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The importance of drug scheduling and recovery phases in determining drug activity: improving etoposide efficacy in BCR-ABL-positive CML cells

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Abstract

K562 leukaemic cells are known to be less sensitive to etoposide than other cell lines, despite having similar *topo II* mRNA levels and cleavable complex formation. We have investigated the effect of etoposide schedule on cell cycle distribution, apoptosis and $p21^{waf1}$ and $cdk1^{p34}$ status in two *bcr-abl*-positive chronic myeloid leukaemia (CML) cell lines (K562 and KU812) and two small cell lung cancer (SCLC) cell lines (H69 and GLC4). During a continuous 5-day exposure, the SCLC cell lines showed a time and concentration-dependent loss of cell viability, with an initial block in the G2/M phase of the cell cycle followed by apoptosis. In contrast, the two CML cell lines showed no significant apoptosis or loss of viability after a similar block in G2/M. However, when K562 or KU812 cells were placed in drug-free medium following a 3-day drug exposure there was marked, concentration-dependent apoptosis (% apoptosis after release at 1 μM etoposide in K562, 10% at 24 h, 30% at 48 h). Our data also show that $p21^{waf1}$ does not increase after etoposide treatment in either H69 or GLC4 (both with mutated-p53). Although K562 and KU812 cells are null-p53, the arrest in G2/M during drug exposure was associated with increased $p21^{waf1}$ and a decrease in cdk1 (both $P < 0.001$ compared with controls). Upon release of these cells from drug-medium, $p21^{waf1}$ gradually returned to control levels, which was associated with an easing of the block at G2/M and an induction of apoptosis. This study highlights the importance of cell cycle regulatory proteins in drug sensitivity and resistance, and suggests that in cells such as K562 and KU812, a pulsed schedule may be more active than a single prolonged exposure.

Keywords: Drug scheduling; Chronic myeloid leukaemia; p21; cdk1; G2-arrest

1. Introduction

Etoposide is a schedule-dependent antitumour agent, displaying greater cytotoxicity in protracted schedules, compared with large single doses [1–3]. However, a reduction in etoposide-induced apoptosis by a drug-induced cell cycle arrest has been reported in a number of cell lines [4,5]. The possibilities of improving drug efficacy by the removal of drug to allow cells to recycle and undergo apoptosis, has been investigated.

Human chronic myeloid leukaemia (CML) is a malignancy of the pluripotent haemopoietic stem cells, characterised by the proliferation and accumulation of myeloid progenitors and increased resistance to numerous cytotoxic agents [6]. The cell lines K562 [7] and KU812 [8] were derived from patients with CML. In particular, the K562 cell line, derived from a patient with CML in erythroid blast crisis, has been shown to be resistant to a wide range of anticancer agents including topoisomerase inhibitors etoposide and camptothecin [9,10]. Reports have suggested that this resistance is mediated by the aberrant suppression of the apoptotic pathway by the product of the Philadelphia chromosome, which results from a reciprocal translocation between chromosome 9 and chromosome 22 [11,12]. This translocation of the breakpoint cluster region (*bcr*) of chromosome 22 and the *abl* gene from chromosome 9 results in a BCR/ABL fusion protein with elevated ABL tyrosine kinase (TK) activity [13]. McGahon and colleagues [10] speculated that the expression of this *bcr-abl* oncogene product contributed to this increased drug resistance. Although the exact mechanism for this *bcr-abl*-mediated inhibition of

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apoptosis is not clear, it is thought to involve the proteins that regulate the cell cycle in the G2/M compartment, as studies have shown that preventing the transit of irradiated murine haemopoietic cells through G2 could delay the apoptotic pathway [14,15]. The maintenance and control of the G2 to M transition depends on the phosphorylation state of several cyclin-dependent kinases (cdks), in particular cdk1 (also known as $p34^{cdc2}$) [16,17], which itself is regulated by a complex series of phosphatases and kinases [18]. Delaying entry into M would therefore not only allow for the repair of DNA damage, but could also delay apoptosis.

Cdks are present in all phases of the eukaryotic cell cycle, their primary function being to regulate entry into each of these specific and distinct phases [19–21]. A dominant regulator of entry into the mitotic phase of the cell cycle involves the conjugation of the kinase cdk1 to the B-type cyclin $p56^{cdc13}$. Through a complex interplay of de-phosphorylation and phosphorylation of amino acid residues on the cdk1 molecule (in particular Thr 161 by $p42^{CAK1}$ kinase), an active maturation-promoting factor (MPF) is formed which initiates mitosis. Apart from this classical function of the complex, a secondary role has been proposed for the MPF complex, which is the regulation of cell entry into S-phase. Cells in G2 do not normally re-enter S-phase, but are able to do so if cdk1/$p56^{cdc13}$ complex levels are low. By maintaining a moderate level of protein kinase activity, the complex maintains diploidy by preventing re-initiation of S-phase without a complete mitotic phase. Loss of protein kinase activity through the gradual degradation of $p56^{cdc13}$ results in exit from mitosis into interphase and concomitant release of the inhibition of S-phase initiation [18].

