Etoposide-induced cell cycle delay and arrest-dependent modulation of DNA topoisomerase II in small-cell lung cancer cells

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Summary As an approach to the rational design of combination chemotherapy involving the anti-cancer DNA topoisomerase II poison etoposide (VP-16), we have studied the dynamic changes occurring in small-cell lung cancer (SCLC) cell populations during protracted VP-16 exposure. Cytometric methods were used to analyse changes in target enzyme availability and cell cycle progression in a SCLC cell line, mutant for the tumour-suppressor gene p53 and defective in the ability to arrest at the G_1/S phase boundary. At concentrations up to 0.25 μM VP-16, cells became arrested in G_2 by 24 h exposure, whereas at concentrations 0.25-2 μM G_2 arrest was preceded by a dose-dependent early S-phase delay, confirmed by bromodeoxyuridine incorporation. Recovery potential was determined by stathmokinetic analysis and was studied further in aphidicolin-synchronised cultures released from G_1/S and subsequently exposed to VP-16 in early S-phase. Cells not experiencing a VP-16-induced S-phase delay entered G_2 delay dependent upon the continued presence of VP-16. These cells could progress to mitosis during a 6-24 h period after drug removal. Cells experiencing an early S-phase delay remained in long-term G_2 arrest with greatly reducing ability to enter mitosis up to 24 h after removal of VP-16. Irreversible G_2 arrest was delimited by the induction of significant levels of DNA cleavage or fragmentation, not associated with overt apoptosis, in the majority of cells. Western blotting of whole-cell preparations showed increases in topoisomerase II levels (up to 4-fold) attributable to cell cycle redistribution, while nuclei from cells recovering from S-phase delay showed enhanced immunoreactivity with an anti-topoisomerase IIα antibody. The results imply that traverse of G_1/S and early S-phase in the presence of a specific topoisomerase II poison gives rise to progressive low-level trapping of topoisomerase IIα, enhanced topoisomerase IIα availability and the subsequent irreversible arrest in G_2 of cells showing limited DNA fragmentation. We suggest that protracted, low-dose chemotherapeutic regimens incorporating VP-16 are preferentially active towards cells attempting G_1/S transition and have the potential for increasing the subsequent action of other topoisomerase II-targeted agents through target enzyme modulation. Combination modalities which prevent such dynamic changes occurring would act to reduce the effectiveness of the VP-16 component.

VP-16 (VP-16-213, etoposide), a semisynthetic derivative of the naturally occurring antimitotic agent podophyllotoxin, has become established as one of the most active agents in the treatment of small-cell lung cancer (SCLC). Preclinical studies have provided evidence of schedule dependency (Dombernowsky & Nissen, 1973; Wolfe et al., 1987) and the critical importance of a prolonged schedule has been confirmed in man by Slevin et al. (1989a,b). It has been suggested that continuous low concentrations of VP-16 are required for optimal activity of the drug when administered as a single agent (Clark et al., 1989), and very prolonged schedules of oral VP-16 have been evaluated and found to be effective (Hainsworth et al., 1989; Clark et al., 1990, 1991; Einhorn et al., 1990; Johnson et al., 1990). Since drug-induced, persistent cytostasis is an important clinical goal for the control of rapidly proliferating tumours, we have studied the dose dependency and kinetics of processes leading to irreversible cell cycle arrest of SCLC cells to VP-16 following protracted exposure in vitro.

VP-16 appears to initiate its cytotoxic action by acting as a specific poison for the cell cycle-regulated protein DNA topoisomerase II (Heck et al., 1988; Liu, 1989). DNA topoisomerase II is a nuclear enzyme that effects unknotting, decatenation or relaxation of supercoiled DNA molecules by a process of introducing transient double-strand breaks through which the strands of an intact helix can pass (Wang, 1985). Topoisomerase poisoning results in the trapping of enzyme molecules on DNA as cleavable complexes and the subsequent generation of potentially lethal lesions (Glisson & Ross, 1987; Liu, 1989). The majority of laboratory studies carried out with VP-16 have involved the use of acute exposures of cultured cells to high doses of the drug. Although this investigational approach may aid the study of the immediate DNA-damaging effects of the agent and its

relationship with topoisomerase II trapping, it does not reflect the pharmacodynamics of the clinical situation in which tumour cells typically undergo protracted exposure to VP-16 (Miller et al., 1990). The intrinsic sensitivity of actively proliferating tumour cells to topoisomerase II poisons appears to depend in part on the availability of the target enzyme (Liu, 1989; Smith & Makinson, 1989). The major type II enzyme, topoisomerase IIα, is cell cycle regulated, and as such its availability increases as cells progress towards mitosis (Heck et al., 1988). Thus, protracted VP-16 exposure would be expected to modulate the availability of the target enzyme as a result of changes in cell cycle progression. The study is pertinent to the use of low levels of VP-16 in the control of tumour growth since changes in the expression of topoisomerase II may play a central role in the inhibition of cell cycle transit (Lock & Ross, 1990), the development of drug resistance associated with low levels of target enzyme and in defining chemosensitivity to other agents used in combination regimens.

