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Original Paper

Cell Cycle Effects of the Novel Topoisomerase I Inhibitor NU/ICRF 505 in a Panel of Chinese Hamster Ovary Cell Lines

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The effect of the novel topoisomerase I inhibitor NU/ICRF 505 (20 μM, approximate IC_{50} concentration) on the cell cycle was studied by flow cytometry in four Chinese hamster ovary (CHO) cell lines. Postdrug treated cells were incubated with optimal concentrations of cytochalasin B to prevent re-entry of daughter cells into the cell cycle. NU/ICRF 505 had no significant effect on cell cycle distribution in the parent cell line (CHO-K1) and two mutants hypersensitive to topo II inhibitors (ADR-1, ADR-3), all of which express similar levels of topo I protein. In the drug-resistant variant ADR-r, which overexpresses topo I 2-fold, a significant accumulation of cells in G_1 phase was recorded. These results are broadly consistent with the cell cycle effects expected in CHO cells by a classic topo I poison (camptothecin) and add weight to the view that NU/ICRF 505 induces cell death primarily through topo I inhibition.

Key words: anthracenyl-amino acids, topoisomerase I, NU/ICRF 505, cell cycle, CHO cells

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INTRODUCTION

NU/ICRF 505 is an *N*-tyrosine conjugate of anthraquinone and is the lead compound from a novel series of anthracenyl–amino acid conjugates shown to exhibit a range of specificities as inhibitors of DNA topoisomerases I and II [1]. NU/ICRF 505 stabilises topoisomerase I cleavable complexes without inhibiting the catalytic activity of the enzyme and does not appear to bind to DNA [1]. The compound exhibits broad spectrum *in vitro* activity against human ovarian and Chinese hamster ovary (CHO) cell lines (IC_{50} 4.4–23.9 μM) and retains activity *in vivo* against human xenografts [2]. In addition, it is non-cross-resistant in multidrug resistant (MDR) and camptothecin (CPT) resistant cell lines and is not recognised by the MDR drug transporter P-170 glycoprotein [3].

In a recent study, a strong correlation was observed between *in vitro* chemosensitivity and the level of expression of topo I protein, suggesting a mechanism of action against cells in culture operating through topo I [2]. To elaborate further on possible mechanisms of cell death, the influence of NU/ICRF 505 on cell cycle distribution in a panel of four CHO cell lines was investigated.

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MATERIALS AND METHODS

Chemicals

NU/ICRF 505 was synthesised through the reaction of α-tyrosine ester with [2*H*,3*H*]-9,10-dihydroxyanthracene-1,4-dione as described in detail (Cummings and Mincher, U.K. patent GB 9205859.3; International Application Number PCT/GB93/00546, published 30 Sep. 1993).

Cell lines

All CHO cell lines were kindly supplied by Drs Ian Hickson and Sally Davies (ICRF Molecular Oncology Laboratory, Institute of Molecular Medicine, Oxford, U.K.). The parent cell line is designated as CHO-K1. Two mutant cell lines exhibiting varying degrees of hypersensitivity to established topo II inhibitors (doxorubicin, etoposide and mitoxantrone) designated ADR-1 and ADR-3 were isolated from CHO-K1 cells in a single-selection protocol after 24 h treatment with the point mutagen ethyl methanesulphonate (300 μg/ml) as described previously [4–6]. The

*Table 1. Characteristics of CHO cell lines used to study the cell cycle effects of the novel topoisomerase I inhibitor NU/ICRF 505**

Cell line	P-170 glycoprotein expression (MRK-16) % positive†	Pgp1 mRNA‡	Topoisomerase I expression (immunoblot analysis)	IC_{50} values NU/ICRF 505 (10^{-6} M)	Camptothecin (10^{-7} M)
CHO-K1	21	1	1	19.9 ± 2.4	2.2
ADR-1	38	ND	1	23.9 ± 5.4	3.5
ADR-3	ND	ND	1	21.2 ± 2.5	7.3
ADR-r	91	14↑	2↑	14.9§ ± 0.9	1.6

*Data taken from [2]; †P-170 glycoprotein expression was determined by flow cytometry as the percentage of cells staining positive after incubation with the MRK-16 antibody; ‡Pgp 1 mRNA levels were measured by an RNase protection assay. §$P < 0.01$ compared to CHO-K1 (Student's paired t-test). ND, not determined.

