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A DNA topoisomerase II-independent route for novobiocin-mediated resistance to DNA binding agents

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Summary. The coumermycin antibiotic novobiocin is currently under investigation as an agent that can modify the toxicity of various anti-cancer drugs, potentially via one of its many pharmacological effects: namely, the interference with type II DNA topoisomerase function. This paper investigates the ability of novobiocin to modify the cellular/nuclear accumulation and toxicity of two types of DNA binding agents (the minor groove ligand Hoechst 33342 and the intercalating anthracycline Adriamycin). We report that novobiocin reduces the cytotoxicity of both agents and that this can be attributed to a reduction in cellular and, consequently, nuclear accumulation of these agents rather than to any effect on cellular export. The antibiotic was also active (at non-toxic concentrations) in delaying the progression of cells into S phase and G_2 phase. This potential for novobiocin to effect rescue from toxicity by disturbance of the delivery of a drug to a potentially important intracellular target, together with the provision of an extended period of cellular recovery prior to the commitment of cells to G_2 + M phase, should be recognised in the design of combination chemotherapy.

Introduction

The coumarin novobiocin has many pharmacological effects [3, 5] other than its known ability to interfere with type II DNA topoisomerase activity by inhibiting the ATPase and energy transduction processes [11, 28]. Recent interest in this antibiotic has centred on its ability to modify the action of various anti-cancer drugs. Novobiocin can potentiate the cytotoxicity of several alkylating agents in vitro [7, 26], and there are indications [8] that combining the antibiotic with *cis*-diamminedichloroplatinum(II); 1,3-bis(2-chloroethyl)-1-nitrosourea; or cyclophosphamide may be a useful clinical strategy. For agents that apparently depend upon DNA topoisomerase II-DNA cross-linking for cytotoxicity [12], novobiocin would be expected to interfere with complex generation [5] and thereby actively rescue cells from cell killing. Fundamental to the rational design of drug-novobiocin combinations is the provision of information on the biological action of novobiocin and its effects on drug accumulation.

The cytotoxic effects of non-intercalative DNA minor-groove ligands (e.g. netropsin, distamycin and the Hoechst bisbenzimidazole dye derivatives) are thought to arise from the direct effects of bound molecules [24] on the processes of DNA transcription and replication [29]. This predominant dependence upon DNA interaction for cytotoxicity is less clear for DNA intercalators such as the anti-tumour anthracycline Adriamycin (ADM), with evidence that cytotoxicity may additionally involve interaction with an intranuclear target (e.g. DNA topoisomerase II [12], the intracellular generation radicals [1] or effects at the level of cell membranes [27]. Bisbenzimidazole dyes (e.g. Hoechst 33258) are minor-groove ligands that show marked fluorescence enhancement upon binding to DNA [17]. The DNA binding of the lipophilic derivative Hoechst 33342 (Ho33342) can be readily monitored by flow cytometry [16, 23]. The uptake and cellular export of Ho33342 has been shown to parallel that of a number of anti-cancer drugs involved in the multi-drug resistance phenotype [14, 15, 18]. Thus, Ho33342 is a useful reporter probe for studies on the pathways of drug uptake and their modification.

A recent investigation [25] into metabolic inhibitors that can affect Hoechst 33342: DNA complex formation revealed that novobiocin could block the initial nuclear accumulation of ligand in a mouse cell line. A similar inhibition of the nuclear accumulation of ADM was also

Abbreviations: Ho33258, 2-[2-(4-hydroxyphenyl)-6-benzimidazoyl]-6-(1-methyl-4-piperazyl)-benzimidazole trichloride; Ho33342, 2′,5′-Bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl) trichloride; HEPES, *N*-2-hydroxyethyl piperazine-*N*-2-ethane sulphonic acid; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; ADM, Adriamycin

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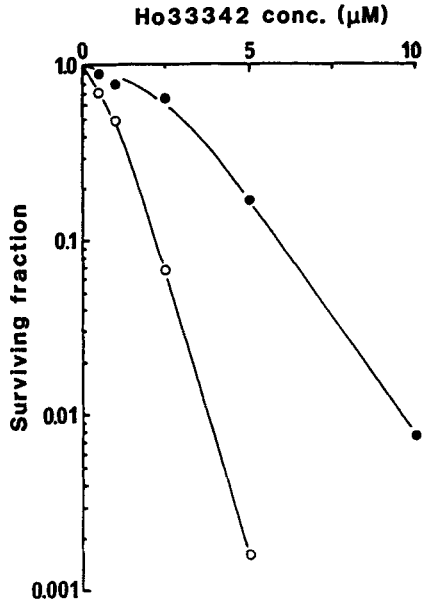