The responses of tumour cells to topoisomerase poisoning are not dependent upon topoisomerase gene expression alone. There appears to be a requirement for the p53 proto-oncogene-encoded protein both in the efficient activation of apoptosis and in cell cycle arrest following exposure to DNA-damaging anti-cancer agents or acute irradiation (Livingstone et al., 1992; Clarke et al., 1993; Lowe et al., 1993). The p53 protein appears to act as an element in the operation of a G_1/S checkpoint (Kastan et al., 1992; Lane, 1992), whereby the induction of DNA damage causes the half-life of the protein to increase, preventing S-phase entry and blocking the replication of damaged DNA. However, somatic mutation of the p53 locus is a frequent occurrence in human tumours (Hollstein et al., 1991), with small-cell lung cancer showing one of the highest rates of p53 mutation (Takahashi et al., 1989, 1991; Levine et al., 1991). Cancers with p53 mutations tend to respond to chemotherapy more weakly than those showing wild-type alleles (Callahan, 1992). Here we have explored the cell cycle arrest responses of a

SCLC cell line, with a defined p53 mutation, to continuous exposure to VP-16. The objective was to determine the effects of drug exposure on target enzyme availability as cells evade the G_1/S checkpoint, in addition to investigating the dose dependency and kinetics of processes leading to irreversible arrest.

Materials and methods

Cell culture, synchronisaton and VP-16 treatments

The SCLC cell line NCI-H69/P (designated H69; originally obtained from a patient with recurrent SCLC treated with doxorubicin) was maintained in suspension culture in RPMI medium supplemented with 10% fetal calf serum, 1 mM glutamine and antibiotics and incubated at 37°C in an atmosphere of 5% carbon dioxide in air. Ancillary experiments confirmed that the H69 cell line used in these studies carried a mutation in exon 5 (G to T at amino acid 171,P. Rabbitts, personal communication). VP-16 (Vepesid; Bristol Myers Pharmaceuticals, Syracuse, NY, USA) was provided as 34 mM stock solutions. Cells in exponential growth phase were diluted $(2 \times 10^5 \text{cell ml}^{-1})$ in fresh growth medium and VP-16 added to cultures following a 24 h growth period. Cultures were resuspended by aspiration using a Pasteur pipette and cell concentrations determined using a Coulter counter. Partial synchrony in early S-phase was achieved by incubating cells with aphidicolin (APC; Sigma) at 1 µg ml⁻¹ for 24 h. Cells were released from early S-phase block by washing cultures in prewarmed fresh medium followed by incubation in fresh medium. When releasing blocked cells into VP-16-containing medium, APC-treated cells were washed with medium supplemented with VP-16.

Cell viability

Cells were plated in a 96-well microtitre plate (100 µl per well) in the presence of varying concentrations of VP-16 and incubated for 1–5 days at 37°C. Viable cell number was assessed in triplicate by a non-separation, chemiluminometric assay (Cytolite Assay; Packard Instrument Company, Meriden, CT, USA) based upon the ability of cells with intact membranes to bind a probe which is activated to produce luminescence. Probe activation occurs in response to the intracellular generation of reactive oxygen species through electron-transferring reactions occurring in viable cells. Briefly, 125 µl of reduced coenzyme plus carrier (amplifier solution) was added to each well and luminescence was generated by the addition of 25 µl of a chemiluminogenic probe (activator solution) and measured in a TopCount Microplate Luminescence Counter (Packard Instruments). A calibration was carried out using a serial dilution of cells from a sister culture to ensure linearity between viable cell number and luminescence measured in counts s⁻¹.

Cell cycle analysis and detection of mitotic subpopulations

Cells were stained with ethidium bromide (50 µg ml⁻¹) plus 0.125% Triton X-100 and ribonuclease (0.5 µg ml⁻¹) for 10 min prior to analysis. DNA fluorescence distributions were analysed by a computer using a cell cycle phase-fitting program, which assumes normal distributions for G_1 and G_2/M phase populations (Watson et al., 1987). A probability function was calculated for the S-phase distribution based upon the means and standard deviations of the G_1 and G_2/M phases. For stathmokinetic experiments, VP-16-treated and control cultures were exposed to colcemid (60 ng ml⁻¹) in order to induce mitotic arrest and low-scatter mitotic populations were analysed as described previously (Epstein et al., 1988).

DNA strand breakage in single cells with respect to cell cycle position

The technique depends upon accurate measurements of the fluorescence intensities (corresponding to cellular DNA con-

tent) and volumes (corresponding to the extent of DNA damage-induced unwinding of nuclear DNA) of nuclei denatured in agarose gels (Smith & Sykes, 1992). An MRC-600 scanning confocal microscope (BioRad, Hemel Hempstead, UK), operating at its minimal confocal aperture, was used to optically section the spherical nucleoid bodies. Volumes were determined from mean diameter measurements of the digitised images accumulated under Kalman filtration to reduce the signal-to-noise ratio. This process was aided by colour-coding pixel intensity ranges above a selected threshold for the scanned image. DNA content was estimated by correcting the integrated fluorescence intensity of the nucleoid section showing the greatest diameter by the factor 2.22 × radius. This procedure was carried out on randomly selected nulceoids for each treatment condition.

DNA synthesis detected by bromodeoxyuridine (BrdUrd) incorporation

Samples of VP-16-treated (24 h exposure) cells $(1-10 \times 10^6$ cells) were pulsed with 20 µM BrdUrd (Sigma) for 1 h under normal growth conditions. Cells were washed twice in phosphate-buffered saline (PBS) before fixing in cold 70% ethanol for 30 min on ice. Fixed cells were treated with 4 N hydrochloric acid for 30 min at room temperature, washed in sodium borate (0.1 M, pH 8.5), resuspended in 20 µl of 0.5% Tween-20/PBS containing anti-BrdUrd antibody (Beckton Dickinson) and held for 30 min at room temperature. Antibody-treated samples were pelleted and resuspended in 0.5% Tween-20/PBS containing FITC-conjugated goat anti-mouse IgG (Tago, Burlingame, CA, USA; 8 µl H + L chains) and held for 30 min at room temperature. Finally, cells were pelleted and resuspended in PBS containing propidium iodide (at a final concentration 5 µg ml⁻¹) to stain nuclear DNA. Subsequent analysis and the use of fluorescence controls has been described previously (Karn et al., 1989).

