

Induction of apoptosis in human cancer cell lines by the novel anthracenyl–amino acid topoisomerase I inhibitor NU/ICRF 505

I Meikle, J Cummings, JS Macpherson and JF Smyth

Imperial Cancer Research Fund Medical Oncology Unit, Western General Hospital, Edinburgh EH4 2XU, UK.

Summary Anthracenyl–amino acid conjugates represent a novel chemical class of topoisomerase (topo) inhibitor. NU/ICRF 505 is a lead compound that stabilises topo I cleavable complexes and is actively cytotoxic at low μM concentrations. In this study, endonucleolytic DNA cleavage was used as a marker of apoptosis to investigate mechanisms of cell death produced by this compound. NU/ICRF 505 ($5 \mu\text{M}$) induced a substantial increase in the level of DNA fragmentation in HL60 cells (up to 30% of total extracted DNA) but only after a 48 and 72 h drug exposure (compared with 6 h after treatment with camptothecin), as determined qualitatively by conventional gel electrophoresis and quantitatively by spectrofluorimetry. This effect was substantially reversed by co-treatment with zinc (1 mM). Subsequent studies with the human lung (NX002), ovarian (A2780) and colon (HT29) cancer cell lines yielded evidence of formation of higher molecular weight DNA fragments in NX002 and A2780 cells in response to NU/ICRF 505 ($5 \mu\text{M}$). Co-treatment with zinc (1 mM) caused a small decrease in DNA fragmentation. These data suggest that the induction of apoptosis may play an important role in the mechanism of cytotoxicity of NU/ICRF 505 in HL60 cells and that other pathways of cell death may also be operative in NX002 and A2780 in conjunction with apoptosis.

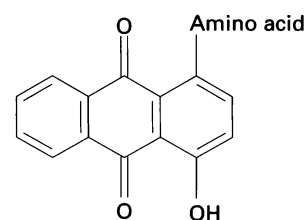
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DNA topoisomerases I and II (topo I and II) are nuclear enzymes that play critical roles in DNA metabolism (Wang, 1985) and appear to be primary targets for a number of clinically active anti-cancer drugs (Schneider *et al.*, 1990; Corbett and Osheroff, 1993; Chen and Liu, 1994). Drugs inhibit these enzymes by stabilising a reaction intermediate, referred to as the 'cleavable complex', where the enzyme remains covalently bound to DNA after strand cleavage (Nelson *et al.*, 1984; Hsiang *et al.*, 1985; Cummings and Smyth, 1993), and there has been much debate as to how such complexes induce cytotoxicity (Zwelling, 1989; Gewirtz *et al.*, 1991; Bertrand *et al.*, 1991). Studies are beginning to focus on the link between topo inhibition and the induction of programmed cell death (or 'apoptosis') (Walker *et al.*, 1991; Bertrand *et al.*, 1991; Hickman, 1992; Onishi *et al.*, 1993), a process which is usually recognised morphologically by a marked condensation of chromatin and shrinkage of the nucleus (Wyllie *et al.*, 1980, 1984; Earnshaw, 1995). However, apoptosis is often characterised by endonucleolytic cleavage of DNA, which can be viewed by electrophoresis as a series of bands, or 'DNA ladder' (Wyllie, 1980; Arends *et al.*, 1990). This particular approach has been employed with a number of topo inhibitors to determine the presence of apoptosis (Kaufmann, 1989; Walker *et al.*, 1991; Roy *et al.*, 1992; Gulliya *et al.*, 1994; Huschtscha *et al.*, 1995). DNA laddering, which can be reversed by zinc (Cohen and Duke, 1984), may not be an ideal marker for apoptosis as there have been a number of reports describing the morphological characteristics of apoptotic cells without internucleosomal cleavage (Cohen *et al.*, 1992; Oberhammer *et al.*, 1993a; Sun *et al.*, 1994). However, it has been reported recently that during early apoptosis DNA is cleaved into larger fragments which may serve as precursors to the smaller oligonucleosomal fragments (Bicknell *et al.*, 1994).

