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Altered cell cycle response of drug-resistant lung carcinoma cells to doxorubicin

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Abstract

The effect of doxorubicin treatment on cell cycle parameters in asynchronous populations of multidrug-resistant human lung carcinoma cell lines was investigated. A sensitive (DLKP-SQ) and three resistant (DLKP-SQ A250 10p#7, DLKP-A2B and DLKP-A5F) variants of a human lung carcinoma cell line DLKP were exposed to equitoxic concentrations of doxorubicin. The latter three were 8-fold, 30-fold and 300-fold resistant to doxorubicin, respectively. Irreversible G2/M arrest in sensitive (DLKP-SQ) cells was observed 24 h after initiation of doxorubicin treatment. In resistant variants, G2/M arrest occurred at 12–16 h with a subsequent bypass of the G2/M arrest to re-emerge and accumulate in G1. This transient G2/M arrest and subsequent progression into G1 indicated an inefficient checkpoint for monitoring DNA damage induced by doxorubicin treatment. Caffeine treatment could bypass the G2/M block in DLKP-SQ cells. Doxorubicin treatment did not alter cyclin B or cdc2 protein levels, the ability of cdc2 to form complexes with cyclin B or the levels of cyclin B bound to cdc2. The G2/M arrest seen in sensitive cells was associated with an increase in inhibitory phosphorylation of Tyr15 on cdc2. In contrast, tyrosine 15 phosphorylation did not change in resistant variants after drug treatment and a general increase in cdc2 kinase activity was seen. Cdc25C levels were not altered following drug treatment.

Keywords: Cell cycle; G2/M arrest; Doxorubicin; Multiple drug resistance; Genetic instability

1. Introduction

Multiple drug resistance frequently emerges after doxorubicin treatment in tumours, and is often associated with overexpression of ATP-dependent membrane pumps such as P-glycoprotein and multidrug resistance-associated protein (MRP), which can actively remove the drug from the cell [1]. Doxorubicin has a pronounced effect on the cell cycle, probably associated with its induction of DNA damage. Doxorubicin treatment of mammalian cells *in vitro* has been shown to induce G1 and G2/M arrests [2], and these arrests are dependent on both the concentration of drug and duration of exposure.

Cell cycle checkpoints can act as surveillance mechanisms [3] which sense DNA damage, arrest the cell cycle and provide the cell with an opportunity to repair damaged DNA. Two main checkpoints exist in mammalian cells, at G1 before entry to S and at G2 before entry to mitosis [3]. G2/M arrest is enforced by a currently incompletely understood pathway involving chk1 [4]. The chk1 signalling pathway culminates in the inhibition of the mitotic kinase cdc2–cyclin B [5].

Before entry to mitosis, cdc2 and cyclin B form a complex with kinase activity, which is activated at mitosis onset by the removal of inhibitory phosphates on cdc2 by a phosphatase enzyme, cdc25 [6]. G2 arrest induced by DNA damage is associated with a decreased kinase activity [7], and the decrease in kinase activity correlates with a failure to remove the inhibitory phosphates on threonine 14 and tyrosine 15 of cdc2 [8–11]. This may be due to a decreased cdc25 phosphatase activity in G2 arrest: e.g. the activity has been shown to decrease in response to nitrogen mustard treatment [12]. In this paper, we describe the different cell cycle responses of drug-sensitive and three drug-resistant variants of the human lung carcinoma line, DLKP, following exposure to equitoxic doses of doxorubicin.

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2. Materials and methods

2.1. Cell lines

All cell lines were cultured in Hams F12: DMEM (1:1) (Gibco BRL, Paisley, UK) supplemented with 5% fetal calf serum and 1% L-glutamine, and grown at 37°C. DLKP-SQ is a clonal population of the DLKP cell line [13]. DLKP-A2B and DLKP-A5F are clonal populations which were developed by exposure to continuous increasing concentrations of doxorubicin and are 30- and 300-fold, respectively, more resistant to doxorubicin than the DLKP cell line [14]. DLKP-SQ/10p is a variant of DLKP-SQ, which was developed by exposing the cells to 10 consecutive weekly pulses (of 4-h duration) of 250 ng/ml doxorubicin [15]. DLKP-SQ A250 10p#7 is a clonal subpopulation of the resulting cell line.

2.2. Chemicals

All media used in cell line maintenance were obtained from Gibco BRL. Doxorubicin was obtained from Farmitalia Carlo Erba (Milton Keynes, UK). Anti-cyclin B1 (sc-245), anti-cdc2 (sc-54), anti-cyclin-E (sc-247), anti-cdk-4 (sc-601) and anti-cdk2 (sc-163) were purchased from Santa Cruz Biotechnology (CA, USA). Anti-cyclin-D1 was purchased from Pharmingen (CA, USA) and phospho-cdc2 (Tyr15) antibody (#9111S) from New England Biolabs (MA, USA). Anti-P-glycoprotein (C-219) was obtained from Centicor (Malvern, PA, USA) and anti-Topoisomerase IIα (6G2) was a gift from Dr G. Astaldi-Ricotti, Italy. All general chemicals were purchased from Sigma (Poole, UK).

2.3. Drug treatment

Doxorubicin was dissolved in sterile water and diluted in medium just before use. Cells were seeded at a density of 2×10^5 per 25 cm^2 flask, 2 days before drug treatment. Medium was removed and drug added for 2 h, after which time the drug was removed, the cells washed twice with sterile phosphate buffered saline (PBS) and refed with medium. At indicated times samples were taken as follows: medium was removed and retained. Cells were detached by trypsinisation and pooled with removed medium, centrifuged and washed twice with ice-cold sterile PBS.

2.4. Crystal violet staining of cells

Assessment of cell kill was by crystal violet staining of the viable/growing cells. Following exposure to various concentrations of doxorubicin, the cells were allowed to grow in drug-free medium for 72 h. The medium was then removed and the cells washed twice with PBS. The cells were fixed to the flask by incubating for 10 min in 10% (v/v) formalin. The flasks were then allowed to air-dry and the cells stained, by incubating for 10 min in 0.25% (w/v) crystal violet solution. Excess stain was removed, the flasks rinsed with water and allowed to air-dry. The stain was eluted from the cells by adding 5 ml of 33% (v/v) glacial acetic acid to each flask. The optical density of the stain was determined at a wavelength of 570 nm. The intensity of staining, relative to the untreated control flasks, was equated to cell growth and the concentration of doxorubicin which resulted in a 50% cell kill was determined.

2.5. TUNEL analysis

Drug-treated cells or untreated (control) cells were trypsinised at the desired times into a single cell suspension, pooled with their relevant growth medium, centrifuged and washed twice with sterile PBS. Cytospins were prepared from a 1×10^6 cells/ml solution and analysed for the presence of apoptotic cells by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTp nick end labeling) analysis. DNA strand breaks were identified using an *In Situ* Cell Death Detection Kit with a fluorescein tag (Boehringer Mannheim).