SDS polyacrylamide gel electrophoresis and Western blotting

Briefly, whole-cell lysates were prepared by direct lysis of cells in hot (65°C) sample buffer (1.0 ml of 0.5 M Tris-Cl, pH 6.8, 0.8 ml of glycerol, 1.6 ml of 10% SDS, 0.4 ml of 2-mercaptoethanol, 0.2 ml of 0.05% bromophenol blue in 8 ml final volume) at a concentration of 1×10^6 cells 100 µl⁻¹. Lysates were boiled for 10 min, forced through a 21-gauge needle four times to shear the DNA, and spun at 13,000 r.p.m. in an Eppendorf centrifuge. The supernatant was retained and 10 µl loaded directly onto acrylamide gels (7.5% acrylamide with a stacking gel of 4% acrylamide). Gels were run at a constant 200 V in electrophoresis buffer (25 mM Tris base, 0.2 M glycine, 0.1% SDS) and either stained with Coomassie blue to check for loading or soaked for 30 min in transfer buffer [25 mM Tris base, 192 mM glycine, 20% (v/w) methanol] prior to transfer to nitrocellulose membranes (Schliecher & Schuell) using a Bio-Rad mini transblot electrophoretic cell apparatus for 1 h at a constant 100 V. Nitrocellulose blots were probed for 30 min with rabbit polyclonal antibody raised against a C-terminal peptide of topoisomerase II (Cambridge Research Biochemicals; 1:100 dilution in TBS). Blots were washed for 3 × 5 min with TTBS (0.1% Tween, 100 mM Tris, 0.9% sodium chloride, pH 7.5) and incubated for 30 min with a 1:200 dilution of biotinylated mouse anti-rabbit antiserum (Vector Laboratories). The biotinylated second antibody was detected using a Vectastain ABC immunoperoxidase kit (Vector Laboratories), with diaminobenzidine and nickel chloride as substrates.

Single-cell analysis of nuclear DNA topoisomerase II content

Samples of VP-16-treated (24 h continuous exposure) cells were taken (approximately 1×10^6 cells) and washed with nucleus buffer and permeabilised using the technique described previously (Minford et al., 1986). Briefly cells were resuspended in nucleus buffer supplemented with 0.35%

Triton X-100 and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF), and agitated for 20 min at 4°C. Permeabilised cells were fixed in 50% methanol (v/v) and agitated for 30 min at 4°C. Fixed cells were washed once in PBS and resuspended in 20 µl of anti-topoisomerase antibody (see above; 1:4 dilution) and held for 1 h at room temperature. Antibody-treated samples were washed once in PBS and resuspended in 20 µl of FITC-conjugated sheep anti-rabbit IgG (1:100 dilution; Sigma Chemicals, whole molecule) and held at room temperature for 30 min. Finally, samples were pelleted and resuspended in PBS containing ribonuclease and propidium iodide (5 µg ml⁻¹) to stain nuclear DNA. Samples were analysed by flow cytometry. Controls samples were processed as above but without the first anti-topoisomerase II antibody treatment. The analysis of samples by flow cytometry has been described previously (Smith & Makinson, 1989) providing dual-fluorescence analysis of cell populations gated for the elimination of debris and cell clumps. The right-angle fluorescence (RF) parameters monitor DNA content (630 nm RF) and second antibody binding (530 nm RF).

Results

Growth inhibition and cell cycle perturbations

Figure 1 shows the effects of VP-16 on H69 culture growth/viability studied over a 5 day period of continuous drug exposure. Doses of 0.25–0.5 µM produced cessation of culture growth within the first 48 h of exposure with no evidence of loss of metabolic function up to 8 µM. However after 5 days' exposure there was a decline in viability at high doses commensurate with the loss of membrane integrity as determined by vital dye staining methods (data not shown).

The results for repetitive cell cycle analyses are shown in Figure 2a and b, in which a value of unity for relative frequency indicates no overall change, with respect to the control, in the percentage of cells within a given cell cycle phase. At both time points, the proportion of cells in G_1 decreased as a function of VP-16 dose up to 1 µM. At 24 h, G_1 emptying at low doses (0.0625–0.25 µM VP-16) was accompanied by delay of cells in G_2. At higher doses (0.5–2 µM VP-16), there was reduced G_2 accumulation owing to the dose-dependent collection of cells in S-phase. By 48 h the cohort of cells initially delayed in S-phase by the higher doses of VP-16 appeared to have progressed through to G_2, with this S-phase emptying effect being most apparent for the 0.5 µM VP-16 dose. Again at 48 h there was a reduced G_2 accumulation at VP-16 doses >0.5 µM owing to the dose-dependent trapping of cells in S-phase.

In the subsequent studies the effects of low (0.25 µM) and high (2 µM) dose levels of VP-16 were compared. At the low dose level, maximum G_2 arrest is observed at 24 h without significant S-phase delay. On the other hand, 2 µM VP-16 allows maximal G_1 emptying but induces significant S-phase delay during the first 24 h exposure.

Stathmokinetic analysis of cell cycle delay and recovery

Using the mitotic spindle inhibitor colcemid, it was possible to investigate further the cell cycle perturbations caused by VP-16 without the complications of cell division and resupply of G_1. Analysis of bivariate plots of right-angle scatter and DNA content of cells permitted the identification of a low light scatter population (LSP) in G_2 representing cells entering mitosis (Epstein et al., 1988). The colcemid exposure of H69 cells was staggered to follow the number of cells attempting mitosis (i.e. escaping the G_2 delay induced by VP-16) in the presence of VP-16 or after release into VP-16-free medium (Table I). The discrimination between G_2 and M populations is not absolute (Epstein et al., 1988), and the average rate of cell cycle traverse may vary in multicellular aggregates during the course of a 48 h incubation experiment involving a centrifugation and resuspension/medium change at 24 h. Thus it is important to compare VP-16-treated

samples with the parallel control included in each treatment group (Table I).