MDR resistant CHO cell line ADR-r was derived by stepwise exposure of CHO-K1 to increasing concentrations of doxorubicin over a 3-month selection period [7]. The chemosensitivity characteristics of the panel to NU/ICRF 505 and CPT is shown in Table 1 together with data on P-170 glycoprotein and topo I expression [2].

Cell culture experimental conditions

All CHO cell lines were maintained and passaged in HAMS F10 mixture with L-glutamine supplemented with 5% heat inactivated fetal calf serum, 5% heat inactivated new born calf serum and a 1% antibiotic mixture as described in detail [2].

In experimental studies, cells were seeded at $4–5 \times 10^5$ cells in 75 cm^2 flasks and allowed to grow for 48 h, after which time they were exposed to 20 μM NU/ICRF 505 for a further 24 h. NU/ICRF 505 was dissolved in pure dimethylsulphoxide (DMSO) immediately prior to use and diluted to the required concentration with medium, producing a final DMSO concentration of 0.3–0.5%. Control cells received the same volume of DMSO and were processed in a manner identical to drug-treated cells. Following drug exposure, drug-containing media were removed, cells were washed in PBS, fresh media containing cytochalasin B (Aldrich Chemical Company, Poole, U.K.) were added, and the cells were incubated for a further 24 h prior to flow cytometry. Cell cycle studies were repeated on 3–4 separate occasions.

Flow cytometry

Culture media were removed, the monolayers were washed with ice cold PBS and cells were mechanically harvested in 3 ml of cold PBS. Cells were pelleted by centrifugation and fixed in 70% ethanol for 30 min after which time they were washed with PBS and incubated with 0.5 ml of RNase A (100 μg/ml in PBS, Type IIIA from bovine pancreas, Sigma Chemical Co.) for 30 min at 37°C. Following a final wash in PBS, cells were resuspended in 1 ml of propidium iodide (25 μg/ml in PBS, Fluka Biochemica, Gillingham, U.K.) and analysed using a FACScan flow cytometry (Becton-Dickson, High Wycombe, U.K.). The percentage of cells in each phase of the cycle was calculated from cell cycle distributions using the Cellfit curve fitting programme (Becton-Dickson).

RESULTS

In a series of preliminary experiments, CHO cells were incubated with varying concentrations of cytochalasin B for 24 h [8] to determine the optimal dose to inhibit cytokinesis without inducing significant levels of cytotoxicity. Progression of cells to 4C and 8C peaks—as determined by flow cytometry—was taken as an indicator of the effectiveness of cytochalasin B. This treatment was adopted to prevent re-entry of daughter cells into the cell cycle after division and thus amplify drug effects [8]. The optimal dose of cytochalasin B was 1 μg/ml for CHO-K1, 3 μg/ml for ADR-1, 2 μg/ml for ADR-3 and 5 μg/ml for ADR-r.

No significant effect on cell cycle distribution was observed in CHO-K1, ADR-1 and ADR-3 (Table 2, Figure 1) after a 24 h incubation with NU/ICRF 505 at a drug concentration (20 μM) close to the IC_{50} for these cell lines (Table 1). There was a tendency towards a G_1 arrest with fewer cells reaching G_2/M but this did not achieve statistical significance. In ADR-4, there was a small but statistically significant accumulation of cells in G_1 (Table 2, Figure 2) coupled to a significant reduction of cells in G_2/M.

DISCUSSION

The aim of this study was to investigate the effect of the novel topo I inhibitor NU/ICRF 505 on the cell cycle in a

Table 2. Effect of 20 μM NU/ICRF 505 on cell cycle distribution in a panel of Chinese hamster ovary cell lines

Cell line	G_1 –	G_1 +	S –	S +	G_2/M + $G1_2$ –	G_2/M + $G1_2$ +
CHO-K1	25.6 ± 4.2	26.6 ± 5.6	18.3 ± 5.1	18.7 ± 2.0	56.0 ± 8.7	54.7 ± 7.5
ADR-1	20.8 ± 6.4	24.2 ± 3.1	24.8 ± 20	27.1 ± 5.8	54.3 ± 15	48.7 ± 7.3
ADR-3	25.6 ± 9.0	29.3 ± 10.4	31.5 ± 11.6	30.4 ± 9.6	42.8 ± 6.5	40.1 ± 5.7
ADR-r	27.3 ± 0.5	33.9 ± 3.9†	16.4 ± 3.0	17.7 ± 1.5	56.2 ± 2.6	48.3 ± 4.3*

–, control cells (no drug treatment); +, drug-treated cells.

Each value represents the percentage of cells in each phase of the cell cycle ± standard deviation from 3–4 repeat experiments.