This intricately balanced system of kinase and phosphatase activity can be affected by cell cycle inhibitors such as the general cdk inhibitor $p21^{waf1}$, which is unregulated in response to DNA damage. The $p21^{waf1}$ protein is a phosphatase that is transcriptionally activated by wild type p53 [22]. Although the major target of $p21^{waf1}$ is the cyclin E/cdk 2 kinase complex, a primary regulator of the G1 to S-phase transition, it has been shown to be universally potent, with activity on all members of the cyclin/cdk family [23]. Specifically, it has been reported that the induction of a cell cycle arrest at G2 could be attributed to a reduction in cdk1-associated kinase activity [24,25]. Therefore, inhibition of the cdks involved in the activation of MPF would not only result in a cell cycle block at the G2/M boundary, but also a loss of S-phase regulation.

Initial studies within this department indicated a clear difference in drug activity between three cell lines. Etoposide was an effective cytotoxic agent in two small cell lung cancer cell lines, but ineffective in K562 cells. Even after a continuous exposure to drug at concentrations up to 10 μM, and duration of up to 5 days, viability did not significantly decrease in these cells (>92% in all cases). Most intriguingly, however, it was found that apoptosis could be increased significantly by applying a 'pulse-like' schedule. Since early observations indicated an induction of a G2/M block in etoposide-treated cells, this study examines the possible causes, such as the effect of etoposide on cdk1 and $p21^{waf1}$, and the effect their levels may have on the sensitivity of the cell lines to the drug.

2. Materials and methods

2.1. Cell culture

Four cell lines, chosen for their known *bcr-abl* status and response to etoposide, were studied. K562 and KU812 (CML, *bcr-abl*-positive and etoposide-resistant) and H69 and GLC4 (small-cell lung cancer (SCLC) cell lines, *bcr-abl*-negative and etoposide-sensitive), were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) Penicillin/Streptomycin (PS), in a humidified atmosphere with 5% CO_2 in air at 37 °C. To study the effect of a continuous exposure of etoposide, cells (1×10^5 cell/ml) growing exponentially were cultured for 5 days with etoposide (Sigma Ltd., Dorset, UK) at a range of clinically relevant concentrations between 0 and 1 μM. Aliquots were removed daily for assessment of viability by trypan blue exclusion, cell cycle distribution and $p21^{waf1}$ and cdk1 levels. Cells were transferred to fresh drug-medium every 24 h due to the instability of etoposide in the medium [26].

The effect of recovery from the drug was studied in K562 and KU812 cells cultured in the same range of etoposide continuously for 1–4 days before release into drug-free medium for a further 4 days. The effect of schedules with multiple recovery phases was studied in K562 cells that were exposed to the drug for 2 days and then transferred into drug-free medium for 2 days; this was then repeated to give a total culture time of 8 days. As with the continuous assay, aliquots were removed daily for assessment, prior to replacing medium with or without drug.

All cell counts were expressed as population doublings (PD) using the following formula:

$$\mathrm{PD} = \frac{\mathrm{Log}\left(\frac{CC_n}{CC_0}\right)}{\mathrm{Log2}}$$

where CC = cell count and n = day of study.

2.2. Flow cytometric analysis of apoptosis

The distinct phases of the cell cycle, including the apoptotic fraction (sub-G1), were distinguished by DNA staining with the fluorescent dye propidium

iodide (PI), and measured by flow cytometry. Cells were washed in ice-cold nucleus buffer (0.15 M NaCl, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM ethylenebis (oxyethylenenitrilo)tetra-acetic acid (EGTA), 0.1 mM Dithiothreitol, 10% (v/v) glycerol in distilled water pH 6.5). Cells (1×10^6) were then re-suspended in 4 ml of freshly constituted permeabilising solution (0.35% (v/v) Triton-X-100, 0.1 mM phenylmethyl sulphonyl fluoride (PMSF) in nucleus buffer), mixed by rotation at 4 °C for 20 min, and then fixed by adding 4 ml of ice-cold methanol and rotated for a further 30 min. Samples were washed with ice-cold phosphate-buffered saline (PBS) before staining with 500 μl of PI stain (50 μg/ml PI and 50 μg/ml RNAse A in PBS). Acquisition of data was performed within 1 h using a Becton Dickinson FACScan machine. Ten thousand cells were analysed for each data-point, and the percentages of cells in the sub-G1, G1, S and G2/M phases were determined using a cell cycle analysis program (WinMDI 2.4). The sub-G1 fraction of cells represented those with a reduced PI stain, but similar morphology, indicative of apoptotic and early-necrotic events [27], and were designated %A.

2.3. Assessment of $p21^{waf1}$ and cdk1 in each cell cycle phase by flow cytometry

Cells (1×10^6) were fixed as previously detailed, before incubation for 1 h with 100 μl of either mouse anti-$p21^{waf1}$ (PharMingen, San Diego, USA—1:60) or mouse anti-cdk1 (Kindly provided by the Imperial Cancer Research Fund, London, UK—1:100). Following a washing step with ice-cold PBS, cells were incubated with 100 μl fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Sigma—1:25 for $p21^{waf1}$ and 1:100 for cdk1). The cell cycle distribution of each sample was assessed by PI staining, and $p21^{waf1}$ and cdk1 fluorescence within each cell cycle phase measured using the WinMDI 2.4 program. This permitted the determination of protein levels in each phase of the cell cycle.