Many recently described topo I and II inhibitors have been demonstrated not to stabilise the cleavable complex but to act on the catalytic activity of the enzyme (Cummings and

Smyth, 1993; Li *et al.*, 1993; Permana *et al.*, 1994). These catalytic inhibitors can also display substantial levels of *in vitro* cytotoxicity (Ishida *et al.*, 1991). Although a number of unique mechanisms of action have been proposed to explain catalytic inhibition of topo I and II, there have been reports that these agents can also induce endonucleolytic DNA cleavage (Onishi *et al.*, 1993; Markovits *et al.*, 1994; Onishi *et al.*, 1994).

Anthracenyl–amino acid conjugates (AACs) represent a novel chemical class of topo inhibitor that can act either through formation of the cleavable complex or by catalytic inhibition (Meikle *et al.*, 1995a) and are actively cytotoxic against Chinese hamster ovary (CHO) and human cancer cell lines at low μM concentrations (Cummings *et al.*, 1994, 1995a; Meikle *et al.*, 1995b). Recent studies using a cytogenetic approach indicated that the topo I cleavage inducer, NU/ICRF 505, and the topo II catalytic inhibitor, NU/ICRF 500, also act as enzyme inhibitors in intact CHO cells (Cummings *et al.*, 1995b). As part of continuing investigations into the mechanism(s) of cell death induced by these compounds, endonucleolytic DNA cleavage was measured in HL60 human leukaemia cells exposed to NU/ICRF 505 (see Figure 1 for structural details). In subsequent experiments DNA fragmentation was assessed in a number of human cancer cell lines: the ovarian cancer line, A2780; the



Codename	Amino acid	C-terminus
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NU/ICRF 505	Tyrosine	— COOC ₂ H ₅
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Figure 1 Structure of the anthracenyl–amino acid conjugate NU/ICRF 505. [2H,3H]-9, 10-dihydroxyanthracene-1, 4-dione was reacted with α -tyrosine ethyl ester to produce the compound.

non-small-cell lung cancer line, NX002; and the colon cancer line, HT29. By simultaneously determining cell number and investigating the effects of zinc on levels of DNA fragmentation induced by AACs, the findings in this study are related to the possible induction of apoptosis.

Materials and methods

Materials

NU/ICRF 505 was synthesised and chemically characterised as described in full in Cummings and Mincher, UK patent Application Number GB 9205859.3; International Patent Application Number PCT/GB93/00546. Camptothecin was purchased from Sigma Chemical Co. (Poole, UK). Stock solutions were made up fresh for each experiment in dimethyl sulphoxide (DMSO) (spectroscopic grade) and the final concentration of DMSO did not exceed 0.5% in cell culture medium. Zinc, which was added as zinc sulphate, was dissolved in sterile distilled water before addition to the culture medium. The fluorescent stain, 4,6-diamidino-2-phenylindole (DAPI), was purchased from Sigma, and the 123 bp ladder was purchased from Life Technologies, Paisley, UK.

Cell culture studies

All cell lines were grown in RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum containing a 1% antibiotic mixture under standard conditions, and were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Cells were plated in 175 cm² flasks so as to achieve a final density of 1–2 × 10⁷ per flask (or 1 × 10⁵ in some growth inhibition studies) at time zero. At this time, medium was replaced with either drug-containing medium, or medium containing 0.5% DMSO (control). At each time point, A2780, HT29 and NX002 cells were trypsinised before counting whereas HL60 cells were harvested by centrifugation immediately. Following two washes in phosphate-buffered saline (PBS), 200 µl of each cell suspension was taken for the determination of total cell number using a Coulter Counter ZM (Coulter Electronics, Luton, UK). For later time points in experiments involving the A2780, NX002 and HT29 cell lines, the culture medium was also subjected to centrifugation to pellet detached cells, and these pellets were treated as separate samples.

DNA extraction/gel electrophoresis

DNA was extracted from cell pellets using the nucleon II DNA extraction kit manufactured by Scotlab Ltd (Coatbridge, UK). The final precipitated DNA fraction was left to rehydrate in sterile distilled water at 4°C overnight, after which time the concentration of DNA was determined spectrophotometrically using a UV2 spectrometer manufactured by ATI-Unicam (Cambridge, UK). DNA samples were analysed by loading 4 µg of each sample onto a 1.8% agarose gel in 40 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA. Each gel also contained 1 µg ml⁻¹ ethidium bromide to enable visualisation of the DNA bands on a UV-light box.