The data show that, although VP-16 alone results in the accumulation of cells in G_2 as a function of dose, there is no evidence of trapping of cells in mitosis. The percentage of cells that have attempted mitosis, in the presence of colcemid, decreases as a function of dose and is approximately 1% at 2 µM VP-16. There is no evidence of significant trapping of percentage of cells in the combined G_1 and S-phase compartment [i.e. 100–(G_2 + M)] for the low dose range (0.0625–0.25 µM VP-16), whereas a high dose (2 µM) of VP-16 induces a significant delay in the delivery of cells to G_2 and a complete block to G_2 exit (condition a in Table I). Parallel
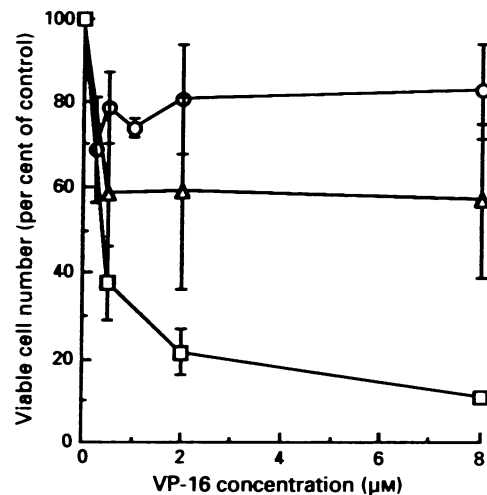

Figure 1 VP-16 dose-dependent changes in H69 viable cell number, relative to untreated controls, for different exposure periods. Symbols: O, 24 h; Δ, 48 h; □, 5 days. Data are mean values (± s.d.) for three experiments.
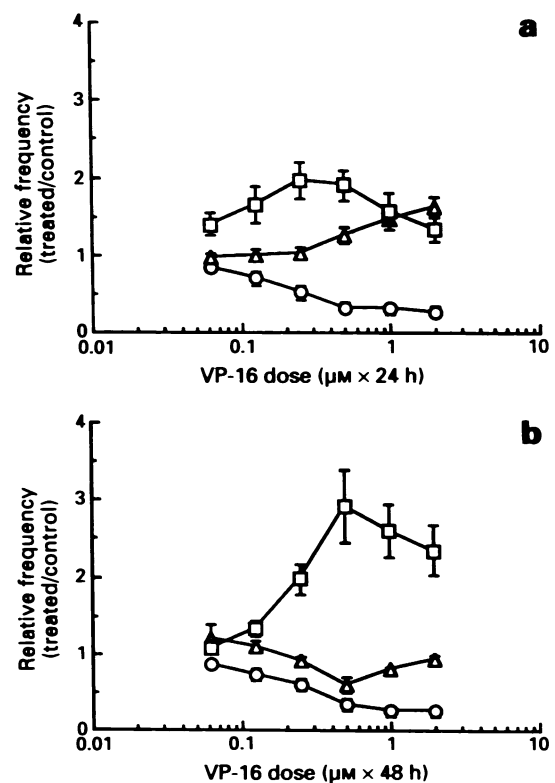

Figure 2 Dose dependency of the H69 cell cycle perturbations induced by continuous VP-16 treatment for 24 h (a) or 48 h (b). Control samples gave mean values (± s.e. for 13 determinations) of 40.5 ± 1.9, 38.4 ± 1.7 and 21.1 ± 0.8% cells in G_1, S phase and G2/M respectively. Data are mean values (± se) for 5 experiments. Symbols: O, G_1; Δ, S-phase; □, G_2.

Table I Stathmokinetic analyses of VP-16-induced cell cycle delay and recovery

VP-16 (µM)	Colcemid	Exposure: Recovery: Compartment:	Total cells in cell cycle compartment for different treatment and recovery periods (%)					
			0–24 h[a] 0 h		0–48 h[b] 0 h		0–24 h[c] 24–48 h	
			G₂	M	G₂	M	G₂	M
0	–		25.3	1.3	21.1	6.6	29.7	1.3
0.062	–		33.3	1.6	34.6	5.4	28.7	1.2
0.125	–		38.6	1.5	56.0	6.1	30.1	1.1
0.25	–		58.6	1.0	79.4	3.3	36.4	1.2
2.0	–		42.7	0.4	76.0	1.0	85.3	0.5
0	+		41.4	46.0	44.5	26.3	41.0	33.3
0.062	+		53.3	33.5	52.7	28.6	41.7	34.5
0.125	+		62.7	23.2	57.6	21.9	43.4	35.2
0.25	+		68.1	15.3	76.1	9.8	56.3	30.1
2.0	+		51.0	1.4	89.7	1.0	86.7	2.9

Data derived from a single representative experiment. [a]24 h continuous exposure to VP-16 (colcemid added at $t = 0$ h). [b]48 h continuous VP-16 exposure; cells resuspended in their own media at 24 h (colcemid added at $t = 24$ h). [c]48 h continuous VP-16 exposure; cells resuspended in fresh media at 24 h (colcemid added at $t = 24$ h).