2.6. Time-lapse videomicroscopy

Time-lapse videomicroscopy was performed using a Nikon Diaphot inverted microscope (Micron Optical, Bray, Ireland) equipped with phase-contrast optics, linked to a Mitsubishi CCD-100 colour video camera. Images were recorded in S-VHS format using a Mitsubishi HS-500 videorecorder with time-lapse capabilities. Recording speed was set at 3.22 s/field (480 h mode) which resulted in an acceleration factor of 160 when played back at normal speed. Temperature was controlled using a Perspex incubator hood (V-Jay Plastics, Dublin) and a Nikon warm air blower.

2.7. Scoring of mitotic and apoptotic events

A random field of 100 cells was chosen for analysis. Mitosis was scored at the time of formation of septa between two daughter cells. Apoptotic events were scored when the classical apoptotic morphology first appeared when cells rounded up just before chromosome condensation, membrane blebbing and apoptotic body formation.

2.8. Flow cytometry

Samples were trypsinised into a single cell suspension, fixed in ice-cold 70% ethanol and stored at 4°C until required. Before analysis, cells were resuspended in 100 μg/ml DNase (Boehringer Mannheim), 40 μg/ml propidium iodide (Sigma) and PBS. Analysis was performed on a Becton Dickinson fluorescent activated cell sorter (FACS)can.

2.9. Western blotting

Cell pellets were resuspended in 250 μl lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF) and incubated on ice for 20 min. If at this stage cells were not lysed as determined by microscopic analysis, sonication was carried out (5 pulses of 0.5 s). Samples were spun, supernatants removed and protein concentration estimated using BioRad protein assay with bovine serum albumin (BSA) standards. 10–50 μg protein was applied to 7.5% (P-glycoprotein and topoisomerase II), 12% (cyclin) or 15% (cdk) sodium dodecyl sulphate (SDS)–polyacrylamide gels. Protein was transferred to nitrocellulose using BioRad semi-dry blotting apparatus. Equal protein loading was verified by staining membrane with Ponceau Red (Sigma) before blocking. Membranes were blocked in 5% non-fat dried milk in 0.1% Tween (T)-PBS for 2 h at room temperature. Membranes were incubated overnight at 4°C with primary antibody (1:1000) and 1 h at room temperature with horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG. Blots were developed using the ECL system (Amersham).

Several exposures of the detection film with the ECL stained blots were performed in all cases. The intensity of the signal appeared linear in all cases (by visual inspection). At no stage were membranes stripped of their primary antibody and reprobed with a different antibody.

Densitometry was not routinely used to quantify band intensity. However, in some cases, where notable unequal protein loading had occurred, it was used to confirm equal expression of protein, based on equal protein loading (using a BioRad GS670 densitometer, with Molecular Analyst software). Although all Western blots were performed a minimum of three times error bars are not available, as densitometry was not performed routinely. In all cases, after accounting for equal protein loading, no variation in protein expression was detected, unless where specifically stated otherwise.

2.10. Immunoprecipitation

One hundred micrograms protein was precleared with 1 μg anti-mouse IgG and the supernatant was incubated with anti-cyclin B for 2 h at 4°C. Ten microlitres protein A-agarose (Santa Cruz) was added and left rocking at 4°C overnight. Laemmli buffer was added, samples boiled and the proteins separated by SDS–polyacrylamide gel electrophoresis. Western blot analysis was carried out as described above, using the anti-cdc2 antibody.

2.11. Cdc2 kinase assay

Kinase assays were enzyme-linked immunosorbent assay (ELISA) based, using a biotinylated synthetic peptide which is specifically phosphorylated by cdc2 kinase, and a monoclonal antibody which recognises the phosphorylated form of the peptide. Assays were carried out using a MESACUPcdc2 kinase assay kit (MBL, distributed by Stratech, UK) according to the manufacturer's instructions. Briefly, cell pellets were resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 0.5 M NaCl, 5 mM EDTA, 0.01% Brij35, 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM beta-glycerophosphate, 1 mM Na-orthovanadate), sonicated and spun at 10 000 *g* for 1 h at 4°C. Supernatant was immunoprecipitated with HCK-gel (supplied with assay) and used as an enzyme source for the kinase assay. Kinase reaction was carried out at 30°C for 30 min. Reaction mixture was then added to microwell strips which were coated with monoclonal antibody (Mab) specific for the phosphorylated form of the biotinylated peptide and levels of phosphorylated peptide were detected using the HRP (horse-radish peroxidase) detection system supplied.

3. Results

3.1. Effect of doxorubicin on cell cycle progression

Cells were treated with equitoxic concentrations of doxorubicin (Table 1) and the cell cycle profiles were analysed using flow cytometry. The toxicity of doxorubicin to the cells was assessed using crystal violet staining 72 h after the 2-h drug exposure, which gave a measure of the cell numbers. TUNEL staining of the doxorubicin-treated cells revealed equal staining, when the cells were analysed 24, 48 and 72 h after the drug treatment (Fig. 1 depicts TUNEL stained cells, 48 h post-treatment), indicating approximately equal levels of apoptosis and DNA breakage in the four cell populations. Cell cycle profile analysis (Fig. 2) revealed that DLKP-SQ cells had an increased number of cells in S phase 12 h after treatment, and by 24 h a G2/M arrest was apparent (Fig. 2a). A few apoptotic cells were present as a sub-G1 peak at 68 h. In DLKP-SQ A250 10p#7 cells (Fig. 2b), doxorubicin treatment caused an accumulation of cells in G2/M at 16 h, after which the cell numbers increased in the G1 phase with a simultaneous decrease in G2/M population. DLKP-A2B also exhibited a temporary G2/M arrest at 24 h with a subsequent re-emergence in G1 but the effect was less prominent than for the DLKP-SQ A250 10p#7 cells (Fig. 2c). DLKP-A5F exhibited a temporary G2/M arrest by 16 h and accumulated in G1 from this time, with a corresponding decrease in G2/M. For each cell line, the cell cycle profile was also determined at each time point in the absence of drug treatment. In each case, the profile was consistently similar to time 0 and no variations were observed (data not shown).

Table 1
The concentrations of doxorubicin which resulted in 50% kill (IC_{50}) following a 2-h exposure to cells in 25 cm^2 flasks[a]

Cell line	Concentration IC_{50}
DLKP-SQ	0.6 μg/ml
DLKP-SQA250 10p#7	2 μg/ml
DLKP-A2B	30 μg/ml
DLKP-A5F	100 μg/ml

[a] These values are the calculated mean from three independent experiments and are the concentrations of doxorubicin used in all assays.

3.2. Time-lapse videomicroscopic analysis

Time-lapse videomicroscopy analysis was used to study apoptotic and mitotic events in the populations after equitoxic doxorubicin treatment (Fig. 3). This technique allows visualisation of morphological events in a randomly selected field of drug-treated cells. Fig. 3 shows the percentage of mitosis occurring in each population over a 72-h period, following doxorubicin treatment. No mitotic events were observed in DLKP-SQ. In the resistant variants, however, mitosis occurs (13% mitosis in A250 10p#7 (Fig. 3b), 15% mitosis in DLKP-A2B (Fig. 3c) and 58% mitosis in DLKP-A5F (Fig. 3d)). Apoptosis is also occurring in these cell lines and it was noted that the daughter cells frequently died by apoptosis shortly after mitosis occurred.