Quantitation of DNA fragmentation

Cell pellets, harvested as described above, were lysed in 0.5% Triton X-100 containing 5 mM Tris-HCl (pH 7.4) and 1 mM EDTA, for 20 min on ice. The percentage of DNA in the supernatant following centrifugation of the lysate was determined using the spectrofluorimetric method described in detail by Onishi *et al.* (1993) in which a Baird Nova spectrofluorimeter (Baird-Atomic Ltd, Braintree, UK) was used to detect the fluorescence of each fraction following the addition of DAPI.

Results

Induction of DNA fragmentation in HL60 cells

Initial experiments investigated whether the topo I inhibitor NU/ICRF 505 (see Figure 1 for structure) could induce endonucleolytic DNA cleavage over a 72 h period in HL60 cells. Simultaneous determination of cell number during this time period showed that NU/ICRF 505 at 5 µM caused a marked reduction to a level similar to 0.1 µM camptothecin (Figure 2). Using a quantitative fluorescence assay, over the 72 h study period camptothecin was demonstrated to induce 50% fragmentation of total extracted DNA compared with 30% for NU/ICRF 505 (Figure 3).

The degree of DNA fragmentation shown in Figure 3 was confirmed by agarose gel electrophoresis of DNA extracts (Figure 4). Camptothecin clearly produced characteristic DNA laddering after only 4 h, which became progressively more intense at 8 and 24 h (Figure 4, lanes 3–6). Similarly,

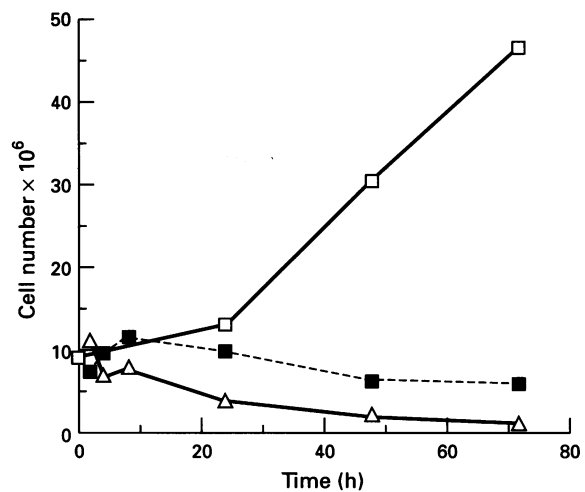


Figure 2 Total number of HL60 cells following exposure to camptothecin or NU/ICRF 505. HL60 cells were exposed to either 0.1 µM camptothecin or 5 µM NU/ICRF 505 for 2, 4, 8, 24, 48 and 72 h, and the total number of cells remaining at each time point determined by Coulter Counting. Control cells were incubated with fresh medium containing 0.5% DMSO. Results represent a typical graph taken from experiments that were repeated on four separate occasions. □-, control; ■-, NU/ICRF 505; △-, camptothecin.

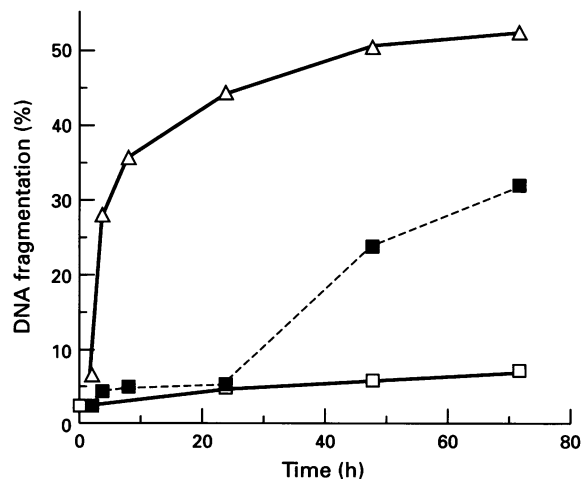


Figure 3 Quantitation of DNA fragmentation levels following exposure of HL60 cells to camptothecin and NU/ICRF 505. As for Figure 2, except at each time point the per cent DNA fragmentation induced by each drug treatment was determined spectrofluorimetrically as described in Materials and methods. □-, control; ■-, NU/ICRF 505; △-, camptothecin.