Table II Flow cytometric analysis of proportions of cells actively engaged in DNA synthesis following 24 h exposure to VP-16

VP-16 (µM)	BrdUrd pulse	Cells in region (per cent of total) at end of 24 h VP-16 treatment			
		G₁	S (active)	S (inactive)	G₂
0	–	41.5 ± 7.2	3.9 ± 1.6	30.8 ± 6.9	23.9 ± 2.0
0	+	37.3 ± 4.7	32.9 ± 0.9	10.2 ± 2.0	19.2 ± 3.6
0.125	+	21.6 ± 4.2	48.1 ± 3.3	7.9 ± 2.8	22.4 ± 4.8
0.25	+	15.7 ± 1.3	54.0 ± 3.4	10.7 ± 0.9	19.6 ± 3.9
0.5	+	16.0 ± 3.1	52.7 ± 2.5	11.0 ± 4.2	19.7 ± 1.9
2.0	+	10.9 ± 1.6	58.9 ± 2.3	11.1 ± 1.2	18.6 ± 3.3

Mean data (± range) derived from two determinations.

studies (data not shown), which included a wild-type p53-expressing human cell line (Smith et al., 1994), confirm that the H69 cell line also shows a defect in arrest at G₁/S following acute X-irradiation (S. Souès & P.J. Smith manuscript in preparation). Collectively, the results are consistent with the evasion of the G₁/S checkpoint by VP-16-treated H69 cells.

During the incubation period of 24–48 h for a continuous (48 h; condition b in Table I) exposure to doses of <0.25 µM VP-16 there is evidence of cells attempting mitosis, whereas at the 2 µM concentration the majority of cells become trapped in G₂. Thus, it appears that at low doses, over the first 48 h, cells are progressing through the cell cycle relatively normally except that there is an increasing probability, with time, of a given cell being trapped in G₂. In contrast, at the higher concentration, over the first 24 h period, a large proportion of cells are slowed down or blocked in S-phase with a very high probability that any cell delivered to G₂ will remain trapped in that cell cycle compartment.

Cells released from a 24 h exposure to low doses of VP-16 (condition c in Table I) re-enter cycle during the subsequent 24 h incubation in fresh medium. This is in contrast to high-dose VP-16-treated cells, which when released at 24 h continue to accumulate in G₂ over the following 24 h period and remain blocked in G₂ with very few cells attempting mitosis.

S-phase delay analysed by BrdUrd incorporation

Flow cytometric measurements of BrdUrd incorporation were used to analyse S-phase delay in order to determine the proportion of cells synthesising DNA in treated and untreated cultures. Gates were set around two regions on contour plots of DNA content and BrdUrd incorporation. Region 1 contained cells that did not incorporate BrdUrd, and this region was analysed by the standard cell cycle phase-fitting programme and the calculated G₁, 'inactive' S

and G₂ percentages converted into percentages of total cells within regions. Region 2 was designated 'active S-phase', as this contained cells that incorporated BrdUrd above background levels. Typical results, shown in Table II, indicate that with increasing VP-16 dose after 24 h there is an increase in the percentage of cells engaged in DNA synthesis. The reduced G₂ arrest observed upon VP-16 treatment may arise from a direct effect of the thymidine analogue on cell cycle traverse. The contour plots show that the extent of BrdUrd incorporation relative to position in S-phase is similar for control and VP-16-treated cells (data not shown). Parallel studies using thymidine incorporation also show that such DNA synthesis detected in VP-16-treated cells is resistant to the inhibitory effects of acute 1 h high-dose exposures to VP-16 despite continued sensitivity to the DNA–protein cross-linking action of such high doses of drug (data not shown). The results consolidate the stathmokinetic results in that at the 2 µM VP-16 dose level initially delayed cells continue to traverse S-phase after a 24 h drug exposure in an apparently normal manner. Importantly, the percentage of cells in 'inactive' S-phase did not increase above the numbers gated in control cultures in response to VP-16 treatment, suggesting that no cells are actively blocked at 24 h in S-phase for any of the doses studied.

Cell cycle delay and recovery in synchronised cultures

Figure 3a and b shows the effects of VP-16 on the traverse of S-phase as monitored by G₂ accumulation. Asynchronous cultures were compared with those released from partial synchrony in early S-phase achieved by aphidicolin treatment. Control cells, released from G₁/S synchrony, traverse S-phase during the first 6 h of release, pass as a cohort through G₂ and re-enter G₁ by 24 h. Following release into 0.25 or 2 µM VP-16 (Figure 3b), cells are delayed in S-phase and accumulate in G₂. After a 24 or 30 h VP-16 exposure only cells exposed to the low dose (0.25 µM) can exit G₂ and
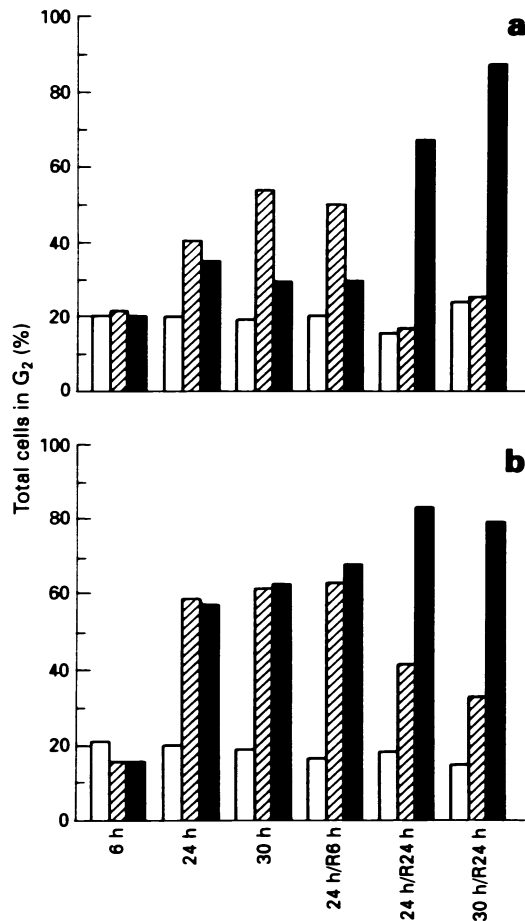
Figure 3 Effects of VP-16 on S-phase traverse, monitored by G₂ accumulation, for asynchronous H69 cells (**a**) and cells released from synchrony G₁/S (**b**). Mean data derived from three independent experiments (s.e. <10%). Cultures were treated for 6, 24 or 30 h with VP-16 at 0 μM (☐), 0.25 μM (▨) or 2 μM (■). Following 24 or 30 h treatments cells were released for the indicated recovery (R) period.