*$P = 0.05$ compared to non-drug treated controls; †$P < 0.05$ compared to non-drug treated control (Student's paired t-test).

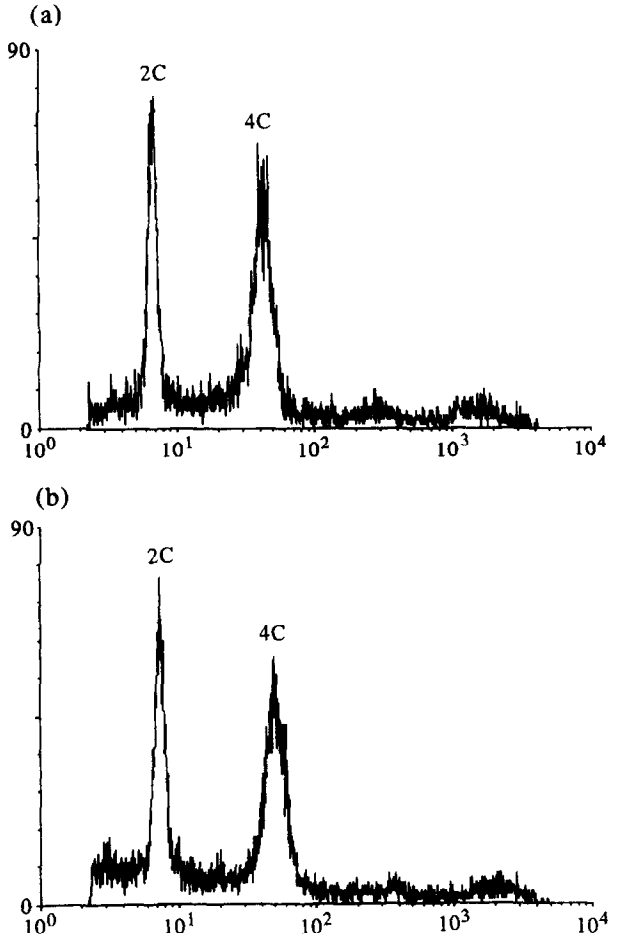

Figure 1. Effect of 20 μM NU/ICRF 505 on cell cycle distribution in the CHO ADR-3 cell line. Cells were pretreated with drug for 24 h and then post-treated with cytochalasin B for 24 h to prevent daughter cells from re-entry into the cell cycle. (a) Control no drug treatment and (b) drug treated. *x*-axis is DNA content of cells and *y*-axis is cell number. 2C represents cells in G_1 phase and 4C represents cells in G_2/M (with a contribution from binucleate cells in G_1).

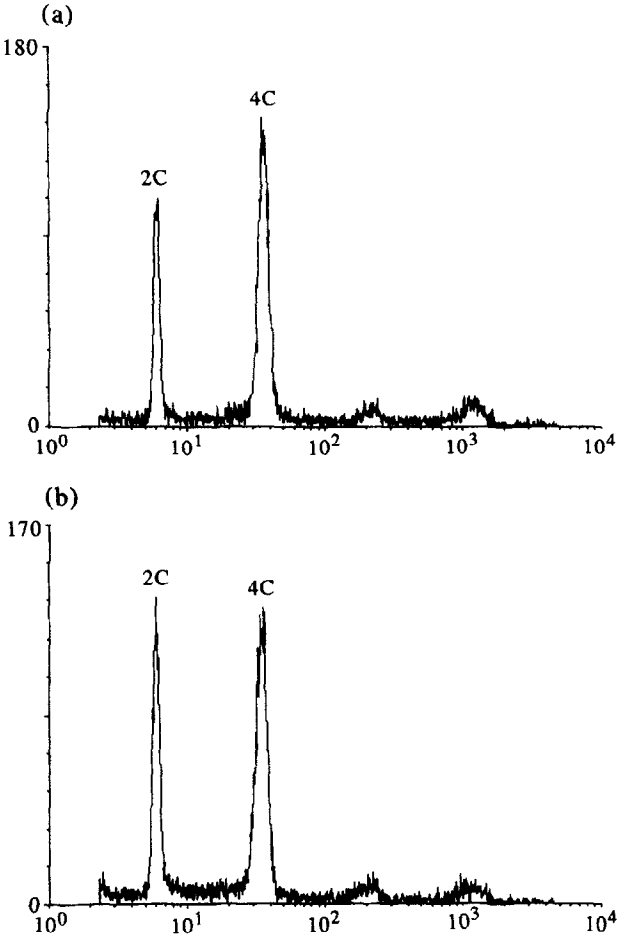

Figure 2. Effect of 20 μM NU/ICRF 505 on cell cycle distribution in the CHO ADR-r cell line. Cells were pretreated with drug for 24 h and then post-treated with cytochalasin B for 24 h to prevent daughter cells from re-entry into the cell cycle. (a) Control no drug treatment and (b) drug treated. *x*-axis is DNA content of cells and *y*-axis is cell number. 2C represents cells in G_1 phase and 4C represents cells in G_2/M (with a contribution from binucleate cells in G_1).