2.4. Assessment of $p21^{waf1}$ by an immunoblot analysis

Whole cell lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide with a 5% stacking gel according to the method of Laemmli [28]. The gels were then electroblotted onto polyvinylidene difluoride membranes (Bio-Rad Lab. Ltd, Hemel Hempstead, UK). Membranes were treated with 1% (w/v) blocking reagent (Boehringer Mannheim, Lewes, UK) in maleic acid buffer (100 mM Maleic acid, 150 mM NaCl, pH 7.5). Primary antibody probing was performed with mouse-anti-$p21^{waf1}$ at a concentration of 0.2 μg/ml (PharMingen). Following a washing step in 0.1% (v/v) Tween in-tris buffered saline (Sigma—100 mM Tris, 150 mM NaCl, pH 7.6), horseradish peroxidase-conjugated anti-mouse IgG antibody was used as the secondary antibody (DAKO Ltd, High Wycombe, UK). Bands were visualised by the enhanced chemiluminescence (ECL) plus detection system (Amersham Life Science Ltd, Little Chalfont, UK).

2.5. Statistical analysis

All statistical analysis was carried out using Minitab version 13 (State College, PA, USA). The effect of concentration and exposure duration of etoposide on cell growth, viability, cell cycle distribution, $p21^{waf1}$ and cdk1 status were investigated using a balanced analysis of variance. Where this analysis indicated an effect of concentration and/or duration, differences between these variables and control cultures were further characterised by the standard paired Student's *t*-test. Control samples were normally distributed as determined by the Shapiro–Wilk test, and parametric tests were used throughout.

Table 1
The concentration and duration-dependent effect of etoposide on population doubling. Results are given for days 1, 3 and 5, and negative values signify a cell number that is lower than that on day 0, indicating a cytotoxic effect. Values are the means and SDs of at least three separate experiments

SCLC	H69			GLC4		
(μM)	1 day	3 days	5 days	1 day	3 days	5 days
0	0.4±0.1	1.3±0.6	2.1±0.5	2.5±0.1	4.0±0.3	4.7±0.1
0.2	0.1±0.2	0.0±0.4	0.1±0.7	1.9±0.4	2.8±0.5	3.3±0.4
0.4	−0.2±0.3	−0.7±0.1	−2.5±1.3	1.4±0.3	1.3±0.4	1.4±0.6
0.8	−0.6±0.3	−0.6±0.6	NC	0.9±1.1	0.8±0.2	0.3±0.4
CML	K562			KU812		
(μM)	1 day	3 days	5 days	1 day	3 days	5 days
0	0.6±0.7	1.5±0.7	2.6±0.4	1.0±0.1	3.0±0.1	4.2±0.1
0.2	0.4±0.3	1.2±0.6	1.6±0.5	1.0±0.1	2.7±0.2	3.5±0.4
0.4	0.3±0.6	0.6±0.4	0.9±0.4	0.5±0.0	1.7±0.2	2.3±0.3
0.8	0.3±0.8	0.6±0.4	0.0±0.4	0.4±0.1	1.2±0.2	0.1±0.7

SCLC, small-cell lung cancer; CML, chronic myeloid leukaemia; NC, no viable cells present.

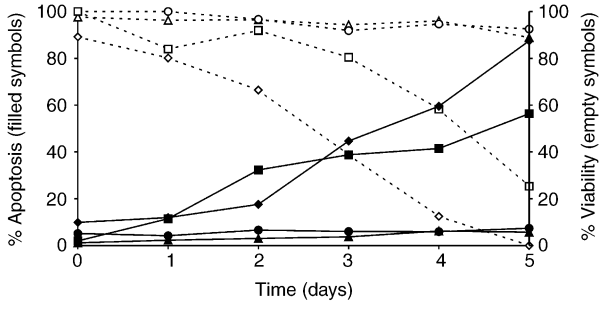

Fig. 1. The effect of 0.8 μM etoposide on cell viability (empty symbols) and apoptosis (filled) in four cell lines (K562—triangles; KU812—circles; H69—diamonds; GLC4—squares). Data is expressed as the means of at least three separate experiments SDs have been omitted for clarity and were <10%.

3. Results

Control cultures of all cell lines followed a normal growth pattern with a doubling time of approximately 24 h and minimal loss of viability. Flow cytometric analysis indicated a normal cell cycle distribution for the four cell lines. Control cell cycle distributions for each cell line were: GLC4, 3% sub-G1 (A), 61% G1, 10% S, 23% G2/M; H69, 1% A, 49% G1, 17% S, 30% G2/M; K562, 2% A, 42% G1, 29% S, 25% G2/M; KU812, 6% A, 52% G1, 17% S, 20% G2/M.