Fig. 1. Effects of novobiocin on the sensitivity of mouse cells to Ho33342. Data points represent arithmetic means of values from two independent experiments (range $< \pm 4\%$). Novobiocin (1 m*M*) was present for 20 min prior to and during a 1 h exposure to Ho33342. L*tk*$^-$ cells without (○) and with (●) novobiocin treatment

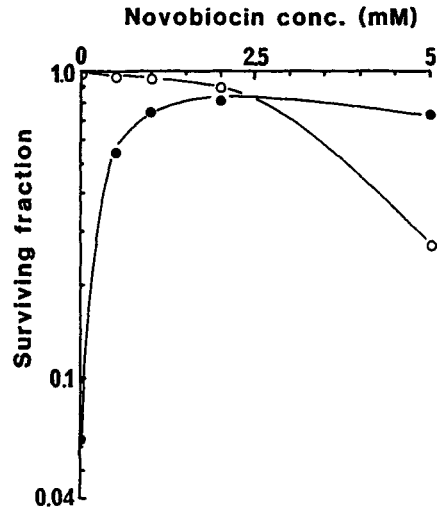

Fig. 2. Concentration-dependence of novobiocin-induced resistance to Ho33342 toxicity. ○, control; ●, 2.5 μ*M* Ho33342 treatment (as detailed in the legend to Fig. 1). Data were obtained from a single representative experiment (SE <5% for quadruplicate determinations)

observed [25]. The novobiocin-sensitive cellular pathway responsible for modifying the nuclear accumulation of Hoechst 33342 and Adriamycin is not known. This paper attempts to determine the effects of novobiocin on drug accumulation and loss together with any modification of cellular sensitivity assayed by changes in clonogenicity and cell-cycle perturbation.

Materials and methods

Cell culture

The murine cell line deficient in thymidine kinase, called L*tk*$^-$ in this paper, originated from L-M (TK$^-$) clone 1D cells and has previously been described [2, 24, 25]. Cells were maintained in monolayer culture in Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum, 1 m*M* glutamine and antibiotics (referred to as growth medium). For the establishment of standard growth conditions, cells were inoculated into culture vessels at an initial density of 10^4 cells cm^{-2} for experimental manipulation after 48 h incubation at 37° C in closed culture in an atmosphere of 5% CO_2 in air.

Drug preparation and treatments

Exact concentrations of filter-sterilised stock solutions (approx. 5 mg/10 ml distilled water) of Hoechst dye 33342 (CP Laboratories, Bishop's Stortford, UK) were determined spectrophotometrically (molar extinction coefficient, 4.1×10^4 M^{-1} cm^{-1} at 340 nm) in buffer (10 m*M* NaCl, 5 m*M* HEPES; pH 7.0). Stock solutions (0.85 m*M*) of ADM (Farmitalia Carlo Erba Ltd, Barnet, Herts, UK) were stored at −20° C. Novobiocin stock solutions in distilled water were prepared immediately before use. All drugs were added directly to culture vessels containing growth medium under standard culture conditions. Following treatment, cells were washed twice with phosphate-buffered saline (PBS) before further manipulation.

Cytotoxicity assay

Cultures in exponential growth phase were detached using trypsin/versene, plated at low density ($0.25-5 \times 10^3$ cells per 6-cm dish) and allowed to attach for a period of 6 h. Drugs were added directly to each plate and cultures were incubated for 1 h before drug removal by washing plates with PBS and subsequent assaying for clonogenic potential by continued incubation (10 days) in fresh growth medium.

Analysis of Ho33342 DNA binding by flow cytometry

Sample preparation. Cellular uptake experiments involved the treatment of exponential growth-phase cultures in 5-cm plastic dishes under standard conditions. Following treatment, with or without a recovery period in fresh medium, all cultures were subjected to a standard protocol [23] for the generation of freeze/thawed (permeabilised) cells directly from monolayer, enabling the rapid resuspension of cells in buffer (10 m*M* TRIS-HCl, pH 8; 100 m*M* NaCl; 10 m*M* EDTA; 1 mg ml^{-1} bovine serum albumin).

