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Novobiocin; an inhibitor of the repair of UV-induced but not X-ray-induced damage in mammalian cells

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ABSTRACT

In addition to inhibiting replicative DNA synthesis in HeLa cells, novobiocin has a severe effect on the cellular response to UV irradiation, reducing the number of breaks made in pre-existing DNA by the excision repair process. The inhibition of UV repair by novobiocin is reflected in enhanced UV-killing of these cells. Rejoining of DNA after X irradiation is not impaired by novobiocin. The recognition and removal of UV damage may require unwinding of the DNA by gyrase, which - in bacteria - is the target for novobiocin.

INTRODUCTION

Novobiocin is well known as an inhibitor of DNA synthesis in bacteria, and its target has been identified[1] as DNA gyrase, the enzyme which introduces negative supercoils into transiently relaxed closed circular DNA. Recently it has been reported[2] that, in mammalian cells too, novobiocin inhibits DNA synthesis, specifically preventing initiation of new DNA strands. It is tempting to suppose that initiation of DNA synthesis requires the insertion by gyrase of negative supercoils in the template DNA molecule[3]. The conversion of the DNA double helix to a slightly "underwound" state may be necessary for recognition or binding by replication enzymes.

We here investigate the possible dependence of two kinds of DNA repair in mammalian cells on such a conformational change in DNA, by examining their sensitivity to novobiocin. The repair which occurs after UV irradiation, i.e. excision repair, consists of a degradative stage (recognition of damage sites, endonucleolytic breakage of the DNA nearby, and excision of a length of DNA containing the damage) followed by a synthetic stage (polymerisation of nucleotides and ligation of the repair patch into the old DNA). If the second stage is blocked by incubating irradiated cells with inhibitors of DNA synthesis such as hydroxyurea (HU) or 1-β-D-arabinofuranosylcytosine (ara C), the two stages are uncoupled, and the breaks introduced in the

degradative stage accumulate. This breakage of pre-existing DNA is readily detected by the reduced rate of sedimentation of DNA on gradients,[4, 5], by the increased rate of DNA unwinding in alkali [6, 7], and by the gross decondensation of metaphase chromosomes prepared as for standard karyotype analysis [4, 8, 9], and it provides an assay of in vivo repair endonuclease activity [5, 10]. In the case of X-ray damage, on the other hand, DNA breaks appear as a direct consequence of the irradiation, without enzymic involvement, and repair is assessed by examining the disappearance of these breaks on incubation.

MATERIALS AND METHODS

Cell culture, synchronisation and radioactive labelling. Hela cells were routinely cultured in suspension in Eagle's minimal essential medium supplemented with 5% foetal calf serum and nonessential amino acids. Synchronised mitotic cells (mitotic index >95%) were obtained by a thymidine-nitrous oxide block as described [11]. Cells in G1 or S phase were taken from a synchronous mitotic population allowed to enter interphase (in suspension or in plastic petri dishes). When cells with prelabelled DNA were required, they were incubated for 8h with 0.04 μCi/ml [Me-^{3}H] thymidine (20 Ci/mmole) (Radiochemical Centre, Amersham) before beginning the synchronisation procedure, or - in the case of experiments with unsynchronised cells - before an overnight change of growth medium prior to the experiment.

Assay of protein synthesis and replicative DNA synthesis. Unsynchronised HeLa cells in duplicate 35 mm dishes were incubated with 0.1 μCi/ml of [^{3}H] thymidine (20 Ci/mmole) or L-[4,5-^{3}H] leucine (54 Ci/mmole) (Radiochemical Centre, Amersham) under the conditions described in the figure legends. After [^{3}H] thymidine incorporation, cells were washed with cold phosphate-buffered saline (PBS), lysed in 1 ml 0.5M NaOH, and acid-insoluble material precipitated by addition of 3 ml 10% (w/v) trichloroacetic acid. After [^{3}H]leucine incorporation, cells washed with PBS were suspended in 1 ml H_2O and 1.5 ml of 50% (w/v) trichloroacetic acid was added. The samples were then cooled and filtered on 2.5 cm Whatman GF/C glass fibre filters (W. & R. Balston, Ltd.), washed with 5% (w/v) trichloroacetic acid and 96% ethanol, and dried. ^{3}H incorporated into DNA or protein was measured using a toluene-based scintillant on a Tri-Carb Spectrometer (Packard Instrument Co.).