3.3. Combination of doxorubicin and caffeine treatment

Caffeine was used in combination with doxorubicin on DLKP-SQ cells in an attempt to recreate the effect seen in resistant variants. Cells were initially exposed to doxorubicin using doses that induce 50% cell kill and treated with 4 mM caffeine either immediately upon removal of the drug or 18 h later (Fig. 4). When the cells were treated with caffeine immediately after removal of the doxorubicin, no cell cycle arrest was observed, with cells subsequently entering G1 phase and apoptosis occurring, as seen by the presence of the sub-G1 peak at $T=36$ h. When treated with caffeine 18 h post-doxorubicin treatment, the cells had begun to arrest in the G2/M phase prior to caffeine exposure, by 24 h cells were still arrested but by 36 h cells had emerged in G1 phase and apoptosis occurred. These results suggest that the caffeine-induced cellular alterations in DLKP-SQ cells are similar to those manifested following doxorubicin treatment in the resistant variants (for example, Fig. 2d).

3.4. Effects of doxorubicin on cyclin B protein levels

Cyclin B protein levels were investigated by Western blot at intervals up to 45 h after doxorubicin exposure (Fig. 5(i)), at which time the sensitive cells experience a G2/M arrest and resistant variants have overcome the G2/M arrest and emerged in G1. Cyclin B protein levels do not change up to 45 h after doxorubicin treatment in any of the cell lines and protein levels remain constant compared with controls (Fig. 5(i)). The G2/M arrest is most prominent from 24 h after drug treatment in DLKP-SQ, as is the G2/M override in the resistant variants, therefore any possible influence on cyclin B protein expression should be apparent at the timepoints that were chosen for the immunoblotting analysis.

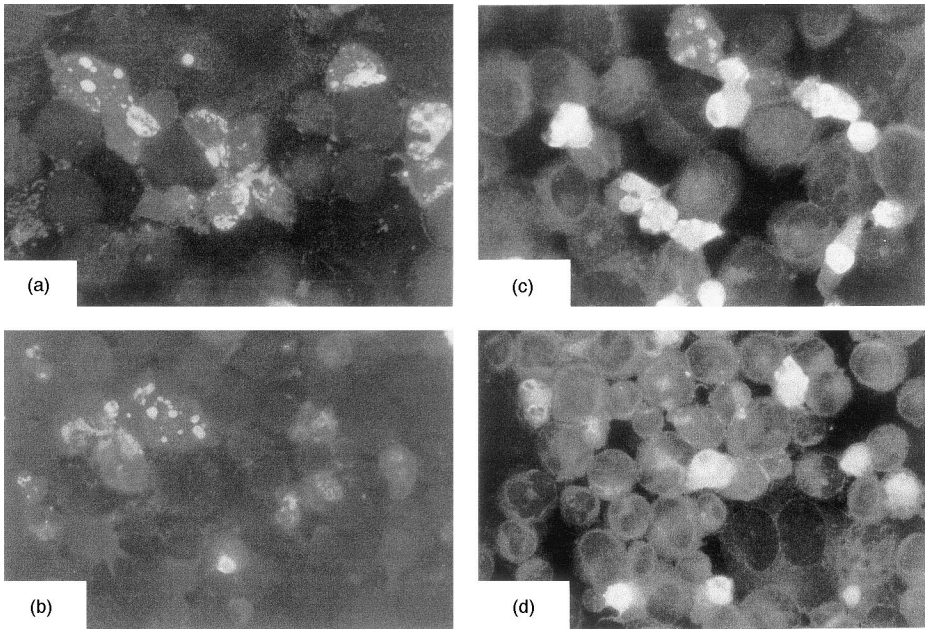

Fig. 1. TUNEL analysis of (a) DLKP-SQ; (b) DLKP-SQ A250 10p#7; (c) DLKP-A2B; and (d) DLKP-A5F, 48 h after doxorubicin treatment. The bright staining is reflective of DNA breaks, as the cells undergo apoptosis.