Flow cytometry. The samples were analysed using a flow cytometer described previously [24]. An Innova 3000K krypton laser (Coherent Corp.; Palo Alto, Calif., USA) was tuned to the 337 line at a light power of 200 mW to excite fluorescence. Ho33342 is a DNA-specific ligand that shows considerable fluorescence enhancement upon binding, the emission spectrum with DNA extending over a range of 400–600 nm. The analysis system at 90° to the intersection of the laser beam with the cell stream monitored fluorescence at 500 ± 5 nm. Forward scatter and 90° scatter signals were used to exclude small debris and clumps. Median fluorescence values were calculated for specified population distributions.

Cell-cycle phase analysis

A rapid, one-step ethidium bromide DNA-staining technique for RNase-digested cells was used for the measurement of cellular DNA content, with subsequent computer evaluation of cell-cycle phase distributions. Details have previously been described [22].

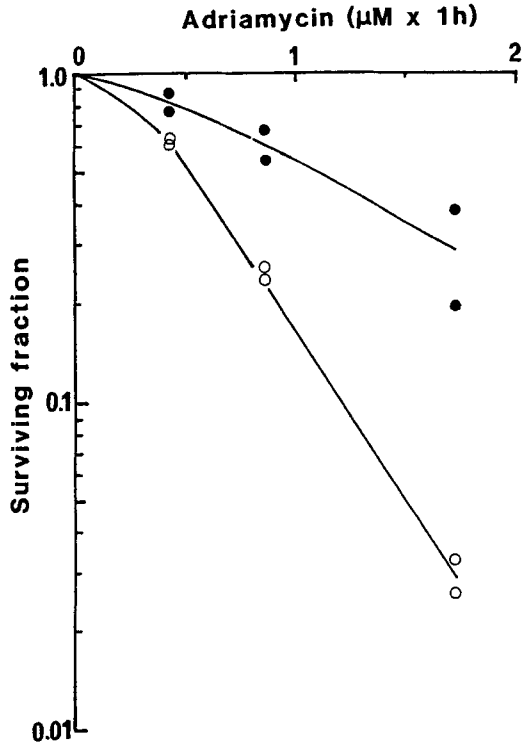

Fig. 3. Effect of novobiocin (1 m*M*) on the sensitivity of mouse cells to Adriamycin. Details and symbols as given in the legend to Fig. 1. Data points were derived from two independent experiments

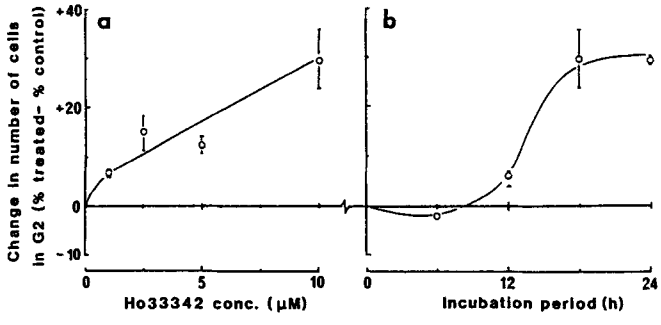

Fig. 4 a, b. Recruitment of *Ltk*$^-$ cells into G_2/M delay as a function of a Ho33342 concentration (1 h dye exposure; delay measured after an 18 h post-treatment incubation period) or b post-treatment incubation period (10 μ*M* Ho33342 × 1 h). Data represent mean determinations of values from 3 independent experiments (error bars denote SE)

Table 1. Effect of novobiocin on Ho33342-induced cell-cycle perturbation in *Ltk*$^-$ cells

Ho33342 treatment (μ*M* × 60 min)	Novobiocin treatment[a] (m*M* × 80 min)	% change in cell number (treated – control)[b]		
		G_1	S	G_2 + M
2.5	0	-2.2	-14.0	+16.2
0	1	-3.2	+ 3.4	- 0.2
2.5	1	+7.4	- 6.4	- 1.0

