiol.* **45**, 105–111.
9. Boritzki,T.J., Wolfard,T.S., Besserer,J.A., Jackson,R.C. and Fry,D.W. (1988) *Biochem. Pharmacol.* **37**, 4063–4068.
10. Hsiang,Y.-H., Hertzberg,R., Hecht,S. and Liu,L.F. (1985) *J. Biol. Chem.* **260**, 14873–14878.
11. Mattern,M.R., Mong,S.-M., Bartus,H.F., Mirabelli,C.K., Crooke,S.T. and Johnson,R.K. (1987) *Cancer Res.* **47**, 1793–1798.
12. Erixon,K. and Ahnström,G. (1979) *Mutat. Res.* **59**, 257–271.
13. Squires,S., Johnson,R.T. and Collins,A.R.S. (1982) *Mutat. Res.* **95**, 389–404.
14. Collins,A.R.S., Squires,S. and Johnson,R.T. (1982) *Nucl. Acids Res.* **10**, 1203–1213.
15. Kaufmann,W.K. and Briley,L.P. (1987) *Mutat. Res.* **184**, 237–243.
16. Reichard,P. (1972) In Weber, G. (ed.), Advances in Enzyme Regulation, Pergamon Press, Oxford, Vol. 10, pp. 3–16.
17. Ikegami,S., Taguchi,T., Ohashi,M., Oguro,M., Nagano,H. and Mano,Y. (1978) *Nature* (London) **275**, 458–460.
18. Covey,J.M., Jaxel,C., Kohn,K.W. and Pommier,Y. (1989) *Cancer Res.* **49**, 5016–5022.
19. Dresler,S.L. and Robinson-Hill,R.M. (1987) *Carcinogenesis* **8**, 813–817.
20. Fry,D.W., Boritzki,T.J. and Jackson,R.C. (1984) *Cancer Chemother. Pharmacol.* **13**, 171–175.
21. Drlica,K. and Franco,R.J. (1988) *Biochemistry* **27**, 2253–2259.
22. DiNardo,S., Voelkel,K. and Sternglanz,R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2616–2620.
23. Yang,L., Wold,M.S., Li,J.J., Kelly,T.J. and Liu,L.F. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 950–954.
24. Mitchison,J.M. (1981) *The Biology of the Cell Cycle*, Cambridge University Press, Cambridge.
25. Hand,R. (1978) *Cell* **15**, 317–325.
26. Vogelstein,B., Pardoll,D.M. and Coffey,D.S. (1980) *Cell* **22**, 79–85.
27. Hamlin,J.L. (1978) *Exp. Cell Res.* **112**, 225–232.
28. Fry,D.W., Besserer,J.A. and Boritzki,T.J. (1984) *Cancer Res.* **44**, 3366–3370.
29. Avemann,K., Knippers,R., Koller,T. and Sogo,J.M. (1988) *Molec. Cell. Biol.* **8**, 3026–3034.
30. Hsiang,Y.-H., Lihou,M.G. and Liu,L.F. (1989) *Cancer Res.* **49**, 5077–5082.
31. Horwitz,S.B., Chang,C.-K. and Grollman,A.P. (1971) *Molec. Pharmacol.* **7**, 632–644.
32. Gilmour,D.S., Pflugfelder,G., Wang,J.C. and Lis,J.T. (1986) *Cell* **44**, 401–407.
33. Stewart,A.F. and Schütz,G. (1987) *Cell* **50**, 1109–1117.
34. Alberts,B., Bray,D., Lewis,J., Raff,M., Roberts,K. and Watson,J.D. (1983) Molecular biology of the cell, Garland Publishing, New York.
35. Collins,A.R.S. and Johnson,R.T. (1984) *Adv. Radiat. Biol.* **11**, 71–129.
36. Uemura,T. and Yanagida,M. (1984) *EMBO J.* **3**, 1737–1744.
37. Andoh,T., Ishii,K., Suzuki,Y., Ikegami,Y., Kusunoki,Y., Takemoto,Y. and Okada,K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5565–5569.
38. Schor,S.L., Johnson,R.T. and Waldren,C.A. (1975) *J. Cell Sci.* **17**, 539–565.
39. Johnson,R.T., Collins,A.R.S. and Waldren,C.A. (1982) In Rao,P.N., Johnson,R.T. and Sperling,K. (eds), Premature Chromosome Condensation: Application in Basic, Clinical and Mutation Research, Academic Press, New York, pp. 253–308.