Figure 4 Induction of DNA damage in individual H69 cells by 24 h exposure to VP-16 as a function of total cellular DNA content determined by nucleoid volume (halo assay). **a**, Control cells, line fitted by linear regression ($y = 0.934 + 0.388x$; $R^2 = 0.718$; reproduced in **b** and **c**). **b** and **c**, 0.25 μM or 2 μM VP-16 × 24 h respectively.

re-enter G₁. Synchronisation increases the number of cells experiencing early S-phase during VP-16 exposure and reduces the number of cells capable of recovery from G₂ arrest compared with asynchronous cultures. Asynchronous cultures contain fewer cells in early S-phase and show a greater recovery from 0.25 μM VP-16 compared with synchronised cultures. Cells exposed to 2 μM VP-16, under either culture condition remain trapped in G₂, indicating the essentially irreversible nature of the arrest for the lowest concentration capable of inducing an overall S-phase delay.

DNA damage as a function of cell cycle position

We have used an adaptation of the 'comet' assay (Singh *et al.*, 1988; Smith & Sykes, 1992) to detect DNA breakage resulting from cleavage of trapped topoisomerase complexes or secondary fragmentation events as monitored by the nucleoid volume measurements. Figure 4 a-c shows the essentially linear relationship between nucleoid volume and DNA content for control cultures and the increase in the proportion of cells with high DNA contents in VP-16-treated cultures. The results reveal heterogeneity in the responses of cells to VP-16, with only a few cells (<15%) showing low levels of damage at 0.25 μM VP-16, whereas the majority of cells (>80%) show substantial damage at 2 μM VP-16. The G₁ fraction in cultures exposed to 2 μM VP-16 was too infrequent for detailed analysis. However, some cells with DNA contents approximating to S-phase showed no significant levels of DNA damage and may represent a subpopulation not undergoing either S-phase delay or elevation of damage levels.
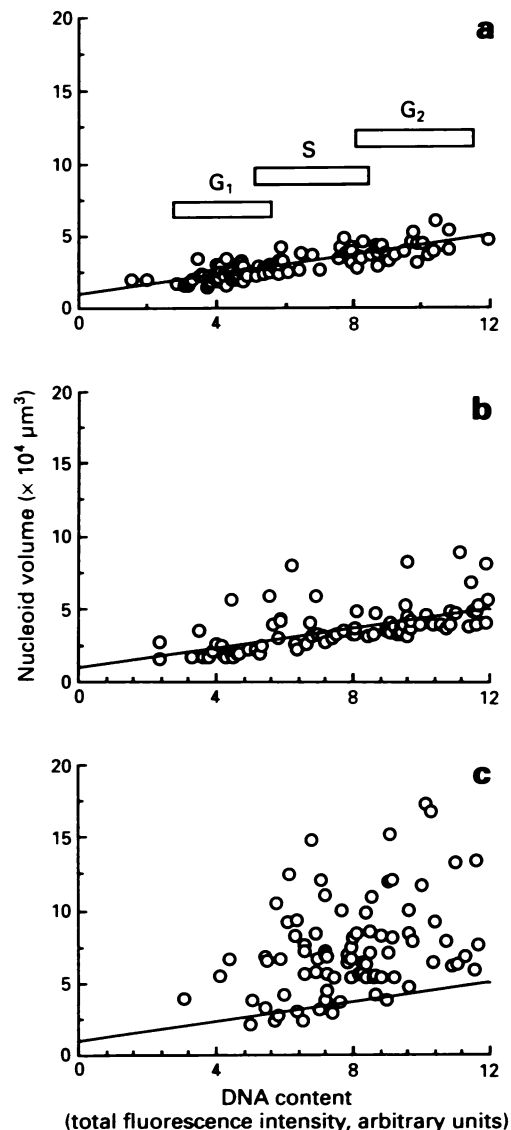
VP-16-induced changes in topoisomerase II availability

Whole population studies Immunoblots of whole-cell preparations of H69 cultures exposed to VP-16 for 24 h showed a major immunoreactive band with a mean molecular weight (± s.d. for ten determinations) of 171.3 ± 2.9 kDa corresponding to that expected for the p170 form of human DNA topoisomerase IIα. An increase in the intensity of the p170 band above control was observed for all VP-16-treated samples, and densitometry (Figure 5) was used to quantify the changes with respect to control samples. The p170 band intensity increased with drug dose and exposure period, reaching a maximum of 3.7-fold at doses of 1-2 μM VP-16 with a reduction in band intensity evident at 8 μM VP-16.