NU/ICRF 505 produced a significant level of DNA laddering but only after 48 h (Figure 4, lane 13) which increased at 72 h (Figure 4, lane 15).

Induction of DNA fragmentation in A2780 and NX002 cells

Following on from initial studies with HL60 cells, NU/ICRF 505 was used in further studies with three different human cancer cell lines. A2780 and NX002 were both chemosensitive to NU/ICRF 505 with 5 μM drug producing a similar effect on cell counts to 0.1 μM camptothecin (illustrated only for A2780 in Figure 5). However, these effects were possibly not

as dramatic as in the HL60 cell line (compare with Figure 2). Experiments with the human non-small-cell lung cancer cell line, NX002, revealed evidence of high molecular weight DNA fragmentation in those cells detached from culture flasks at 24, 48 and 72 h (Figure 6a, lanes 6, 8 and 10 respectively). Those cells remaining attached to culture flasks did not show any evidence of fragmentation (Figure 6a, lanes 3–5, 7 and 9). The medium from 72 h control cells did not contain a sufficiently high number of detached cells to enable extraction of DNA for electrophoresis, although cells remaining attached to the flask at this time did not show noticeable DNA fragmentation (Figure 6a and b, lane 11). A

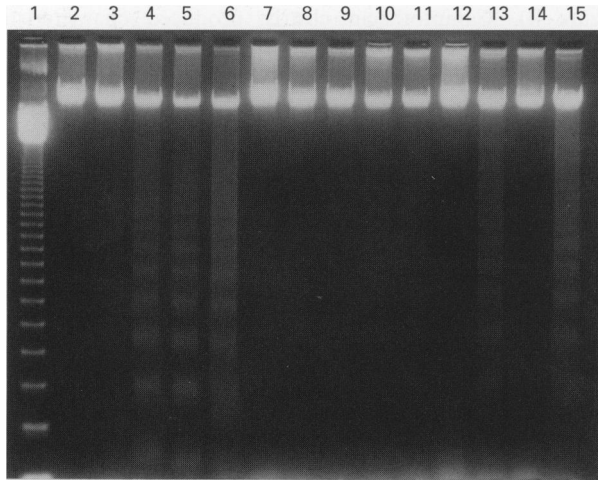


Figure 4 DNA fragmentation in HL60 cells exposed to camptothecin and NU/ICRF 505. As for Figure 2 except that the cell pellets obtained at each time point were used to extract total cell DNA. DNA extracts (4 μg each) were subjected to gel electrophoresis on 1.8% agarose gels as described in Materials and methods with the following gel loadings: lane 1, 123 bp DNA ladder; lane 2, 0 h control; lanes 3–6, 2, 4, 8 and 24 h camptothecin exposure respectively; lane 7, 24 h control; lanes 8–11, 2, 4, 8 and 24 h NU/ICRF 505 exposure respectively; lane 12, 48 h control; lane 13, 48 h NU/ICRF 505; lane 14, 72 h control; lane 15, 72 h NU/ICRF 505. Results represent a typical example for $n=3$ replicates.