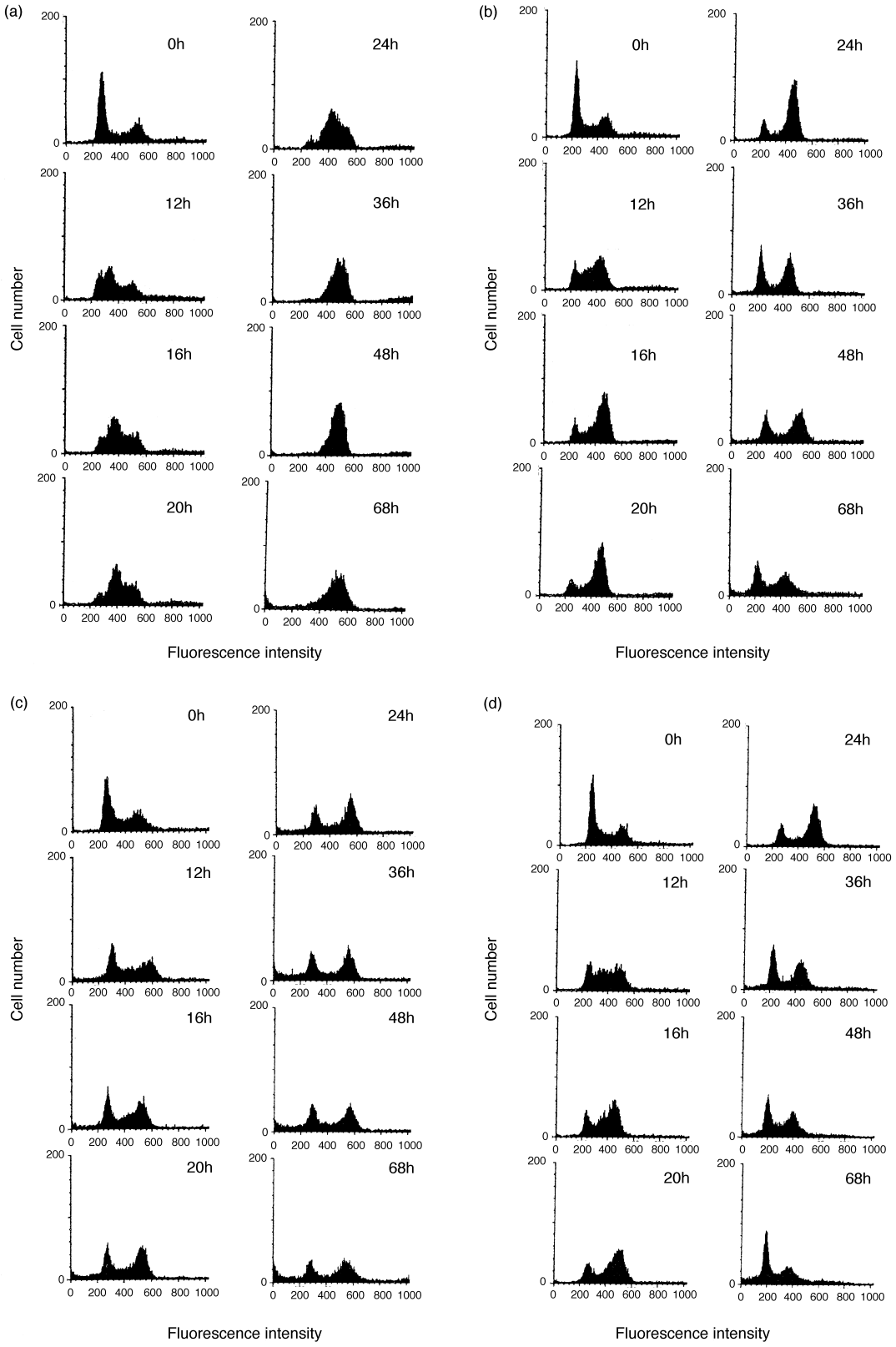

Fig. 2. Effect of the IC_{50} concentration of doxorubicin on the cell cycle profile of (a) DLKP-SQ; (b) DLKP-SQ A250 10p#7; (c) DLKP-A2B; and (d) DLKP-A5F. Histograms represent population distributions of cells at the indicated times 0, 12, 16, 20, 24, 36, 48 and 68 h following drug treatment. 10 000 cells were counted for each sample. The experiment was carried out in triplicate.

3.5. Effect of doxorubicin on cdc2 protein levels

Levels of cdc2 protein were examined after doxorubicin treatment and in untreated controls in sensitive (Fig. 5(ii)a) and resistant variants (Fig. 5(ii) b, c and d) to investigate if the G2/M override in resistant variants was associated with altered cdc2 expression. Results indicate no alterations in the cdc2 protein level.

3.6. Cdc2 complex levels

Cyclin B–cdc2 complex levels were then studied to test their involvement in the G2/M bypass in resistant DLKP cells. Complex levels were analysed by immunoprecipitation and results are shown in Fig. 6. Doxorubicin treatment does not affect the levels of cyclin B bound to cdc2 in either sensitive or resistant lines, thus the complex-forming ability of cdc2 apparently remains intact following doxorubicin treatment. To demonstrate specificity, a negative control was carried out where the primary antibody was omitted and the immunoprecipitation performed with mouse IgG (data not shown).

3.7. Phosphorylation status of Tyr15 on cdc2

Investigation of inhibitory phosphorylation on Tyr15 of cdc2 was carried out using a Tyr15-specific anti-cdc2 antibody (Fig. 7(a) and (b)). Cells were monitored for up to 48 h (the time at which flow cytometry analysis shows the greatest drug effects). Levels of Tyr15 phosphorylation in sensitive DLKP-SQ cells were higher in doxorubicin-treated cells compared with controls, with an increase in phosphorylation seen at 24 h, coinciding with the timing of the G2/M arrest seen in flow cytometry (Fig. 2). In fact, there appears to be a slight decrease in phosphorylation in the untreated DLKP-SQ cells (Fig. 7a(i) and 7(b)); perhaps this reflects their increasing growth rate in culture, with more cells entering mitosis.

In contrast, levels of cdc2 Tyr15 phosphorylation in resistant cells remain constant over 48 h following drug treatment, similar to the result seen in drug-resistant untreated controls. Time-points were studied where G2/M arrest was detectable in the resistant cell lines, and at later time-points, where G2/M bypass had occurred. An increase in tyrosine 15 phosphorylation on cdc2 was detectable in the G2/M arrested DLKP-SQ cells, whereas no alterations in phosphorylation levels were detectable in the other three cell lines, even at time-points where temporary G2/M arrest had occurred (as an example, DLKP-SQ A250 10p#7 24 h post treatment; compare Fig. 2b, 24 h and Fig. 7a(ii), lane 5). This suggests that the phosphorylation state of Tyr15 on cdc2 has key involvement in the G2/M override in

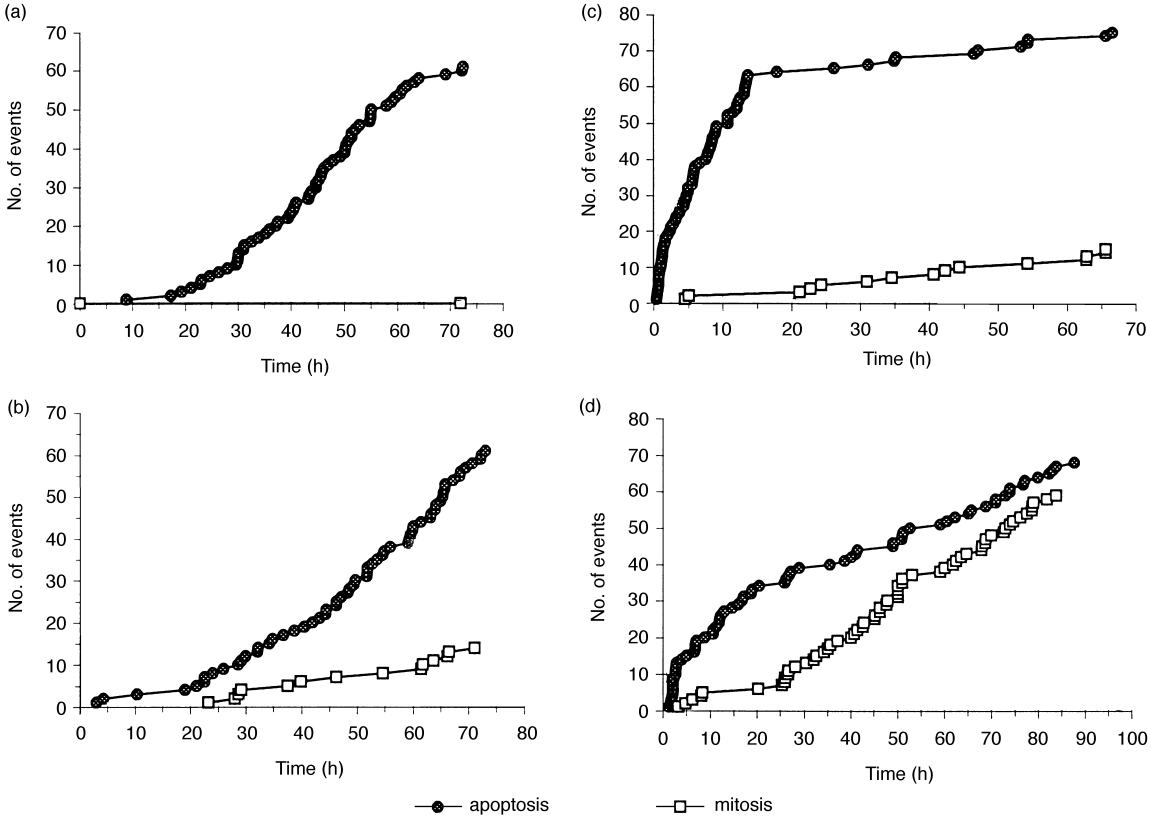

Fig. 3. Time-lapse videomicroscopy analysis of (a) DLKP-SQ; (b) DLKP-SQ A250 10p#7; (c) DLKP-A2B; and (d) DLKP-A5F following a 2-h pulse with equitoxic doses of doxorubicin.

the resistant variants. The relatively high constant level of Tyr15 phosphorylation seen in control cells was as expected for an asynchronous population of exponentially growing cells.