[a] Drug given 20 min prior to Ho33342 treatment

[b] Changes in cell-cycle distributions were measured after 18 h incubation in drug-free medium. Values represent arithmetic means of two determinations (range ±2%). %G_1, %S and %G_2+M values are 39.2, 47.3 and 13.5, respectively, for exponential-phase cultures

Estimation of cellular accumulation of ADM

The extraction procedure has been described in detail by Schwartz [19]. Briefly, exponentially growing cultures of cells in 6-well plates were detached by either trypsin/versene or freeze/thawing (see above) following ADM treatment in the presence or absence of novobiocin. Cells were centrifuged at 200 *g* for 5 min at 4° C and the pellet (5×10^5 cells) was resuspended in 100 μl 0.1% solution of sodium lauryl sulphate (BDH Chemicals) on ice. Silver nitrate solution (33% w/v; 100 μl/sample) was added and the samples were shaken vigorously for 10 min at 4° C. Ice-cold isoamyl alcohol (2 ml/sample) was added to the lysates and samples were shaken for 10 min at 4° C. Extracts were centrifuged at 200 *g* for 5 min and the ADM content of the alcohol fraction was measured by fluorimetry in an MPF-4 Perkin-Elmer spectrofluorimeter (excitation at 490 nm and emission measured at 590-nm wavelength). Untreated cell lysates receiving known amounts of ADM provided calibration standards for the extraction procedure.

Results

Effect of novobiocin on Ho33342 and ADM cytotoxicity

The mouse cell line (*Ltk*$^-$) demonstrated a significant decrease in Ho33342 sensitivity in the presence of novobiocin (10% survival dose increased from 2.2 to 6 μ*M* × 1 h; Fig. 1). The shape of the survival curve suggests that novobiocin acts on the majority of the cells in the population in a similar manner and as a dose-modifying agent. The novobiocin dose dependency of the rescue effect was examined (Fig. 2). Novobiocin concentrations of >0.5 m*M* were found to be equally effective in reducing Ho33342 toxicity in *Ltk*$^-$ cells. Interestingly, a combination of Ho33342 at 2.5 μ*M* (survival, <7%) and novobiocin at 5 m*M* (survival, <27%) resulted in dramatic cell rescue (survival, >70%). Novobiocin-mediated rescue could also be observed in ADM-treated *Ltk*$^-$ cells (Fig. 3), suggesting that the coumarin can modify responses to different classes of DNA-binding agents.

Cell-cycle effects

In an attempt to follow the early effects of novobiocin on cellular responses to Ho33342, the induction of cell-cycle delay was monitored. Figure 4a demonstrates a dose-dependent delay of cells in G_2 phase of the cell cycle, this being a toxicity-related phenomenon. At least a 12-h interval between the time of dye treatment and analysis is required (Fig. 4b) for the expression of G_2/M delay response. Having defined appropriate conditions for the analysis of G_2/M delay in this cell line, we analysed the effects of novobiocin alone and in combination with dye (Table 1). The results show that after 18 h post-treatment incubation, a non-toxic (Fig. 2) concentration of novobiocin had no significant effect on cell-cycle distribution. However, the marked G_2/M delay induced in *Ltk*$^-$ cells by Ho33342 was abolished by co-treatment with novobiocin.

To analyse the origin of this rescue effect of novobiocin more closely for *Ltk*$^-$ cells, we adopted a stathmokinetic approach using colcemid to block cells in G_2/M (Fig. 5a, b). Control cultures treated with colcemid alone showed a steady, time-dependent loss of cells from G_1