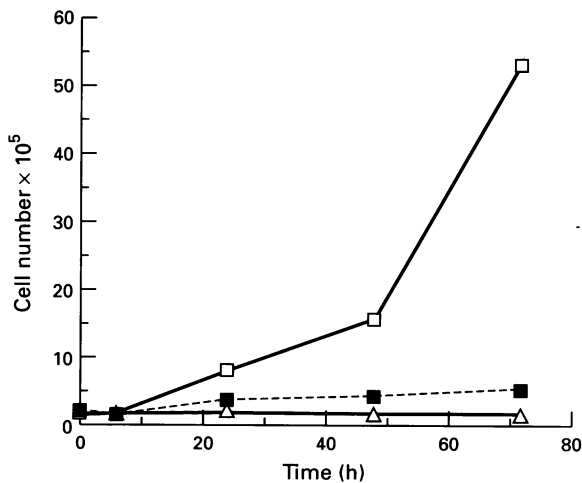


Figure 5 Total number of A2780 cells following exposure to camptothecin and NU/ICRF 505. Cells were exposed to either 0.1 μM camptothecin or 5 μM NU/ICRF 505 for 6, 24, 48 and 72 h, and the total number of cells remaining at each time point determined by Coulter Counting. Control cells were incubated with fresh medium containing 0.5% DMSO. Results represent a typical graph taken from experiments that were repeated on four separate occasions. -□-, control; -■-, NU/ICRF 505; -△-, camptothecin.

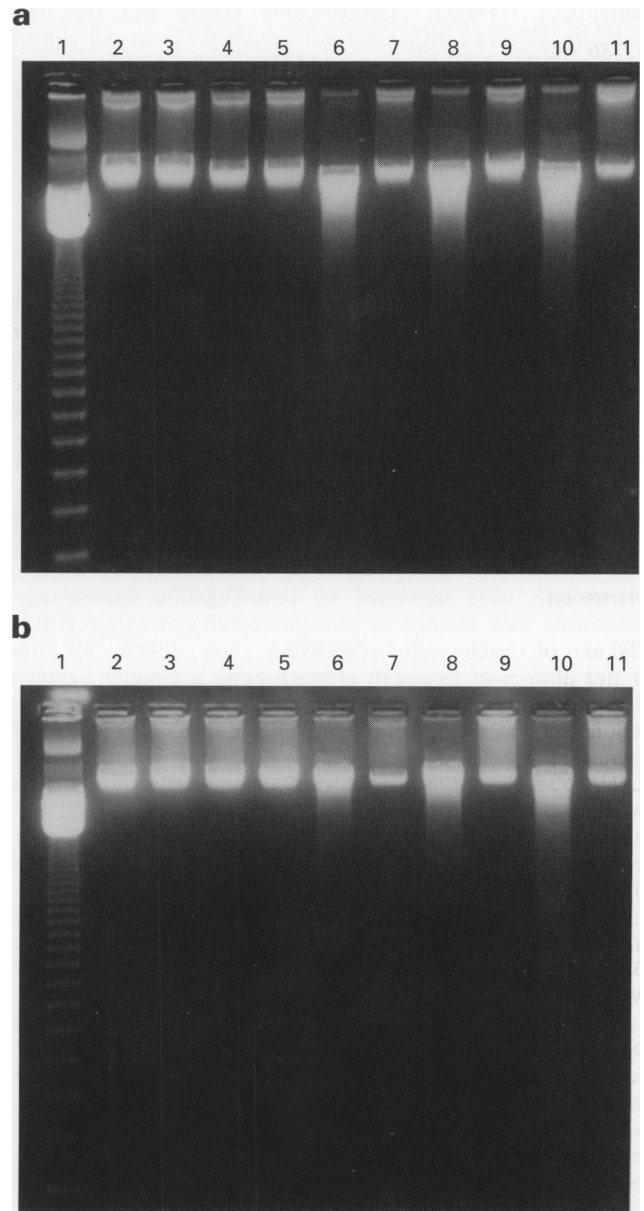


Figure 6 DNA fragmentation in human non-small-cell lung and ovarian cancer cells exposed to NU/ICRF 505. DNA was extracted from cells following various exposure times to NU/ICRF 505 and analysed by gel electrophoresis as described in Materials and methods. Upper gel (a), NX002 lung cancer cells; lower gel (b), A2780 ovarian cancer cells. Gel loadings as follows (4 μg per sample): lane 1, 123 bp ladder; lane 2, 0 h control; lanes 3, 4, 5, 7 and 9, DNA from attached cells following 4, 8, 24, 48 and 72 h exposure to NU/ICRF 505; lanes 6, 8 and 10, DNA from detached cells following 24, 48 and 72 h exposure to NU/ICRF 505; lane 11, 72 h control. Results represent a typical example for $n=3$ replicates.

very similar pattern was obtained with the human ovarian cancer cell line, A2780. Cell pellets obtained from centrifugation of the culture medium at 24, 48 and 72 h yielded evidence of high molecular weight DNA fragmentation (Figure 6b, lanes 6, 8 and 10 respectively).