3.1. Continuous exposure

A concentration and exposure duration dependent reduction in cell proliferation, expressed as population doubling (PD), was observed in the SCLC cells cultured with etoposide continuously for 5 days (Table 1). This reduction in PD was mirrored by a concomitant decrease in viability (Fig. 1). In fact, there were no viable cells in H69 cultures of 1 μM etoposide beyond day 4 (at 1 μM etoposide on day 5, viability was 0% in H69, 7% in GLC4). Similarly, there was a concentration-dependent decrease in the rate of PD in the CML cells (Table 1); however, there was no reduction in cell viability at any concentration or duration (>88% in all cases), indicating a cytostatic response (Fig. 1).

There was a concentration-dependent cell cycle block in G2/M in all four cell lines after 24 h of drug exposure, which was transient in the H69 and GLC4 cells (data not shown). For K562 and KU812 cells, however, this block persisted in the presence of drug (change in%G2/M: +16.2±0.2% and +11.3±0.2% on day 1 versus +21.7±0.2% and +29.6±0.7% on day 5,

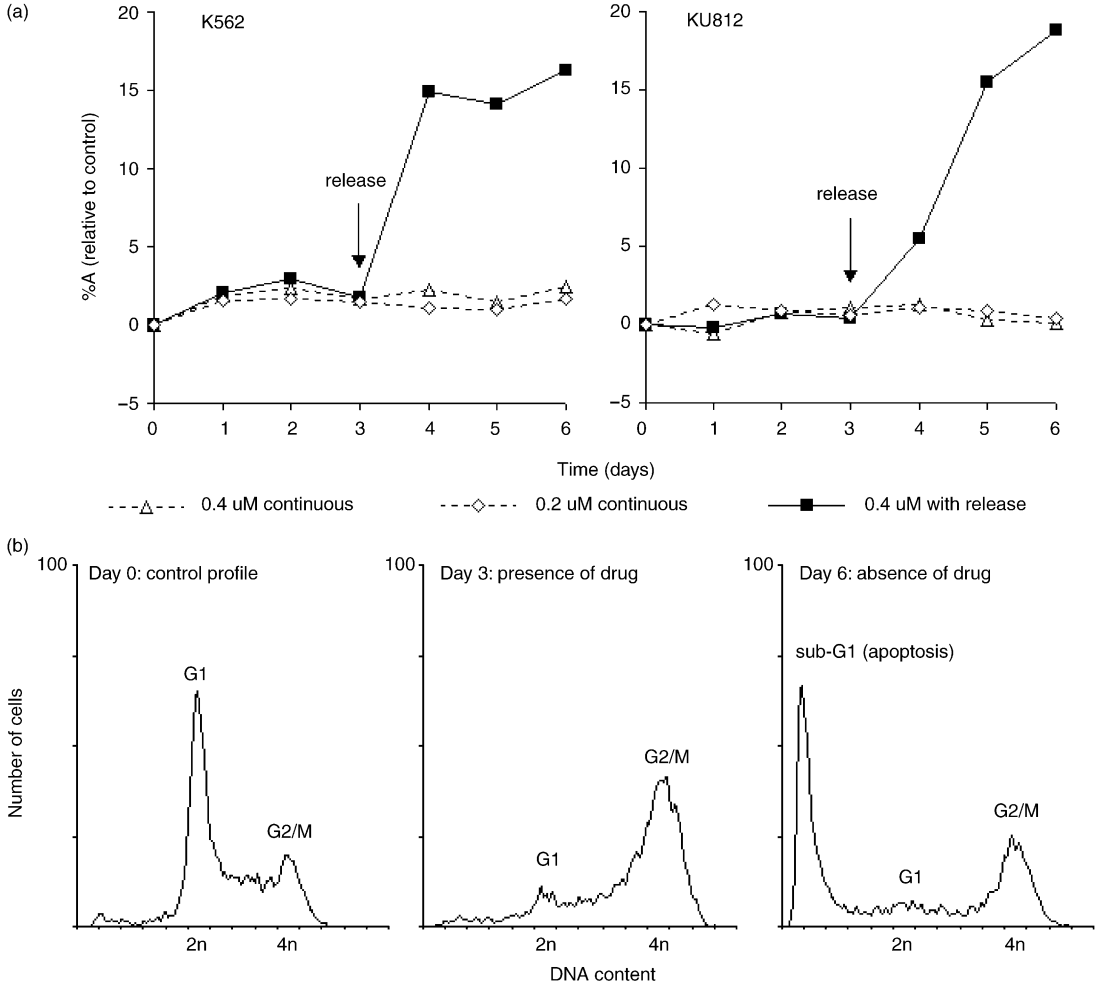

Fig. 2. (a) The effect of a recovery phase on the cell cycle in CML cells. Cells were cultured according to one of three schedules: 0.4 μM continuously (△), 0.2 μM continuously (◇), or 0.4 μM etoposide for 3 days followed by drug-free medium for 3 days (■). The last two schedules were of an equal exposure duration (ED: duration×concentration). Data are expressed relative to control cells without treatment, which showed no significant increase in % apoptosis (A) over the 6 days (<5% in all time-points). Data-points represent the means of three separate experiments, and SDs have been omitted for clarity and were <10%. (b) Typical DNA histograms of K562 cells cultured with 0.4 μM etoposide for 3 days followed by recovery in drug-free medium for a further 3 days—on day 0 (normal DNA profile), day 3 (presence of drug) and day 6 (absence of drug).

respectively, in cells treated with 0.8 μM; all $P<0.001$ compared with controls with no added drug). There was a concentration and duration-dependent increase in %A in the H69 and GLC4 cells (Fig. 1), whereas there was no significant change in the two CML cells at any concentration and duration. More accurately, %A in the K562 cells was $<5\%$ in all of the cultures of duration <3 days (Fig. 1).