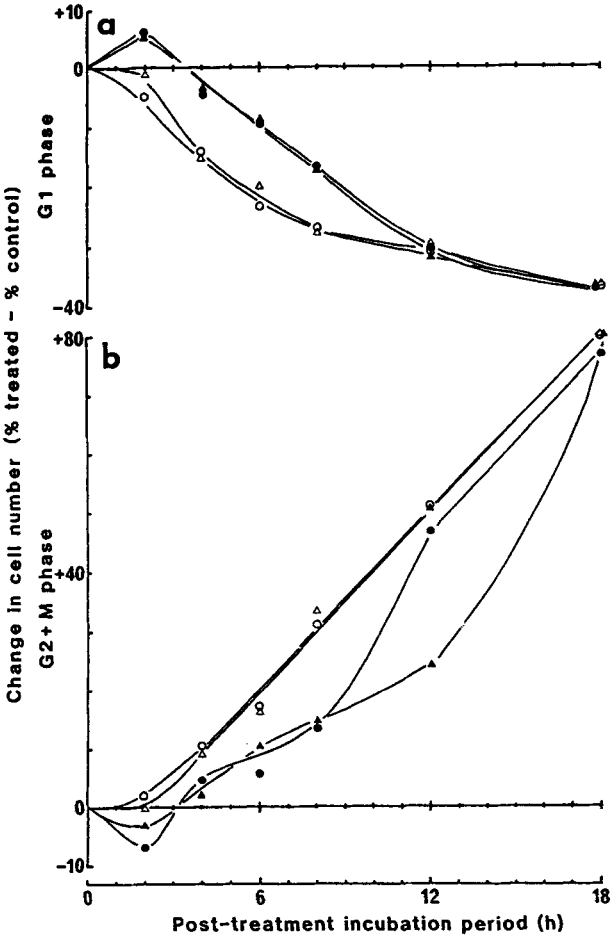

Fig. 5a, b. Stathmokinetic analysis of Ho33342-, novobiocin- and Ho33342 + novobiocin-induced perturbations in cell-cycle transit in asynchronously growing L*tk*⁻ cells. Data represent mean values derived from a single representative experiment carried out in duplicate. a G_1 phase emptying and b G_2/M accumulation curves are shown for asynchronously growing cultures blocked at mitosis with colcemid (60 ng/ml) after the specified treatments. O, control; Δ, exposure to 2.5 μ*M* Ho33342 for 1 h; ▲, exposure to 1 m*M* novobiocin for 80 min; ●, exposure to novobiocin and Ho33342 as detailed in the legend to Fig. 1

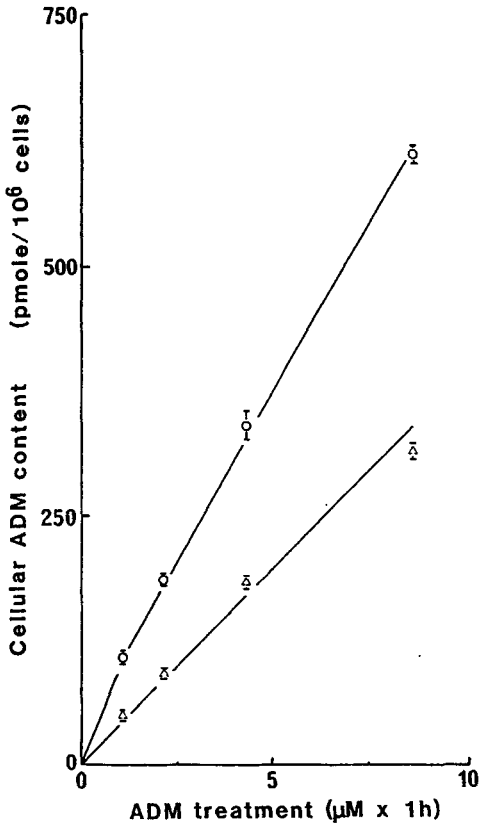

Fig. 6. Accumulation of ADM by L*tk*⁻ cells. O, control; Δ, novobiocin (1 m*M*) treatment during the 1-h ADM uptake period

phase and the accumulation of cells in G_2/M. There was a delay of 1–1.5 h in the initial ability of colcemid to effect metaphase arrest. The treatment of cells with Ho33342 caused only a minor delay in G_2/M accumulation, although there was a 1-h delay, compared with the control, in the exit of cells from G_1 phase. This effect of cells on G_1/S transition is consistent with the ability of Ho33342 to inhibit DNA synthesis [21, 22]. the similarity between the control and treatments with Ho33342 alone suggests that the major cell-cycle effect of Ho33342 is to prevent cells from exiting G_2 [21, 22].