The HT29 colon cancer cell line was chemoresistant to NU/ICRF 505 at 5 μM and, correspondingly, no DNA fragmentation was apparent at any of the time points studied (results not shown).

Possibly owing to the high molecular weight nature of the DNA fragmentation observed, the spectrofluorimetric method used to quantitate levels of DNA fragmentation in HL60 cells was not sufficiently sensitive to give reliable values with the NX002 and A2780 cell lines. This method was consequently not used in studies with these cell lines.

Effect of zinc on DNA fragmentation and cell number in HL60, A2780 and NX002 cells

The values for cell counts at 48 h showed that the addition of zinc substantially enhanced the survival of HL60 cells exposed to both 1 μM camptothecin (23.7% *vs* 1.6%) and 20 μM NU/ICRF 505 (17.7% *vs* 3.3%). This was confirmed in Figure 7 (lanes 11 and 12) in which the level of DNA fragmentation was clearly reduced in the zinc-treated cells (camptothecin-treated samples are not shown in this figure as there was an insufficient number of cells present at this time for DNA extraction). The effect of zinc addition on cell numbers in A2780 and NX002 was less marked, where both camptothecin and NU/ICRF 505 showed similar small increases in survival (3.2–4.9% increase). Analysis of DNA from zinc-treated NX002 cells revealed a reduction in the level of DNA fragmentation in camptothecin-treated cells (Figure 7, lanes 7 and 8), which was also apparent in NU/ICRF 505-treated cells (Figure 7, lanes 9 and 10). These effects were less obvious in A2780 cells (Figure 7).

Discussion

The aim of the present study has been to determine whether or not anthracenyl–amino acid conjugates, a new chemical class of topo inhibitor, can induce apoptosis through endonucleolytic cleavage of DNA. One of the most promising candidates from this class was investigated: NU/ICRF 505, which stabilises topo I cleavable complexes (Meikle *et al.*, 1995a). Initial experiments were carried out in HL60 human promyelocytic leukaemia cells as previous reports have demonstrated that this line exhibits particularly high levels of DNA fragmentation in response to known topo inhibitors (Kaufmann, 1989; Del Bino *et al.*, 1991; Solary *et al.*, 1993) without the relatively high levels of spontaneous DNA fragmentation observed in untreated thymocytes (Wyllie, 1980; Onishi *et al.*, 1993).

Using camptothecin as a positive control, characteristic DNA laddering was detected following only a 4 h drug exposure and this is consistent with previous reports (Kaufmann, 1989). Characteristic DNA laddering was also detected with NU/ICRF 505 in HL60 cells – suggesting that this compound can also induce apoptosis – but only after a 48 h drug exposure. Such a long delay and the reduced level of DNA fragmentation may be explained by the fact that NU/ICRF 505 is less effective at stabilising topo I cleavable complexes compared with camptothecin (Meikle *et al.*, 1995a).

Treatment of HL60 cells with zinc (which is known to antagonise endonuclease activation; Cohen and Duke, 1984) appeared to markedly enhance cell survival to both camptothecin and NU/ICRF 505. In addition, this was associated with a clear reduction in the level of DNA fragmentation providing further evidence that NU/ICRF 505 induces programmed cell death in HL60 cells.

Following on from the findings with HL60 cells, a number of other human cancer cell lines were treated with NU/ICRF