panel of CHO cell lines. Results obtained suggest that NU/ICRF 505 had little effect on cell cycle distribution with a tendency towards a G_1 arrest. Camptothecin (CPT) is considered the classic topo I poison and its affect on the cell cycle has been well documented. CPT is selectively cytotoxic to S-phase cells (by up to 1000-fold) [9] due to collisions between drug-stabilised topo I cleavable complexes and the replication machinery [10, 11]. The major consequence of the ensuing DNA damage on the cell cycle is a prolonged G_2 arrest mediated by inhibition of dephosphorylation of the cdc2/cyclin B complex necessary for the onset of mitosis [12, 13]. In contrast, a more immediate response to CPT-induced double-stranded DNA breaks (DSB) has been reported in the HL-60 cell line where cells rapidly enter into programmed cell death possibly due to a transient activation of cdc2 [14]. At frankly cytotoxic concentrations of CPT, a greater proportion of cells can be observed in the G_1 phase, but this is not due to cell cycle arrest, rather through the selective disappearance of S and G_2 phase cells by apoptosis [15].

In CHO-K1 cells (as used in the present study), a different sequence of events has been described after CPT treatment [16]. Double-stranded breaks are induced with high frequency but are also repaired quickly. As a consequence, cells are not blocked in G_2 through DNA damage recognition checkpoint control and enter into mitosis. However, the rapid DNA repair is error prone producing gross chromosomal aberrations resulting in cell death during failed and catastrophic mitosis [16]. Several reports have demonstrated high degrees of chromosomal aberrations in CHO cells treated with CPT and an absence of a classic apoptotic response [17, 18]. Nevertheless, CHO cells possess the capability of responding to DNA damaging agents (such as the topo II inhibitor etoposide or UV radiation) through prolonged G_2 arrest [19]. Thus, the lack of G_2 arrest observed with NU/ICRF 505 is entirely consistent with the cellular response of CHO cells to a topo I inhibitor.

Although NU/ICRF 505 and CPT share a common mechanism of action as topo inhibitors [1], several differences have emerged between the compounds. NU/ICRF

505 is significantly less effective in stabilising the cleavable complex and is two orders of magnitude less cytotoxic than CPT [2]. In HL-60 cells which are particularly sensitive to topo I inhibitors [14], CPT induces apoptosis as early as 6 h after drug incubation, but with NU/ICRF 505 there is a latent period of at least 24 h before evidence of endonucleolytic cleavage of DNA is seen [20]. In the present study, we now report a different property, namely that NU/ICRF 505 at its IC_{50} value can arrest CHO cells in G_1 phase (when topo I levels are elevated), whereas no such phenomenon has been reported for CPT [16].

The basis for this difference could be due to the fact that NU/ICRF 505 produces only a limited degree of DNA damage [1]. This level may not be sufficient to induce early entry into apoptosis [14, 20]. These limited DNA lesions may, however, be recognised by a more sensitive DNA damage surveillance mechanism resulting in the observed G_1 block. From this point of view, we have recently reported in human cell lines that p53 status is an important determinant of chemosensitivity to NU/ICRF 505, and that in cell lines containing wild type p53 (A2780) the drug produces a distinct G_1 block associated with induction of both p53 and $p21^{waf1}$ [21]. p53 induction only occurred after a latent period of 24 h, whereas induction of p53 by CPT was evident after only 6 h. In cell lines containing mutant p53 (HT-29), no induction of p53 or p21 occurred and no cell cycle perturbations were observed [21].

In summary, we describe for the first time the effects of the novel topo I inhibitor NU/ICRF 505 on progression through the cell cycle in a panel of CHO cells. In the parental cell line (CHO-K1) and in two mutants which express wild type levels of topo I, no evidence of cell cycle arrest was observed, consistent with CPT. In a drug-resistant cell line that overexpresses topo I, a small but significant accumulation in G_1 was recorded which may be mediated via a p53-dependent DNA damage recognition pathway.

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