3.2. Recovery in drug-free medium

To investigate whether this protracted G2-block observed in the CML cells persisted even in the absence of etoposide, these cells were cultured in etoposide for 1, 2, 3 or 4 days before removal of the drug and recovery in drug-free medium for a further 4 days.

There was no significant effect on the population doubling and cell viability status of the K562 or KU812 cells in the 1-day etoposide schedule. However, those cultures in which cells were exposed to drug for 2, 3 or 4 days before recovery showed an increasing loss of viability only after removal of drug and subsequent incubation in drug-free medium. For example, the percentage cell viability on day 7 for K562 and KU812 cells treated with the schedule involving 3 days of 0.8 μM etoposide followed by 4 days drug-free was $57\pm1\%$ and $52\pm6\%$, respectively (compared with $97\pm2\%$ and $90\pm3\%$ in the control cultures with no added drug; $P<0.001$).

There was a cell cycle arrest at G2/M in the presence of etoposide, which was released upon drug-removal. In the 1-day schedule, cells slowly returned to a cell distribution comparable to control, with no increase in cell kill. In contrast, the 2-, 3- and 4-day exposure schedules caused a significant concentration-dependent increase in %A upon release from drug exposure in both cell lines ($P<0.001$). In particular, %A in the 3- and 4-day exposure schedules continued to rise significantly in the absence of drug (Fig. 2a and b).

3.3. Multiple recovery phases

To further highlight the importance of recovery phases in enhancing etoposide cytotoxicity in CML cells, a single-dose schedule (4 days exposure followed by 4 days drug-free) and a double-dose schedule (2 days exposure followed by 2 days drug-free twice) was directly compared in K562 cells.

During the recovery phase of the single-dose schedule, there was a gradual easing of the block in G2/M and a significant increase in the %A (Fig. 3a). This increase was reflected by a dose and duration dependent reduction in viability to 41.2% on day 8. In the double-dose schedule, the first phase of recovery occurred after 2 days exposure to etoposide. During this recovery phase, the drug-induced block in G2/M was eased and %A was significantly increased (at 0.8 μM, $5.1\%\pm1.2\%$ on day 2 to $28.6\%\pm3.4\%$ on day 4, $P<0.001$). Re-exposure to drug resulted in a second G2/M block, and a plateau in the apparent rate of%A. The removal of drug as part of the second recovery-phase following this second round of drug-exposure resulted in a re-release of the G2/M-block and a further increase in %A (Fig. 3b).

Calculation of the area under the %A-time curve (AUC) revealed that the accumulative %A was on average 2-fold greater in the double-dose schedule than in the single-dose schedule, even though there was an equal exposure duration to etoposide in both schedules ($P<0.001$) (Fig. 4a). More importantly, the number of cells remaining at the end of the double-dose schedule was significantly lower than that seen in the single-dose schedule ($P<0.001$) (Fig. 4b; the cell concentration of control cultures with no added etoposide was $34.2\pm1.5\times10^5$ cell/ml).

3.4. $p21^{waf1}$ and cdk1 levels

Quantification of $p21^{waf1}$ and cdk1 levels was primarily assessed by flow cytometry, with $p21^{waf1}$ levels confirmed by immunoblot analysis only in K562 cells. All results from the flow cytometric analyses relate to the cellular protein levels within the G2/M phase of the cell cycle.

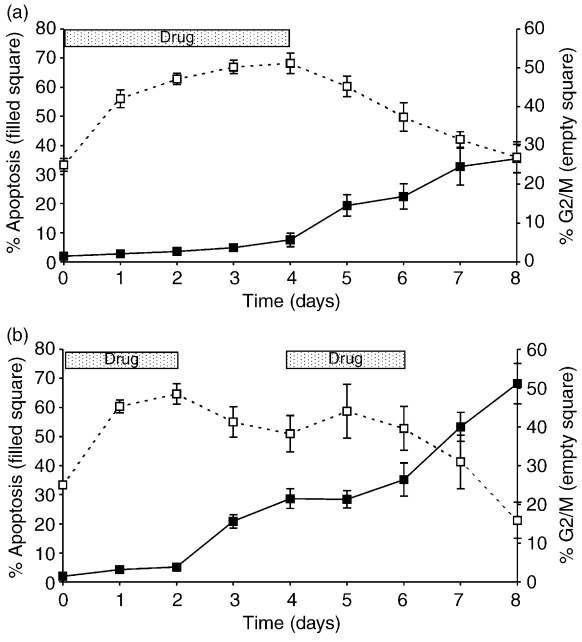

Fig. 3. (a) The effect of a single-recovery phase in K562 cells. Cells were cultured in an 8-day schedule involving a 4-day exposure to 0.8 μM etoposide and 4-day recovery. Data-points represent the means and SDs of at least three separate experiments. (b) The effect of a double-recovery phase in K562 cells. Cells were cultured in an 8-day schedule involving a 2-day exposure to 0.8 μM etoposide and 2-day recovery repeated twice. Data-points represent the means and SDs of at least three separate experiments.