3.8. Cdc2 kinase activity

Cdc2 kinase activity is a critical factor in entry to mitosis, and a decrease in activity coincides with a G2/M arrest [8,16]. DLKP-SQ cells arrested in G2/M experienced a slight decrease in kinase activity compared with controls, especially notable at 24 and 48 h post drug treatment (Fig. 8). Similar results were obtained in several repeat experiments, in agreement with results reporting decreases in cdc2 kinase activity associated with the G2/M arrest induced by chemotherapeutic drugs such as doxorubicin [10], etoposide [7,8] and camptothecin [9]. In general, doxorubicin-treated resistant cells, however, possessed a slightly elevated kinase activity compared with the controls (Fig. 8), suggesting an increased activation of cdc2 kinase, which can push the cells past the checkpoint. The increase in cdc2 kinase activity in drug-resistant cells represents an important cellular response to doxorubicin treatment. A comparison of kinase activity in untreated resistant variants and the sensitive DLKP-SQ cells showed that the levels of inherent cdc2 kinase activity did not account for the resistance of the cells to doxorubicin or their ability to overcome the temporary G2/M arrest. The main cell cycle-associated alterations in resistant variants appear to occur only after the cells are exposed to doxorubicin, so the resistant variants

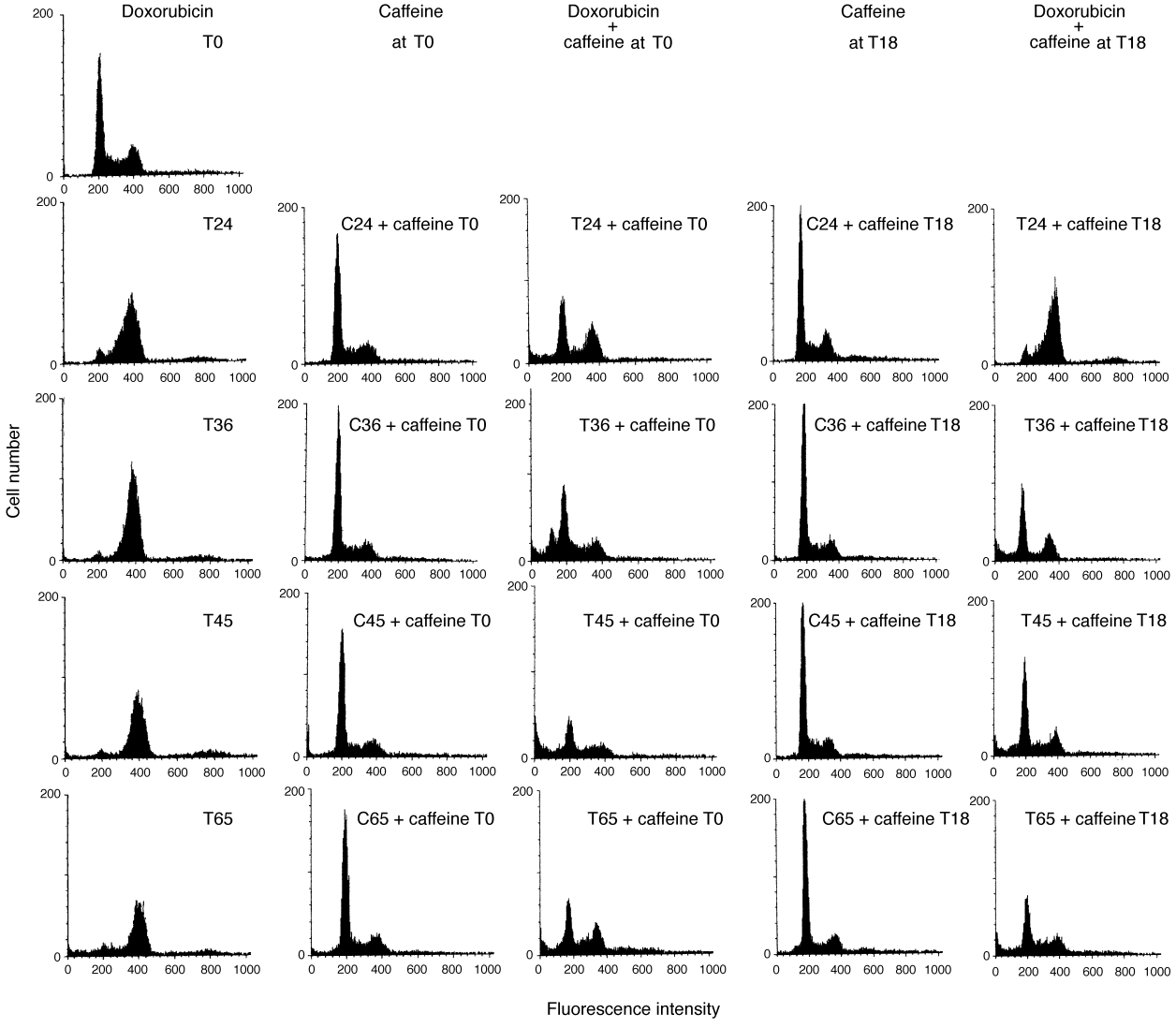

Fig. 4. Caffeine abrogates doxorubicin-induced G2/M arrest in DLKP-SQ. Cells treated with doxorubicin for 2 h undergo a G2/M arrest (doxorubicin). When treated with 4 mM caffeine, either at the time of doxorubicin removal (doxorubicin + caffeine at T0) or 18 h following drug removal, when cells had already arrested in G2/M (doxorubicin + caffeine at T18), the cells bypassed the G2/M arrest. Controls were carried out using 4 mM caffeine in the absence of doxorubicin treatment (caffeine at T0, caffeine at T18). Samples were taken at the indicated times (0, 24, 36, 45 and 65 h post-doxorubicin treatment). The experiment was carried out in triplicate.