Direct microscopy and light-scatter measurements (performed simultaneously during flow cytometric analyses; technique described in [9]) confirmed that the ligand does not arrest cells in mitosis. On the other hand, novobiocin was found to induce a more prominent delay (2 h longer than the control value) in the exit of cells from G_1 and the accumulation of cells in G_2/M. In keeping with the non-toxic nature of the novobiocin treatment (Fig. 2), the L*tk*⁻ cultures appeared to recover from such effects within 12–18 h, there being no evidence of residual subpopulations undergoing long-term delay. Interestingly, the combination of dye and inhibitor gave responses that were very similar to the effect of novobiocin alone, except for an apparent prolongation effect on the delay of cells entering G_2/M. Taken together, the results indicate that the overriding effect of novobiocin is to delay G_1–S and S–G_2 transitions, whereas the main effect of Ho33342 is to block G_2 exit. When the two agents were given in combination there was an S–G_2 transition delay, imposed by novobiocin, with subsequent full recovery (by 18 h) of the delayed population and an ability to exit G_2 (see Table 1).

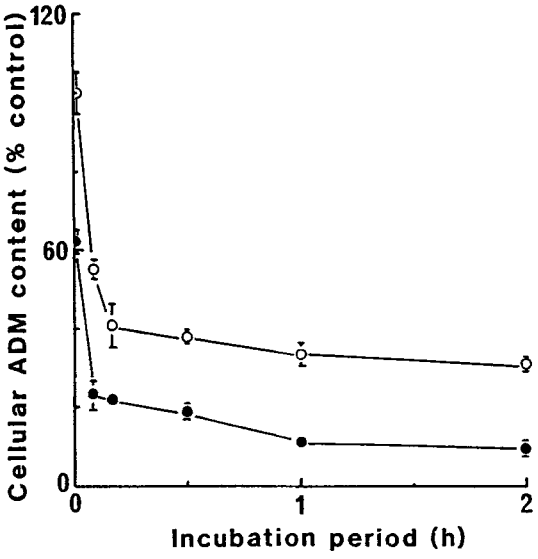

Fig. 7. Loss of ADM *Ltk*⁻ cells following exposure to 8.6 μ*M* drug for 1 h. ○, control; ●, novobiocin (1 m*M*) treatment

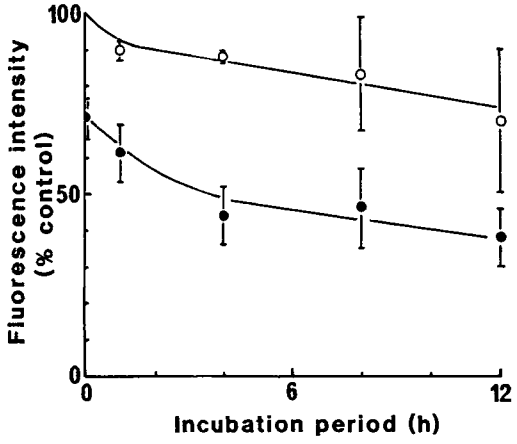

Fig. 8. Effect of novobiocin (1 m*M*) on the initial level and subsequent loss of Ho33342-DNA fluorescence (500 nm wavelength) in *Ltk*⁻ cells as monitored by flow cytometry. ○, control; ●, novobiocin (1 m*M*) treatment during the Ho33342 uptake (2.5 μ*M* dye × 1 h) period

Drug uptake and nuclear binding effects

A previous study [25] showed that novobiocin reduced the whole-cell accumulation of Ho33342 by *Ltk*⁻ cells. Flow cytometric measurements of Ho33342 and ADM uptake also showed that novobiocin reduced the nuclear accumulation of the DNA binding drugs [25]. The present study shows that novobiocin can also reduce the whole-cell accumulation of ADM (Fig. 6) without modifying the kinetics of cellular loss of the drug (Fig. 7). Furthermore, Fig. 8 shows that novobiocin reduces the initial level of Ho33342 bound to cellular DNA rather than having any significant effect on the subsequent loss (approx. 40% over a 12-h period) of fluorescence. These observations provide clear explanations for the reduced cytotoxicity of these two types of DNA binding agents in novobiocin-treated cells, namely, reduced cellular accumulation. The magnitude of this effect correlates well with the 2-fold dose modification observed for cytotoxicity (see above).

Discussion

This paper reports that mammalian cells co-treated with moderate (>0.25 m*M*) but non-toxic concentrations of the coumarin novobiocin show unusual resistance to the cytotoxic effects of either a minor-groove binding drug (Ho33342) or a DNA intercalating drug (Adriamycin). Novobiocin-induced resistance related to a reduction in the initial levels of drug accumulation. Although novobiocin has previously been used as a topoisomerase II inhibitor [3, 5], our results provide a further caveat for the use of this non-specific inhibitor, especially in attempts to identify the involvement of DNA topoisomerase II in the cytotoxic action of a given drug.