There were no significant changes in the fluorescence intensity of p21^{waf1} in the G2/M phase in H69 or GLC4 cells treated with 0.4 μM etoposide, as measured by flow cytometry. However, there was a steady and significant increase in p21^{waf1} levels with time ($P<0.001$) in both K562 and KU812 cells (Fig. 5a).

As a result of this observation, cdk1 levels were assessed. Levels remained constant in the control cultures with no added etoposide. There was no significant change in the level of cdk1 in both the GLC4 and H69 cells; however, there was a duration-dependent decrease in staining in the G2/M phase of the cell cycle of K562 and KU812 cells. Cells treated with 0.4 μM etoposide showed a gradual sustained decrease in the cdk1 level over time, which was significantly reduced in cells cultured for $\geqslant 2$ days ($P<0.001$) (Fig. 5b).

To determine whether the G2/M block during drug exposure and release following re-culture in drug-free medium was mediated by p21^{waf1}, their levels in K562 cells exposed to etoposide for 3 days followed by 3 days recovery, was assessed. In the presence of etoposide, p21^{waf1} levels were elevated, but upon removal of the drug, there was a significant reduction in p21^{waf1} after 3 days recovery ($P<0.001$) (Fig. 6a). The same pattern of p21^{waf1} staining was observed in KU812 cells, with the initial etoposide-mediated increase in levels falling upon removal of drug and returning to control levels by day 6. This pattern of p21^{waf1} expression was also confirmed