Irradiation. For survival studies, the required number of cells was

plated in growth medium and the cells allowed to attach for 30 min before being UV irradiated in PBS using a Philips germicidal tube emitting at 254 nm. The dose rate was 1 $Jm^{-2}s^{-1}$.

Cells already growing in plastic dishes for the assay of repair were UV irradiated in situ after removal of growth medium and addition of 0.5 ml PBS at room temperature; the amount of inhibitor required for incubation after UV was present at this stage, control experiments having detected no sign of direct interaction with UV. 1.5 ml of growth medium was added after irradiation.

X irradiation was performed on cells at 0°C, suspended in growth medium at 3 x 10^5/ml. 1500 rads were delivered, at a rate of 1400 rads/min at 16 MeV.

Assay of repair by alkaline lysis. Prelabelled cells, irradiated and incubated as described in the figure legends, were scraped into suspension with a silicone rubber policeman, centrifuged, washed with cold (4°C) PBS, centrifuged again, and suspended in cold PBS at 6 x 10^6/ml. 50 µl samples were lysed for 15 min in alkaline sucrose (5% w/v sucrose, 0.3M NaOH, 0.5M NaCl, 0.01M EDTA, pH 12.7) on ice, and the proportion of single-stranded DNA (reflecting the number of unwinding points or breaks in DNA, introduced by X irradiation, or by excision repair after UV irradiation) was measured by hydroxyapatite chromatography[6]. Calibration on the basis of known rates of breakage of DNA by X-rays has been described[10].

Survival studies. After irradiation, cells in quintuplicate plastic Petri dishes were incubated with novobiocin (Boehringer Mannheim) at various concentrations in growth medium. After 1 to 4h, depending on the experiment, cells were washed twice with growth medium, and left in medium for ten days, at the end of which colonies were fixed, stained and scored. Colonies around the edge of the dish which would have been shaded from UV were discounted.

RESULTS AND DISCUSSION

To confirm the effect of novobiocin on replicative DNA synthesis, we incubated unsynchronised HeLa cells with [^{3}H]thymidine for 30 min in the presence of novobiocin at several concentrations (Figure 1a). Incorporation of thymidine into DNA is inhibited by novobiocin at 10^{-5}M or above, with 50% inhibition at about 1.6 x 10^{-4}M. In contrast, protein synthesis is only inhibited by novobiocin at concentrations above 10^{-4}M, and to achieve