Single-cell analysis and cell cycle distribution To relate the cell cycle perturbations to the changes in topoisomerase IIα we have utilised a flow cytometric technique (Smith & Makinson, 1989), which provides a simultaneous analysis of DNA content and nuclear DNA topoisomerase II. Initial studies established the cell cycle distribution of topoisomerase II. Bivariate plots of DNA versus topoisomerase II content revealed the expected distribution of the p170 form of DNA topoisomerase II throughout the cell cycle: low levels in G₁,
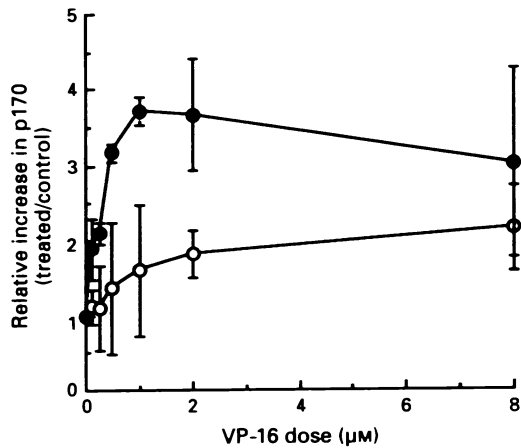
Figure 5 Densitometric analysis of the p170 topoisomerase II band for Western blots for H69 whole-cell preparations. Cultures were exposed to VP-16 for 24 h (O) or 48 h (●). Data are mean values (± s.d.) derived for four independent blots.
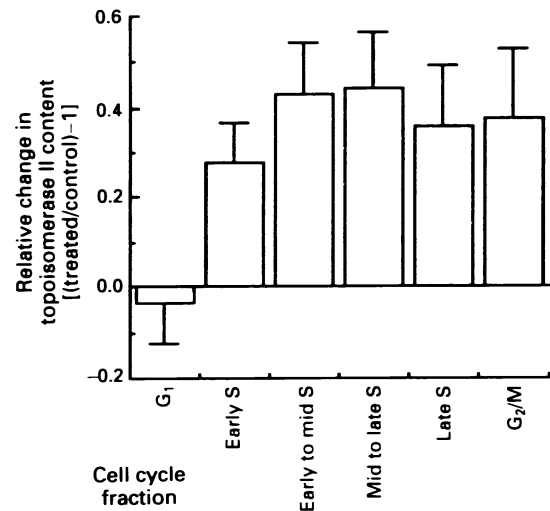

Figure 6 Histograms of VP-16-induced changes in topoisomerase II, relative to control levels, in cells within gated cell cycle compartments measured 24 h after continuous exposure to 2 μM VP-16 for 24 h. Data are means (± s.e.) of the corrected median values derived from four independent experiments. Median 530 nm RF values were background corrected and expressed as a ratio of the value obtained from unperturbed control cells such that a value of zero indicates no change in topoisomerase II-associated immunofluorescence compared with the control. S phase has been divided into four equal compartments of increasing DNA content.

levels increasing through S-phase, high levels in G_2 with a subset in G_2 showing the highest levels before recycling into G_1. Setting the anti-topoisomerase II antibody staining of G_1 cells at unity, signal was increased 1.7 ± 0.18-fold and 3.47 ± 0.61-fold for S-phase and G_2/M cells respectively (± s.d.; three experiments). Extensive redistribution of cells in the cell cycle by exposure to colcemid (see above) for 24 h gave corresponding values of 1.85 ± 0.28 and 3.47 ± 0.19 for S-phase and G_2/M cells respectively, showing that the analysis for immunoreactivity is independent of the proportions of cells within the gated regions. The control data (not shown) demonstrate the progressive increase in nuclear enzyme content through the cell cycle and support the interpretation that the changes seen above by Western blotting represent, at least in part, cell cycle redistribution. DNA content analysis for drug-treated cells (2 μM VP-16 for 24 h) clearly showed the expected VP-16-induced accumulation of cells in G_2 and S phase. DNA distributions were divided into six compartments of increasing DNA content representing: G_1, early S, early to mid S, mid to late S, late S and G_2/M fractions respectively (Figure 6). Median 530 nm fluorescence intensities were calculated for each gated compartment, corrected for background fluorescence and expressed as a value relative to control samples (Figure 6). No increase above control values was observed for cells exposed to 0.25 μM VP-16 (data not shown). At 2 μM VP-16 there is an unscheduled increase in nuclear topoisomerase II content in S-phase and in G_2/M.

Discussion

This study has demonstrated the strict dose dependency of the cell cycle perturbations underlying the cytostatic action of VP-16 on a human SCLC cell line. The disruption of the traverse of S-phase, in particular a delay in early S-phase progression appears to be a key component in the irreversible cytostatic action of VP-16. Continuous exposure to low doses of VP-16 (≤ 0.25 μM) results in significant enrichment of cells within cell cycle compartments which normally express high levels of topoisomerase II. VP-16 induced a cell cycle block in G_2 with a significant delay of cells in S-phase being apparent at high VP-16 concentrations. Similar observations have been reported for other human cells including transformed fibroblasts (Smith et al., 1986), lymphoblasts (Kalwinsky et al., 1983) and breast tumour cells (Epstein et al., 1988).