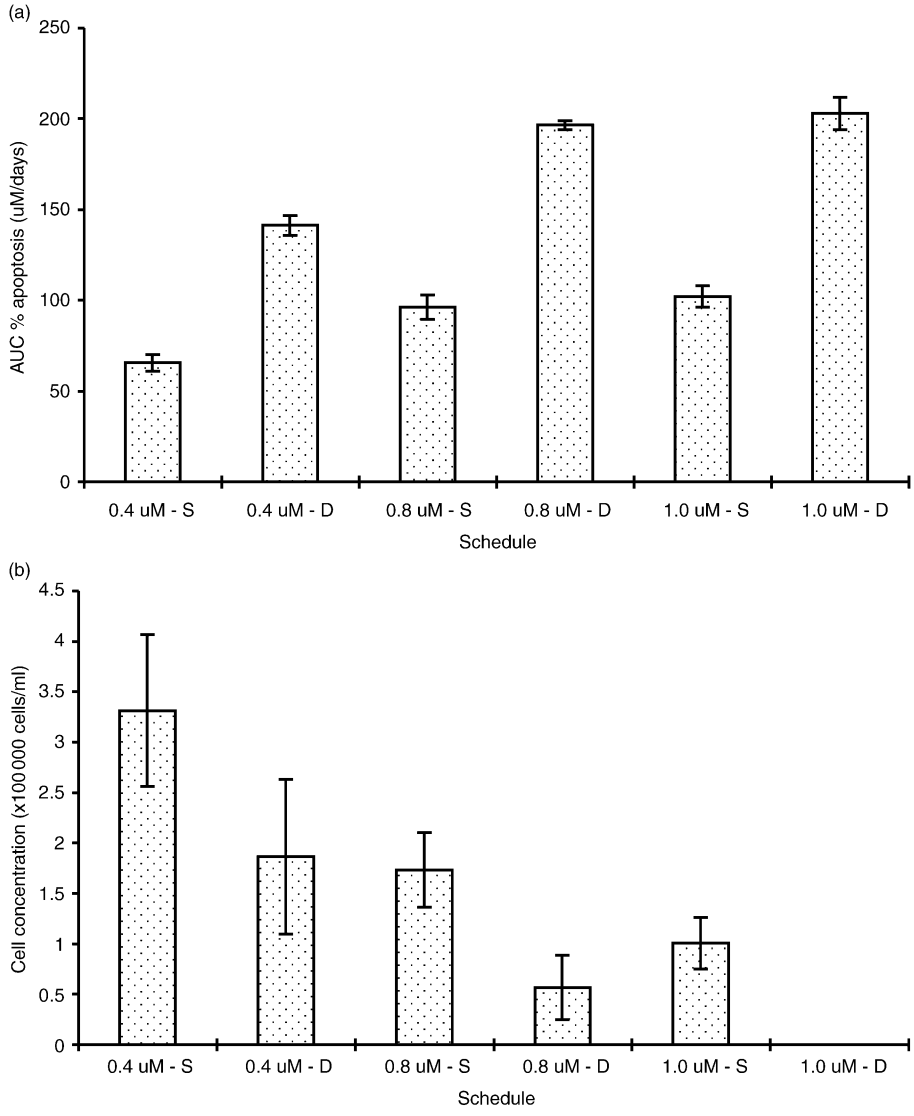

Fig. 4. (a) The comparison of AUC%apoptosis of two schedules in K562 cells. Cells were cultured in one of two 8-day schedules involving exposure to 0.8 μM etoposide. Data-points represent the means and SDs of at least three separate experiments. AUC, area under time curve. (b) The comparison of cell numbers remaining on day 8 after culture in the two schedules in K562 cells. Cells were cultured in one of two 8-day schedules involving exposure to 0.8 μM etoposide, given either as a single (S) 4-day dose followed by a 4-day recovery phase or as a double (D) 2-day dose and 2-days recovery schedule. There were no viable cells seen in cultures in the 1 μM D-schedule. Data-points represent the means and SDs of at least three separate experiments.

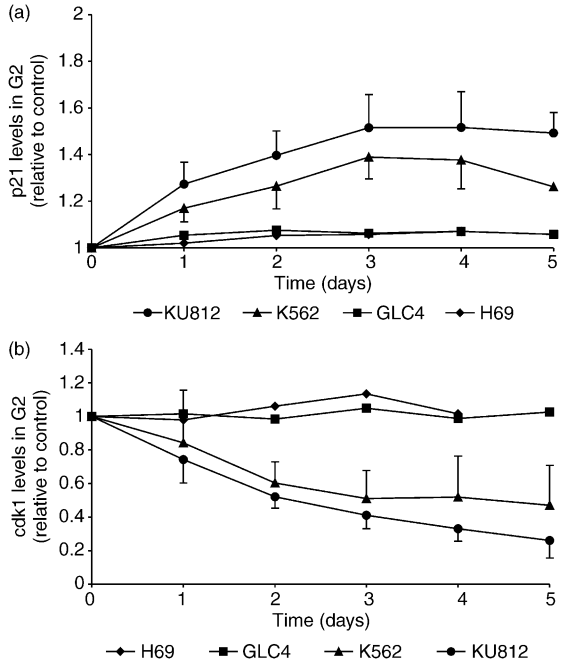

Fig. 5. (a) Flow cytometric analysis of p21 levels in four cell lines incubated continuously with 0.4 μM etoposide. Data-points represent the means of at least three separate experiments, and SDs are only shown in the CML cell lines. (b) Flow cytometric analysis of cdk1 levels in the cell lines incubated continuously with 0.4 μM etoposide. Data-points represent the means of at least three separate experiments, and SDs are only shown in the CML cell lines.

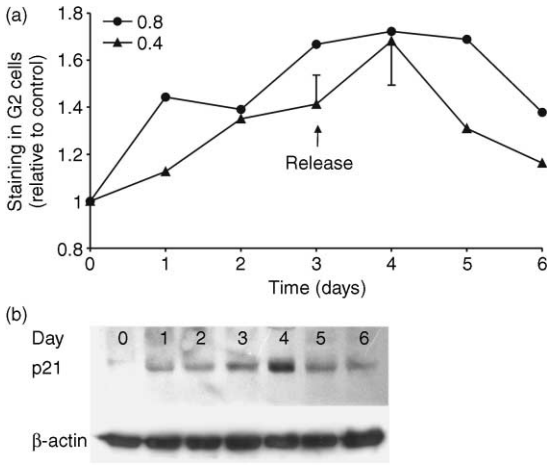

Fig. 6. (a) Flow cytometric analysis of p21 levels in CML cells treated with etoposide on the 3-day schedule. Data-points represent means of three experiments, however, SDs have only been shown for days 3 and 4, which highlight no significant difference in the levels between these two time-points ($P=0.147$). (b) p21 levels in CML cells treated with etoposide on the 3-day schedule.

by western blot analysis of whole cell extracts of K562 cells treated with etoposide on the same drug schedule (Fig. 6b).

4. Discussion

This study was undertaken to investigate the effect of drug scheduling on the cytotoxicity of etoposide in four cell lines, with distinct sensitivities and responses to the drug. To this end, we have performed flow cytometric and immunoblot analysis of cell cycle proteins involved in the control of cell cycle arrest and apoptosis in these cell lines. The most intriguing finding was the observation that although a continuous 5-day exposure of etoposide was ineffective in both of the CML cell lines studied, the release of these cells, from an initial exposure of more than 2 days, into drug-free medium resulted in an increase in apoptosis.

In the first part of this investigation, we determined the effect of a continuous and a non-continuous recovery etoposide schedule on the growth and cell cycle distribution in four cell lines. Continuous etoposide was an effective cytotoxic agent in the SCLC cells lines. Conversely, a continuous culture of the *bcr-abl* positive CML cells with etoposide caused a protracted arrest at G2/M, but no significant cell kill. Most importantly however, cell kill was only significantly increased when these cells were released into drug-free medium, following an initial continuous exposure of at least 2 days. The use of a schedule involving two recovery phases (alternation of: 2 days drug followed by 2 days drug-free for 8 days) was also shown to increase cytotoxicity in this cell type. The arrest at G2/M that was eased with resulting %A in the first drug-free period was re-induced during the second drug exposure. Subsequent re-release of cells from drug resulted in another easing of cell arrest at G2/M and concomitant increase in %A. In addition, direct comparison of this schedule with a schedule involving a 4 day exposure followed by 4 days drug-free indicated significantly higher accumulative %A in the former schedule.