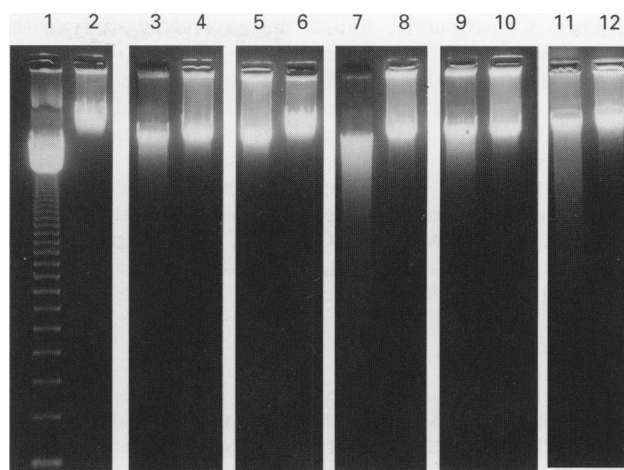


Figure 7 DNA fragmentation in human cancer cells co-treated with zinc and camptothecin or NU/ICRF 505. A2780, NX002 and HL60 cells were either exposed to 20 μM NU/ICRF 505 or 1 μM camptothecin alone or in the presence of 1 mM zinc sulphate. Following 48 h drug exposure, total cell DNA was extracted and analysed by agarose gel electrophoresis as described in Materials and methods. Sample identification as follows: lane 1, 123 bp ladder; lane 2, 48 h control; lanes 3 and 5, A2780 cells exposed to camptothecin and NU/ICRF 505 respectively; lanes 4 and 6, as for 3 and 5 except in the presence of zinc; lanes 7 and 9, NX002 cells exposed to camptothecin and NU/ICRF 505 respectively; lanes 8 and 10, as for 7 and 9 except in the presence of zinc; lane 11, HL60 cells exposed to NU/ICRF 505; lane 12, as for lane 11 except in the presence of zinc.

505 in order to determine levels of DNA fragmentation. Both the non-small-cell lung cancer cell line, NX002, and the ovarian cancer cell line, A2780, responded with a marked reduction in cell number after 72 h. Although there was an absence of distinct low molecular weight nucleosomal size DNA fragments, there was evidence on gels of higher molecular weight DNA fragmentation. Importantly, this effect was observed only in those cells which had detached from the culture flasks. There have now been several recent reports recognising that the formation of high molecular weight DNA can arise as a consequence of treatment of cells with topo I and II inhibitors (Fillipski *et al.*, 1990; Bicknell *et al.*, 1994; Sun *et al.*, 1994; Cohen *et al.*, 1994). The formation of high molecular weight DNA is of particular significance as this can occur in cases of apoptosis in which low molecular weight internucleosomal DNA cleavage is absent (Oberhammer, 1993b). However, during apoptosis large fragments are thought to be formed initially, and it is from these that the characteristic DNA ladders are derived (Cohen *et al.*, 1994). The apparent appearance of only higher molecular weight fragments following prolonged periods of drug exposure (after 24 and 48 h for example) in the A2780 and NX002 cells reported here, however, suggests that this transition did not appear to occur in these two lines, although both do show a noticeable increase in smaller DNA fragments following the 72 h drug exposure. The technique of pulsed-field gel electrophoresis has been applied in the analysis of high molecular weight DNA fragments formed during apoptosis (Walker *et al.*, 1991; Roy *et al.*, 1992) and this would be of value in the present study to determine if these fragments can be resolved into the distinct species characteristic of apoptosis (Cohen *et al.*, 1994).

High molecular weight DNA smearing has also been ascribed to cells undergoing necrosis (Huschtscha *et al.*, 1995), and experiments were performed in which A2780 and NX002 cells were co-treated with NU/ICRF 505 and zinc in an initial attempt to distinguish between the two different mechanisms (Cohen and Duke, 1984). The small increase in cell number in the presence of zinc for both camptothecin and NU/ICRF 505 corresponded well with a small, though noticeable, reduction in levels of DNA fragmentation in both

cell lines. Consequently, unlike the equivalent study with HL60 cells described above, the results of zinc co-treatment of A2780 and NX002 cells are much less obvious. Endonuclease activation is possibly not the sole cause of DNA fragmentation in these cell lines and other mechanisms of cytotoxicity are now also under consideration.

In summary, we have employed endonucleolytic DNA fragmentation as a marker to provide evidence that the AAC topo I cleavage inducer, NU/ICRF 505, induces apoptosis in

HL60 cells. Similar investigations in human lung and ovarian cancer cells suggest apoptosis may participate in the induction of cytotoxicity in these lines, however, additional work is required to clarify its precise role. These results provide important information on mechanisms of cell death induced by this class of compound and will be of benefit in the further development of AACs as potential anti-cancer drugs exhibiting novel properties.

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