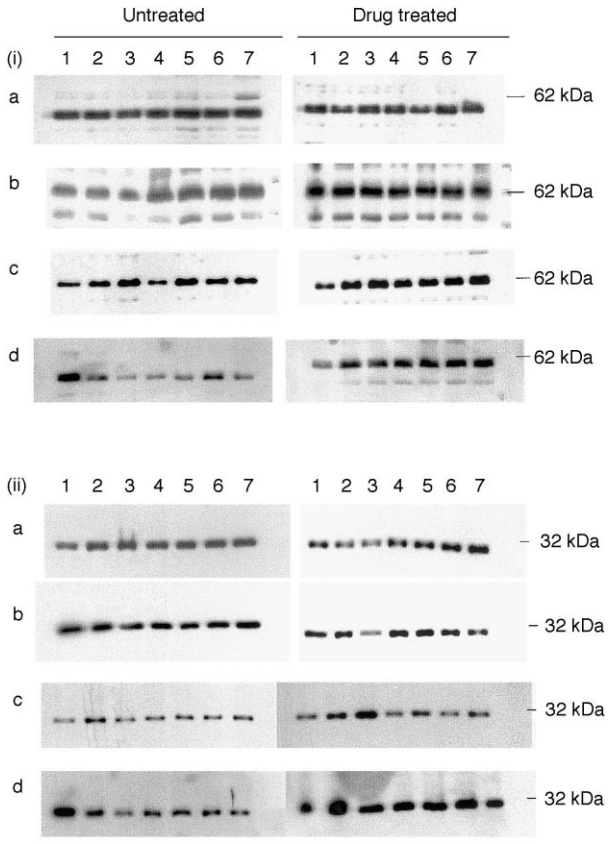

Fig. 5. Doxorubicin treatment does not alter (i) cyclin B; or (ii) cdc2 protein expression in (a) DLKP-SQ; (b) DLKP-SQ A250 10p#7; (c) DLKP-A2B; and (d) DLKP-A5F. Drug-treated samples were compared with untreated controls. Extracts were prepared at 0 h after drug removal (lane 1); 12 h (lane 2); 16 h (lane 3); 20 h (lane 4); 24 h (lane 5); 36 h (lane 6); and 45 h (lane 7). Ten micrograms of protein were loaded per lane.

have the ability to evolve an adaptive response in the form of a G2/M override, which allows the resistant cells to continue to divide.

3.9. Cdc25B and cdc25C expression in DLKP cell lines

Cdc25B and cdc25C expression in the sensitive and doxorubicin-resistant cell lines was investigated using Western blotting and the findings are presented in Fig. 9. The four DLKP cell lines expressed equal levels of cdc25B and cdc25C proteins and their level of expression was unaltered by doxorubicin treatment.

3.10. P-glycoprotein and topoisomerase II expression

Western blotting analysis was carried out to examine P-glycoprotein and topoisomerase II levels in the four DLKP cell lines. Altered expression of both proteins have been associated with the acquisition of drug resistance by cells and downregulation of topoisomerase II has in turn been shown to influence the progression of cells through the cell cycle [1,17]. It can be seen from Fig. 10 that the resistant cells, DLKP-SQ A250 10p#7, DLKP-A2B and DLKP-A5F, expressed higher levels of P-glycoprotein, with respect to the drug-sensitive DLKP-SQ cells, but the resistance level of the cells cannot be fully attributed to the level of expression of the drug efflux pump. Neither can the resistance profile of the cells, nor the altered cell cycle progression noted in the resistant versus sensitive cells, be attributed to altered topoisomerase II. When equal protein loading was accounted for, by Ponceau staining of the membrane (since the DLKP-A2B lane had significantly higher protein loading), DLKP-SQ, DLKP-A2B and DLKP-A5F all expressed approximately equal levels of topoisomerase II, with the DLKP-SQ A250 10p#7 cells expressing slightly but not significantly less. Therefore, altered cell cycle progression cannot be accounted for by lower topoisomerase II levels in the resistant cell lines.

4. Discussion

Cells that are resistant to chemotherapeutic drugs, such as doxorubicin, can proliferate in the presence of high drug concentrations and defects in cell cycle signalling checkpoints could potentially become contributing factors in resistance development.

Barlogie and colleagues [2] demonstrated that G2 arrest was dependent on doxorubicin concentration and drug exposure time, and with higher concentrations and longer exposure times there was a G1 arrest, a delay in S phase transit and an irreversible G2/M arrest. The cell cycle arrest in DLKP-SQ cells reflects this "classic" profile of irreversible G2/M arrest (with G1 arrest seen at only very high doxorubicin concentrations, data not shown). This is in contrast to the resistant variants, which exhibit a weak and transient G2/M arrest and reaccumulation in G1.

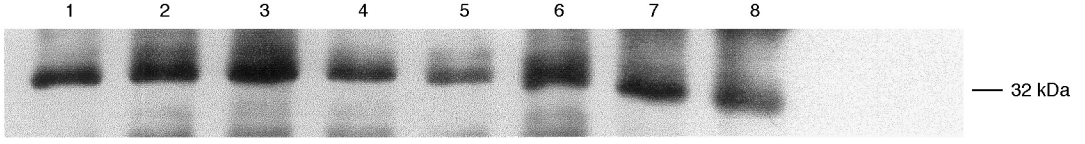

Fig. 6. Doxorubicin treatment does not alter complex-forming ability of cyclin B–cdc2 in DLKP cell lines. Lanes 1, 3, 5 and 7 represent untreated extracts from DLKP-SQ, DLKP-SQ A250 10p#7, DLKP-A2B and DLKP-A5F, respectively. Lanes 2, 4, 6 and 8 represent DLKP-SQ, DLKP-SQ A250 10p#7, DLKP-A2B and DLKP-A5F, respectively, extracted 48 h after doxorubicin treatment. The experiment was carried out in triplicate.

As the resistant cells overexpress the drug efflux pump, P-glycoprotein, they possess the ability to survive exposure to higher concentrations of doxorubicin, by their ability to pump the toxin out of the cells. In addition to this, equitoxic drug doses were chosen for the four DLKP cell lines, as opposed to equicellular concentrations, which would be difficult to estimate accurately due to the sequestration of doxorubicin in intracellular vesicles by DLKP-A5F [18]. However, the altered cell cycle profile observed in the resistant variants is unlikely to be simply a consequence of the higher drug concentration used. There is only a 3.3-fold higher concentration of doxorubicin used with DLKP-SQ A250 10p#7 compared with DLKP-SQ, yet a very high level of apoptosis was seen in the DLKP-SQ, with absolutely no mitotic events occurring. The concentration of doxorubicin used throughout the experimental work (Table 1), resulted in equal inhibition of cell growth and/or kill in the four cell lines, when cell growth was measured three days after doxorubicin treatment. The crystal violet staining of the cells remaining at this time would account for any cytotoxic and cytostatic effects, which may have occurred as a result of the doxorubicin treatment. TUNEL analysis, a specific measure of apoptosis, revealed equal levels of cytotoxicity in the four cell lines. Therefore, the dissimilarity in the doxorubicin-mediated cell cycle profiles of the four cell lines are not linked to possible differences in the ratio of cytotoxic to cytostatic events occurring in the cell lines.