Both K562 and KU812 cells are derived from patients with CML, and the characteristic expression of *bcr-abl* in CML has been widely reported to increase the resistance to cell death via apoptosis. Although studies have implicated the involvement of caspase activation events and of the proteins that regulate the cell cycle, the precise mechanism of this inhibition of apoptosis has not been elucidated [10,29,30]. In particular, a recent report from Martins and colleagues [30], suggested that *bcr-abl* kinase delayed rather than prevented the initiation of the active phase of apoptosis. However, our data showed an increase in apoptosis only when in the absence of drug. This increase could not be attributed to a delayed apoptotic response since control cultures treated continuously for the same exposure duration did not show significant increases in apoptosis. To confirm the significance of the BCR-ABL fusion product in this model, the *bcr-abl*-positive CML cell line KU812 was

studied. Like K562 cells, KU812 cells were resistant to a continuous exposure to etoposide, with cytotoxicity only significantly increased in drug schedules with a recovery phase.

We next investigated the effect etoposide may have on the proteins that regulate the cell cycle. It was important in this model to establish the functional status of p53 in these cells, because etoposide-induced DNA damage normally causes a p53-mediated activation of $p21^{waf1}$ [31,32]. The cell lines studied were either mutated or null in p53, as confirmed by immunoprecipitation (data not shown), and so activation of $p21^{waf1}$ by this upstream regulator was not possible. However, recent studies have suggested a p53-independent pathway of $p21^{waf1}$ activation in some cells, including K562 [33–35]. This was clearly seen in our data, where a significant concentration- and duration-dependent increase in the level of $p21^{waf1}$ was seen in the G2/M phase of the cell cycle in both the K562 and KU812 cells (% increase on day 5 at 0.4 μM of 80% and 92%, respectively, over control cultures; $P<0.05$). By comparison, we showed that $p21^{waf1}$ levels did not change significantly in the H69 and GLC4 cells during a 5-day continuous exposure.

As this increase in $p21^{waf1}$ was coupled with a protracted arrest in G2 without significant increases in apoptosis, the status of cdk1—a $p21^{waf1}$ target and a protagonist of S-phase regulation, was assessed in the K562 cells. Our results showed that there was indeed a reduction in cdk1 associated with the increase in $p21^{waf1}$ in this cell line.

In order to ascertain whether this prolonged cell cycle block at the G2/M boundary, mediated by cdk1 and $p21^{waf1}$, was responsible for etoposide resistance in the CML cells, K562 cells were cultured with etoposide for up to 4 days and then released into drug-free medium. Our results indicated a relationship between the $p21^{waf1}$ level and amount of apoptosis, as the removal of etoposide from the cells, caused an easing of the G2/M block which was synchronous with a reduction in $p21^{waf1}$ levels and concomitant increase in apoptosis. However, the reduction in $p21^{waf1}$ did not occur immediately after the removal of drug, and was only significant after 3 days of culturing in the absence of etoposide ($P<0.001$). This was seen most clearly in the 3-day exposure to 0.4 μM etoposide, which indicated a plateau in $p21^{waf1}$ level during recovery for 1 day before returning to control levels by day 6. This time lag in falling $p21^{waf1}$ could possibly be attributed to the interaction between etoposide and DNA topo II [36]. It is possible that upon release of drug and subsequent exposure of aberrant fragments of DNA previously hidden by drug-stabilised topo II/DNA adducts, a prolongation of the $p21^{waf1}$ response is seen.

These data provide a possible model of drug effects in CML cells, detailing the complex interaction of the nuclear proteins involved in maintaining cell cycle integrity. To recapitulate, a continuous exposure to etoposide was found to be ineffective in CML cell lines. We showed, in the presence of drug, a prolonged G2 block coupled with the inhibition of apoptosis and concomitant increase in $p21^{waf1}$ and reduction in cdk1. Most substantially, we showed that cytotoxicity was only significantly increased once the cells had been removed from etoposide. Culturing these cells in the absence of etoposide, allowed for the cycle block at G2/M to be eased, with the resultant effect of either apoptosis from G2/M or cell death from mitotic catastrophe. The possibility that the efficacy of other cytotoxic agents could be improved through a similar approach is exciting, and warrants further investigation.

Overall, these data reaffirm the importance of schedule in determining drug efficacy. In tumours with characteristics similar to K562 and KU812, a typical continuous 5-day treatment schedule would result in no apoptosis during the time of treatment, as apoptosis would only increase in the absence of drug. However, if these tumours were pulsed with etoposide on an alternating 2-days-on and 2-days-off schedule, a higher degree of apoptosis may be achieved in a similar time period to that above, with the added advantage of a reduced drug exposure. In conclusion, altering treatment regimens to involve multiple 'recovery-phases', and thus allow for increased cell kill episodes, may prove more effective than the use of a single prolonged exposure in CML cells that are typically resistant to cytotoxic drugs.

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