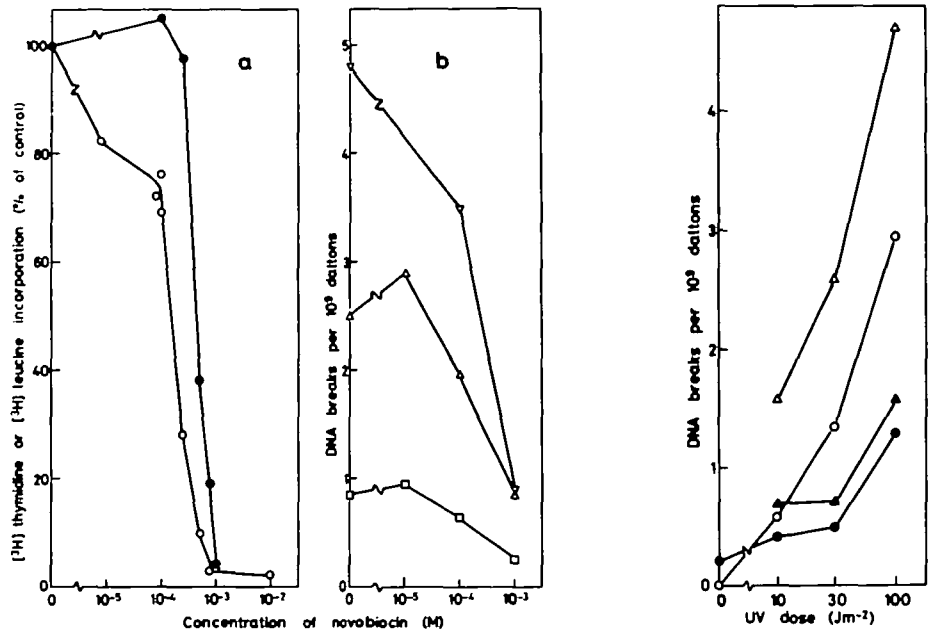

Figure 1. Novobiocin dose responses: (a) Inhibition of replicative DNA synthesis and protein synthesis. Unsynchronised HeLa cells were incubated for 15 min with novobiocin before adding [^{3}H]thymidine (o) or [^{3}H] leucine (●) for 30 min. Incorporation of ^{3}H into acid-insoluble material was measured. (b) Inhibition of UV-induced repair. Late G1 phase HeLa cells were labelled with [^{3}H]thymidine before synchronisation. Novobiocin, ara C (Sigma) and HU (Sigma), when present, were added 15 min before UV irradiation and remained during the 30 min incubation which followed UV. DNA breaks due to repair were measured by alkaline lysis and hydroxyapatite chromatography. □, UV dose of 30 Jm^{-2}: △, UV dose of 10 Jm^{-2}, incubated with 10^{-5}M ara C: ▽, UV dose of 10 Jm^{-2}, incubated with 10^{-2}M HU. All points represent means of duplicate samples.

Figure 2. Ability of novobiocin to block UV repair at different UV doses. Unsynchronised HeLa cells were incubated for 15 min with 3 x 10^{-3}M novobiocin (●), or 10^{-5}M ara C (△), or 3 x 10^{-3}M novobiocin and 10^{-5}M ara C (▲), or with no addition (o). After UV irradiation, incubation continued for 30 min with inhibitors present as during the preincubation. DNA break frequencies were then determined by alkaline lysis and hydroxyapatite chromatography.

50% inhibition requires almost three times the concentration necessary in the case of DNA synthesis (Figure 1a). Mattern and Painter[2] reported inhibition of thymidine incorporation in Chinese hamster ovary (CHO) cells by novobiocin at 50 μg/ml (i.e. 8 x 10^{-5}M) or above. We find that the

inhibition of replicative DNA synthesis by this drug is associated with reduced survival of early G1 or early S phase HeLa cells, although only at concentrations above 10^{-4}M (results not shown).

Figure 1b shows the effect of novobiocin on excision repair following UV irradiation. G1 phase HeLa cells incubated with either ara C or HU for 30 min after irradiation (10 Jm^{-2}) show a high frequency of breaks in prelabelled DNA. Incubation with 10^{-3}M novobiocin in addition to ara C or HU results in a drastic reduction in the break frequency, and novobiocin has a significant effect at 10^{-4}M - as it does in the case of replicative DNA synthesis. Similarly, when mitotic HeLa cells are UV-irradiated (240 Jm^{-2}) and incubated in the presence of ara C and novobiocin (10^{-4}M), the massive chromosome decondensation normally seen after UV and incubation with ara C (see for example, Figure 1c of ref. 9) is greatly reduced. We conclude that the enzymic breakage of DNA at damage sites is prevented by novobiocin: i.e. the action of this drug is at or prior to the incision step of repair.

A smaller number of breaks is seen if cells are incubated after UV without ara C or HU; they may represent the transient existence of excision breaks during normal repair[13]. Novobiocin reduces the number of these breaks (Figure 1b).

The action of novobiocin on UV repair is observed over a range of UV doses. Figure 2 shows the UV-dose-dependent accumulation of breaks in unsynchronised HeLa cells, in the presence or absence of ara C; with novobiocin included in the incubation there are fewer breaks at all doses. We have found that novobiocin inhibits the accumulation of UV repair breaks in other cell types; in CHO-K1 cells at various stages of the cell cycle[13], and in cultured cells of Microtus agrestis[14].

If replicative DNA synthesis in HeLa cells is blocked for 30 min with novobiocin, and the cells are then washed free of inhibitor, [^{3}H]thymidine incorporation into DNA over the following hour is restored to 80% of the level in untreated cells - i.e. the inhibition is largely reversible (Figure 3a). Reversal of the inhibition of repair is shown in Figure 3b. UV-irradiated HeLa cells were incubated with or without novobiocin for 30 min. The break frequencies in DNA were measured at intervals over the next 3h - in some cases following 30 min pulse-treatments with ara C. Addition of ara C to cells washed free of novobiocin results initially in an increased break frequency compared with cells remaining in novobiocin; but the level of breakage does not reach that seen in cells incubated only