Topoisomerase IIα levels in the four cell lines are relatively unaltered, indicating that doxorubicin resistance in the cells is not conferred by downregulation of topoisomerase II. Topoisomerase II levels have been shown to influence progression of cells into mitosis [17], however, cellular levels of topoisomerase II cannot account in our study for the altered cell cycle progression, in response to doxorubicin.

DLKP-SQ cells arrest in G2/M after doxorubicin treatment indicating an intact G2/M checkpoint in these

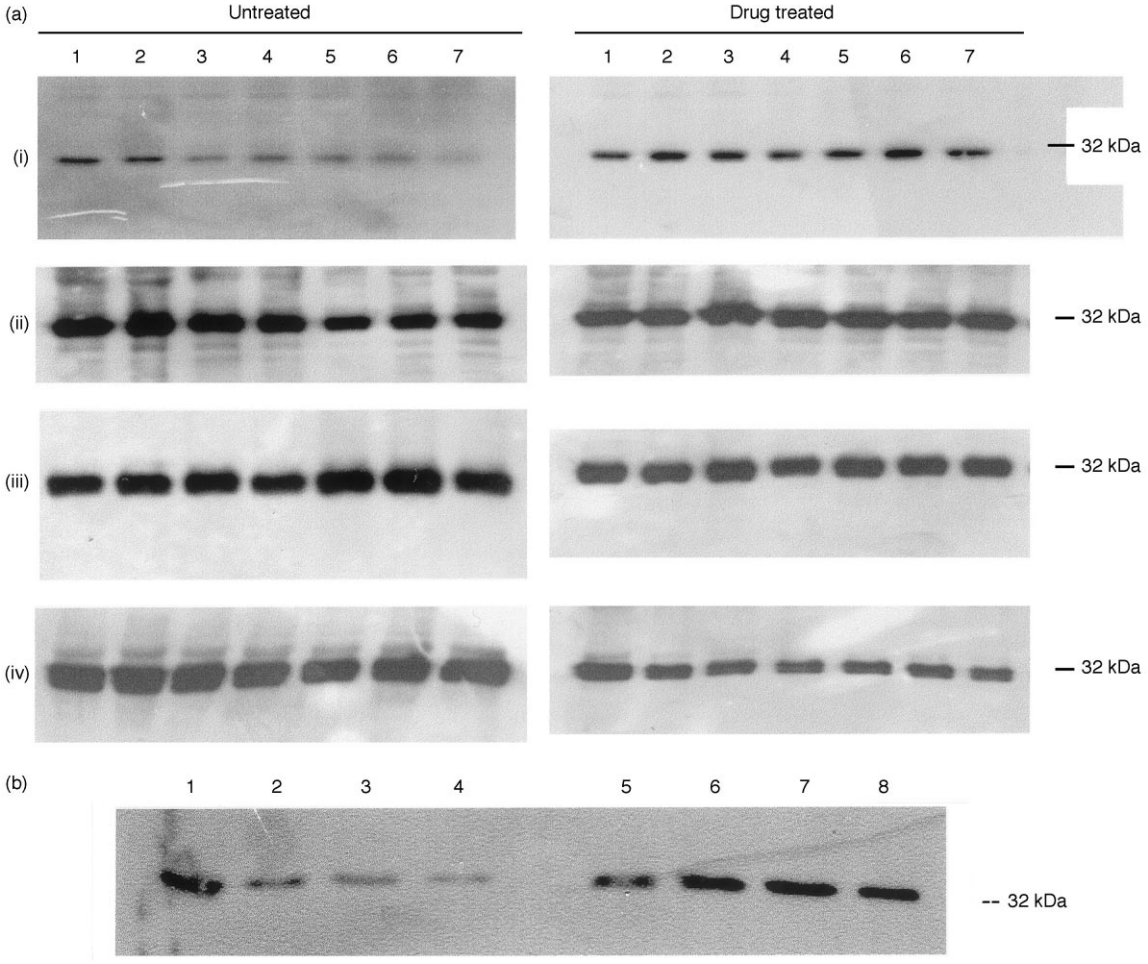

Fig. 7. (a) Levels of Tyr15 phosphorylation on cdc2 in: (i) DLKP-SQ; (ii) DLKP-SQ A250 10p#7; (iii) DLKP-A2B; and (iv) DLKP-A5F in the absence of and following doxorubicin treatment. Extracts were prepared at 0 h after drug removal (lane 1); 12 h (lane 2); 16 h (lane 3); 20 h (lane 4); 24 h (lane 5); 36 h (lane 6); and 48 h (lane 7). Ten micrograms of protein were loaded on each lane. (b) Tyr15 phosphorylation on cdc2 in untreated and doxorubicin-treated DLKP-SQ. Lanes 1, 2, 3 and 4 refer to extracts from untreated DLKP-SQ prepared at 0, 12, 24 and 36 h, respectively. Lanes 5, 6, 7 and 8 represent extracts prepared from doxorubicin-treated cells at 12, 24, 36 and 48 h after drug treatment.

cells. Tumour cells normally arrest in G2 to provide themselves an opportunity to repair DNA damage before mitosis occurs [3]. The results presented here suggest the presence of a defective G2/M checkpoint in resistant cells which allows them to bypass this control and undergo mitosis. The resistant cells do, however, exhibit a transient arrest in G2/M. The G1 peak emerges after 24 h for DLKP-SQ A250 10p#7 and sooner for DLKP-A2B and DLKP-A5F, but a subpopulation of cells still remain arrested in G2/M. It is notable that caffeine treatment bypasses the G2/M block in DLKP-SQ cells; this is consistent with the results of Musk and Steel [19] who showed abrogation, by caffeine treatment, of the G2/M checkpoint, in cells that have experienced DNA damage. Caffeine has been shown to allow cells to bypass a drug-induced G2/M arrest, when present either simultaneously with, or immediately following, drug treatment [20] by the formation of complexes between