Dysfunction of the p53 proto-oncogene would be expected to contribute to a loss of the ability of cells to arrest at G_1/S in response to DNA damage. X-irradiation of the p53 mutant cell line H69 resulted in no detectable arrest of cells

at G_1/S but progressive accumulation at the G_2/M boundary (S. Souès & P.J. Smith, unpublished data). The results obtained with protracted VP-16 exposure also reveal an inability to arrest at G_1/S. In viewing the relevance of the concentration dependency of VP-16 effects, it is important to relate the drug doses used in this model study with those found in clinical practice. In a study by Slevin et al. (1989a,b), pharmacokinetic measurements on previously untreated patients with SCLC demonstrated that a 5 day oral regimen could maintain plasma VP-16 levels above 1.7 μM. Thus the present study describes cellular effects at clinically relevant doses of VP-16. The relationship between S-phase delay and subsequent cellular recovery from G_2 arrest was studied in synchronised cells exposed to VP-16 upon release from the G_1/S transition point. Cells not experiencing a VP-16-induced S-phase delay entered a long-term G_2 arrest dependent upon the continued presence of VP-16. Removal of VP-16 resulted in the progression of the majority of these cells through mitosis during the 6–24 h period after drug removal. Cells experiencing a significant VP-16-induced S-phase delay remained in long-term G_2 arrest with no evidence of progression from G_2.

Continuous exposure to an irreversibly cytostatic concentration of VP-16 also results in an increased immunoreactivity of nuclei to an anti-topoisomerase II antibody. We suggest that two effects can account for this observation. First, the principal effect is an unscheduled increase in topoisomerase II levels. This increase arises from the delay of cells in S-phase during a period in which there is a scheduled increase in topoisomerase IIα. Accordingly, there may be no cellular feedback to link cell cycle progression to topoisomerase levels once a cell is committed to active DNA synthesis. Such a model should be examined with DNA synthesis-inhibiting agents that are not discrete topoisomerase poisons and for the modulation of other cell cycle-regulated proteins. Second, a minor component of the increase may represent enhanced stabilisation of cleavable complexes. In support of this latter possibility is the observation that the sensitive nucleoid expansion method, performed under conditions which cleave DNA at trapped complexes, reveals significant levels of DNA fragmentation in delayed cells. However, the levels of cleavage detected are commen-

surate with only low levels of complex trapping detectable by the conventional K⁺SDS precipitation method (P.J. Smith & S.J. Falk. unpublished data).

It does not appear that the fragmentation revealed by nucleoid scanning represents typical apoptosis given the results of the viability measurements and our observation that acridine orange-stained preparations showed that <1% of cells displayed typical apoptotic nuclei. Furthermore, extractions of low molecular weight DNA fractions from VP-16-treated cell populations showed no detectable levels of nucleosome laddering as assessed by conventional agarose gel electrophoresis (P.J. Smith. unpublished data). The lack of early induction of apoptosis is not surprising since it has been reported that SCLC cell lines may differ in their ability to express VP-16-induced apoptosis (Okamoto-Kubo *et al.*, 1994) and the potential requirement of functional p53 for efficient induction (Clarke *et al.*, 1993; Lowe *et al.*, 1993). Additional studies are required to determine whether the damage visualised by nucleoid scanning represents target enzyme trapping or a secondary process of preapoptotic, perhaps DNA domain-limited, fragmentation.

We suggest that the minimal cytotoxic dose threshold for low-dose, protracted VP-16 exposures is defined by the dose intensity required to impose an early S-phase delay rather than effect G2 arrest *per se*. In the *in vivo* situation, the maintenance of a low but bioactive drug concentration would allow the continued recruitment of tumour cells into S-phase delay as they opt to enter the cell cycle since it is unlikely that the G₁/S checkpoint is functional in SCLC and G₁ emptying is not affected even by high drug doses. It is evident that SCLC cells can overcome the initial restriction to early S-phase traverse even in the continued presence of VP-16 and cells eventually enter a G₂ delayed state with enhanced availability of topoisomerase II.

Clinical studies have supported the concept that prolonged low-dose VP-16 treatment offers the combined benefits of efficacy and low toxicity. On the other hand, it is clear that such treatment will not break new ground in terms of increasing response duration or survival either when used alone

(Slevin *et al.*, 1989*a,b*) or as part of a conventionally designed regimen (Murphy *et al.*, 1992). However, prolonged schedules of VP-16 treatment may be a foundation for novel combination regimens which capitalise on the consequences of continuous topoisomerase II poisoning such as cell cycle synchronisation or modulation of topoisomerase II levels. This hypothesis was evaluated by exposing H69 cells to VP-16 for 24 h prior to treating washed cultures with selected agents and assaying growth potential using the conventional MTT assay (data not shown). The results indicated that VP-16 pretreatment results in a greater than 2-fold enhancement of growth inhibition potential for cisplatin, Amsacrine (*m*AMSA) and VP-16, while the other agents (camptothecin, mitoxantrone and doxorubicin) gave values of less than 1.3-fold enhancement.

The absence of an effect on sensitivity to the topoisomerase I poison camptothecin is consistent with the non-cell cycle-regulated nature of the target enzyme (Heck *et al.*, 1988). VP-16 pretreatment did not interfere with the cytotoxic potential of the topoisomerase poisons doxorubicin and mitoxantrone, suggesting that the initial capacity to induce topoisomerase II cross-linking not being an important factor in the cytotoxic action of anthracyclines and related drugs (Fox & Smith, 1990; Smith *et al.*, 1990). The >2-fold enhancement of cytotoxicity observed for the topoisomerase II poisons *m*AMSA and VP-16 is itself consistent with the effects of topoisomerase II modulation. The observations of enhanced cisplatin sensitivity is interesting given the capacity of this agent to induce DNA–DNA and DNA–protein cross-linking in what may be topologically compromised DNA molecules in VP-16-pretreated cells.

The implications for cancer chemotherapy are that dynamic changes in cell cycle distribution and target enzyme presentation in tumour cells exposed for protracted periods to low doses of VP-16 may offer novel opportunities for the introduction of other agents in combined regimens. The single-cell analytical approach described here offers a method of monitoring the effects of chronic VP-16 exposure *in vivo* within defined tumour target populations.

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