Ho33342 and novobiocin were found to differ in their effects on cell-cycle progression. There was little effect of Ho33342 on S-phase entry and exit in *Ltk*⁻ cells, whereas novobiocin delayed both processes. The accumulation of cells in G_2 phase was found to be the major cell-cycle effect of Ho33342, and novobiocin abolished Ho33342-induced G_2 block. The novobiocin-related perturbation predominated when both agents were applied simultaneously. Novobiocin inhibits bacterial gyrase (the eukaryotic counterpart of which is topoisomerase II) by blocking the ATPase activity of the B subunit [5, 11]. Novobiocin is also known to disrupt a variety of protein-mediated processes, including histone-DNA interactions [20]. Previous studies have suggested that cellular recovery from the effects of novobiocin are generally complete within 3 h [4] and this is in keeping with the recovery of cells from G_1–S and S–G_2 transition delays within 4 h of removal of the inhibitor. These effects could be viewed as providing an additional period for cellular recovery prior to the potential expression of Ho33342-induced G_2 arrest. It is possible that the cell-cycle delay induced by novobiocin is in part related to a disturbance in DNA topoisomerase II activity, although it is more likely that the changes in cellular ATP supply [3] can account for the disturbances in S-phase transit.

The extensive fluorescence enhancement that occurs when Hoechst 33342 binds to cellular DNA enables the specific interaction of dye molecules with nuclear DNA to be followed in single cells without any significant interference from unbound ligand. A small but significant loss of Ho33342-DNA fluorescence could be detected in the parental cell line and the kinetics are consistent with a biphasic process, with a rapid loss of up to 15% of the initial fluorescence [6]. A previous study indicated that the whole-cell and nuclear accumulation of Ho33342 in the *Ltk*⁻ cell line was reduced significantly by the presence of novobiocin [25]. The immediate (<1 h) loss of ligand from the whole cell [25] or nucleus (see Results), together with the slow, long-term removal of ligand from DNA (see Results), were unaffected by the presence of novobiocin

during the Ho33342 uptake period. Clearly, several factors could explain the origin of novobiocin-induced Ho33342 or ADM resistance, but the most direct explanation is reduction in the initial level of ligand bound to DNA as a result of decreased cellular uptake. The results point to the existence of a novobiocin-sensitive pathway for drug accumulation.

It is thought that Ho33342 is taken up into cells by unmediated diffusion and actively excluded by an energy-dependent mechanism [15]. Since acute exposure to novobiocin normally reduces cellular ATP levels [4], it is unlikely that the inhibitor acts to enhance the energy-dependent efflux of drug from cells. A modification of drug uptake could involve disturbances in the plasma membrane. The reduction of ADM accumulation and cytotoxicity by novobiocin has similarities with the previously reported ability of caffeine to reduce ADM uptake and cytotoxicity in Chinese hamster V79 cells [13]. This suggests that perhaps similar transport-related biochemical processes are affected by both agents. Interestingly, caffeine-mediated reduction in ADM accumulation and cell kill can be reversed by trifluoperazine [10], a calmodulin inhibitor that can sensitize multi-drug-resistant cells to ADM by increasing intracellular drug accumulation. Multi-drug-resistant (MDR) cells that exhibit an unusually active, membrane-located drug-efflux pathway for structurally unrelated drugs (e.g. vinca alkaloids and anthracyclines) have also been found to be resistant to Ho33342 [14, 15, 18]. It would be of interest to investigate the expression of novobiocin-sensitive pathways for drug uptake in such MDR cells.

The present observations caution against the use of novobiocin to investigate the involvement of DNA topoisomerase II in some biological or biochemical processes. Thus, the effects of novobiocin on the rescue of cells from topoisomerase II-interactive drugs may be explained by changes in the initial level of drug bound to cellular DNA (e.g. Adriamycin) and the imposition of a period of cell-cycle delay rather than a putative inhibition of the ability of a drug to stimulate the formation of stable complexes between DNA and the target enzyme.

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