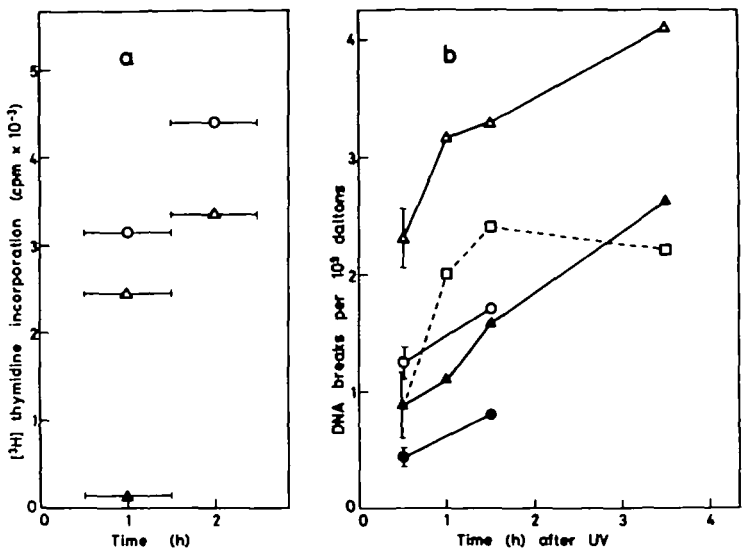

Figure 3. Partial reversal of the effects of novobiocin on (a) replicative DNA synthesis, and (b) UV-induced repair. (a) Unsynchronised HeLa cells were pulsed for 1h with [^{3}H]thymidine. Novobiocin (10^{-3}M) was present for 30 min before and also during the pulse (▲); or for a 30 min period at the end of which cells were washed twice with 1.5 ml of warm (37°C) growth medium and further incubated, [^{3}H]thymidine pulses being given immediately after release from novobiocin or after an interval of 1h (△). Control cultures (i.e. with no inhibitor treatment) were also pulsed (○); the later of these experienced a wash with warm medium at the same time as the cultures with novobiocin. The bars indicate the pulse-periods. (b) Unsynchronized HeLa cells were prelabelled with [^{3}H]thymidine and UV irradiated ($30Jm^{-2}$). They were then incubated with ara C(10^{-5}M) for 30 min periods ending at the times indicated (△,▲), or without ara C (○, ●). Solid symbols indicate the presence of novobiocin (2.5×10^{-3}M) from 15 min before irradiation and throughout the post-UV incubation. To examine reversal of the novobiocin effect, some samples incubated with novobiocin were washed free of the inhibitor 30 min after irradiation and given 30 min pulses of ara C during further incubation (□, broken line). DNA break frequencies were determined by alkaline lysis and hydroxyapatite chromatography.

with ara C. The inhibition of repair by novobiocin is thus partially reversible. In time, cells incubated in the continued presence of novobiocin and pulsed with ara C appear to escape from the novobiocin block. However, a brief exposure of UV-irradiated cells to novobiocin does have a lasting effect, demonstrated by a reduction in cell survival (Figure 4). HeLa cells in early G1 phase were UV irradiated and incubated for 1h in the presence of novobiocin. Enhanced killing was observed only

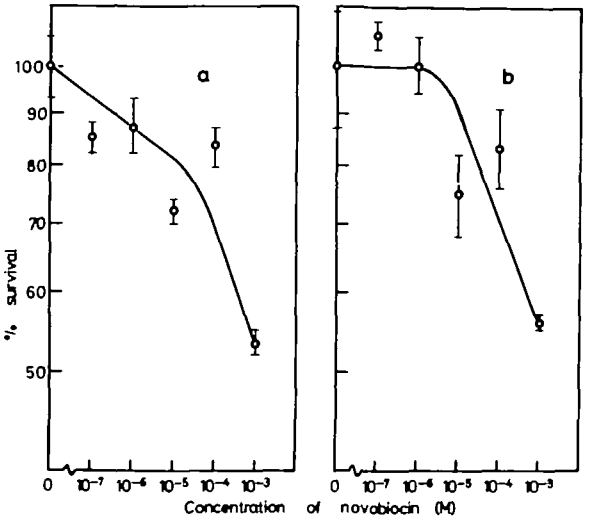

Figure 4. Effect of novobiocin on survival after UV. HeLa cells in early G1 phase were UV irradiated with 5 Jm^{-2}(a) or 10 Jm^{-2}(b) and incubated with novobiocin for 1h. The absolute plating efficiencies were (a) 6.7% and (b) 2.75%. Bars indicate standard error of the mean.

after 10^{-3}M novobiocin, but this was substantial.

It is possible that the inhibition of repair by novobiocin is secondary to a general cytotoxic effect of the drug. This might be inferred from the incomplete reversal of the inhibition and from our observation (unpublished) that, at 10^{-3}M, novobiocin prevents the attachment of suspended HeLa cells to a plastic substratum. However, DNA synthesis and UV repair are clearly more sensitive to novobiocin than is, for example, protein synthesis (see Figure 1). The following observations also argue against a general poisoning effect of the drug: DNA breaks accumulated after UV in the presence of ara C are rapidly rejoined if deoxycytidine is provided to compete with the ara C[9], and we find that this rejoining still occurs if novobiocin is present with the deoxycytidine. This is true for mitotic HeLa cells (examined for changes in chromosome morphology) and for unsynchronised HeLa cells (analysed by alkaline lysis). For example, after 1 hour with ara C, the number of DNA breaks following 30Jm^{-2} was 3.3 per 10^9 daltons; with deoxycytidine present for the last half hour, the number was 2.1, and with novobiocin as well as deoxycytidine, 1.9. In terms of chromosome morphology, after 90 minutes with ara C, the chromosome

decondensation index (see ref 9 for definition) following 240Jm^{-2} was 70%; with deoxycytidine present for the last half hour it was 41%, and with novobiocin as well as deoxydytidine, 47%. Thus only the initial stages of UV repair are inhibited by novobiocin.

An alternative explanation of the results of Figures 1b, 2 and 3b is that novobiocin somehow fixes DNA in a state such that DNA breaks are not revealed by the techniques we have used. The fact that, in UV-irradiated unsynchronised HeLa cells incubated for 75 min with ara C to uncouple repair, the number of DNA breaks(2.3 per 10^9 daltons) is not reduced by the presence of novobiocin for the last 15 min of incubation, indicates that this explanation is unlikely. It is conceivable, however, that novobiocin might bind to chromatin and prevent enzymic attack.

The number of DNA breaks detected immediately after X irradiation of G1 HeLa cells is not reduced by preincubation with novobiocin (Table 1). The effect of incubating X-irradiated cells at 37°C is also shown in Table 1. Almost all the X-ray-induced DNA breaks are rejoined in 45 min, and over 90% of this rejoining which presumably requires DNA synthesis occurs when novobiocin is present.

This marked difference in the sensitivities of UV repair and X-ray repair to inhibition by novobiocin further points to a specific effect of the drug. It is generally accepted that, in mammalian cells, different mechanisms exist for repair of UV damage ("long patch" repair) and X-ray

Table 1. X-ray damage and repair in the presence of novobiocin.

	DNA breaks per 10^9 daltons after 1500 rads		
	Control	+ novobiocin (2×10^{-4}M)	+ novobiocin (10^{-3}M)
Without post-irradiation incubation	3.75	3.75	4.10
Incubated at 37°C for 45 min after X irradiation	0.48	0.73	0.97

Prelabelled HeLa cells were synchronised in G1. Novobiocin was added to some cultures, and after 15 min (at 37°C) the cells were X irradiated. Either with no further incubation or following 45 min at 37°C, samples of cells were analysed for DNA break frequencies by alkaline lysis and hydroxyapatite chromatography. (The extent of unwinding of DNA in alkali seen in the control irradiated cells here was as expected from our standard calibration curve.)

damage ("short patch" repair)[15]. The recognition and removal of distortions of the DNA double helix produced by UV damage is likely to involve processes which are not necessary for repair of the breaks directly induced by X-rays, and it appears that one of these processes is sensitive to novobiocin and may therefore involve the insertion of negative supercoils into the DNA by gyrase. It remains to be seen whether novobiocin sensitivity is a diagnostic property of "long patch" repair in general.

Whether or not novobiocin (or the related drug coumermycin) inhibits repair in bacteria is not clear. Schneck and Staudenbauer[16] found that in toluenised Escherichia coli DNA repair after UV irradiation was not inhibited by novobiocin. But Ryan[17] described 80% inhibition of UV-induced repair DNA synthesis by coumermycin in E. coli cells, and when repair was assayed by the recovery of infectivity of UV-irradiated phage λ in bacteria[18], coumermycin did not prevent it.

We are investigating the possibility that the novobiocin-sensitive step in UV repair is rate-limiting, and that variations in DNA repair capacity, such as are seen during the mammalian cell cycle[8,13,19], may be the result of variations in gyrase activity.

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