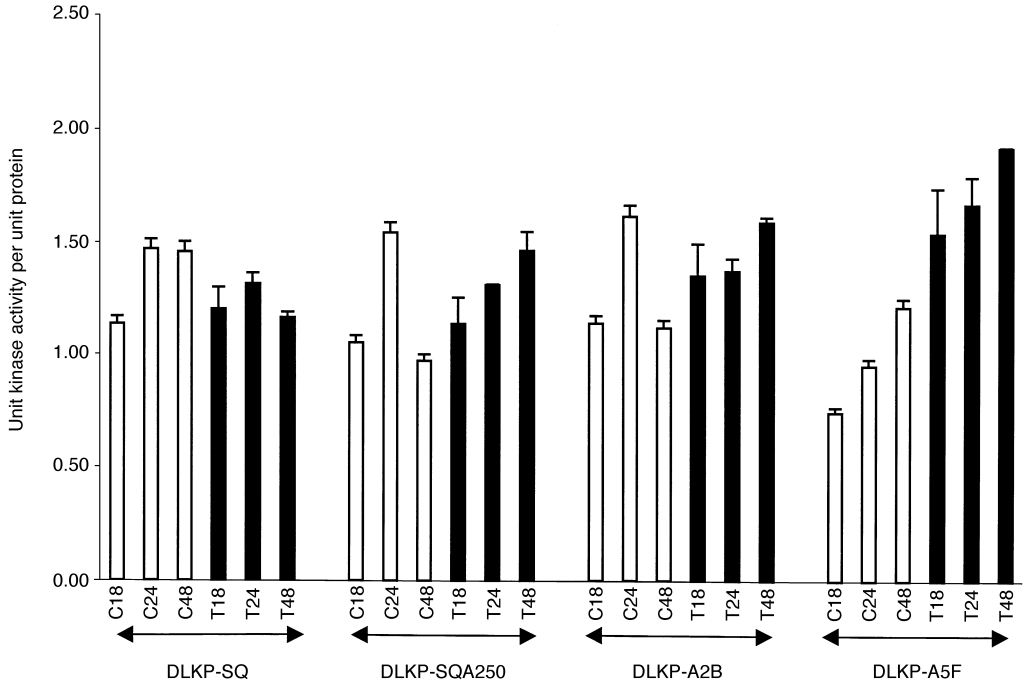

Fig. 8. Cdc2 kinase activity in DLKP cells. Kinase assays were carried out using 100 μg of protein extracted at 18, 24 and 48 h following doxorubicin treatment (■; T) on the four cell lines. Control extracts (□; C) were also prepared at the same times from untreated cells. The graph depicts a representative group of data, with the level of kinase activity (per unit protein) shown on the *y*-axis and cell lines arranged on the *x*-axis.

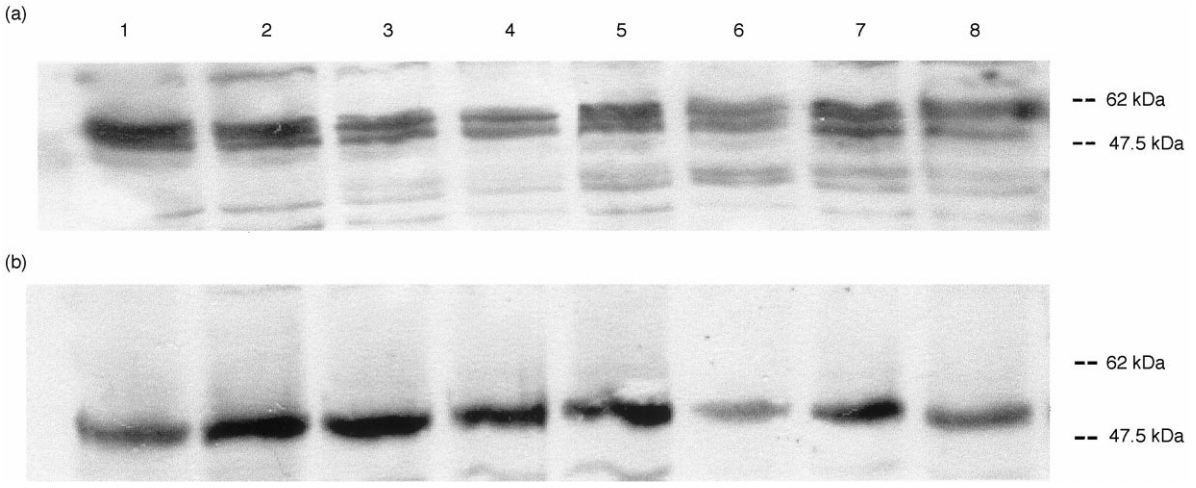

Fig. 9. Cdc25 protein expression is not altered following doxorubicin treatment. Western blots show the effects of doxorubicin on: (a) cdc25B; and (b) cdc25C protein expression in DLKP cell lines. Lanes 1, 3, 5 and 7 represent untreated extracts from DLKP-SQ, DLKP-SQ A250 10p#7, DLKP-A2B and DLKP-A5F, respectively. Lanes 2, 4, 6 and 8 represent cells 48 h after doxorubicin treatment. Twenty micrograms of protein were loaded per lane, and equal protein loading was monitored by Ponceau staining of the membranes. There was slightly less protein loaded in lane 6 in (b) accounting for the weaker band.

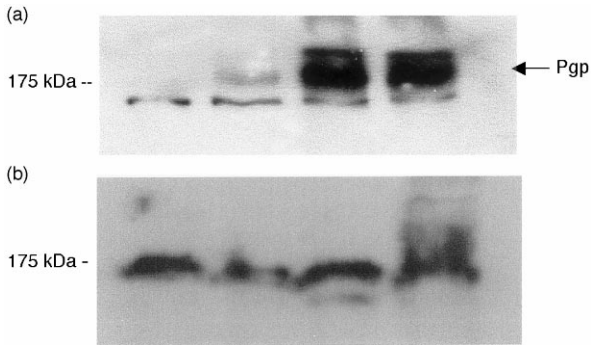

Fig. 10. Western blot analysis of: (i) P-glycoprotein; and (ii) topoisomerase IIα expression in DLKP cell lines. Lanes 1, 2, 3 and 4 represent DLKP-SQ, DLKP-SQ A250 10p#7, DLKP-A5F and DLKP-A2B, respectively.

caffeine and the aromatic ring structures of the chemotherapeutic drugs [21]. However, treatment of DLKP-SQ cells with caffeine permits them to overcome the doxorubicin-induced cell cycle arrest, whether added immediately after the doxorubicin treatment, or 18 h later, when the cells have already been committed to undergo arrest. The ability of caffeine to overcome the G2/M arrest in DLKP-SQ, when the cells are treated immediately upon removal of the doxorubicin, may be partially explained by formation of caffeine–drug complexes and therefore the decreased availability of doxorubicin to intercalate with the cell's DNA. However, in the DLKP-SQ cells, even caffeine treatment 18 h post-doxorubicin exposure, when the cells are beginning to arrest, allows the cells to subsequently overcome the cell cycle arrest. This effect is independent of caffeine–drug complex formation, as high performance liquid chromatography (HPLC) analysis shows very low levels of doxorubicin present in drug-sensitive variants of DLKP, 5 h after removal from drug (data not shown). This residual doxorubicin may be DNA-intercalated drug.

Cyclin B protein levels remained unchanged after doxorubicin treatment, compared with controls. This is consistent with a report by Germain and associates [22] who found cyclin B protein stabilisation after DNA damage. Cells arrested in G2/M by doxorubicin [10] and camptothecin [9,23] do not experience altered cyclin B levels. Cdc2 protein expression was not altered in any of the DLKP cell lines following doxorubicin treatment. Ling and colleagues [10] investigated doxorubicin-induced G2/M arrest in P388 cells and concluded that doxorubicin did not affect cyclin B or cdc2 protein expression. Levels of cyclin B–cdc2 complex after drug treatment were not altered, indicating that alterations in cell cycle response in the resistant variants are not due to deregulated cyclin B–cdc2 complex formation.

Following doxorubicin treatment, DLKP-SQ experienced a slight decrease in cdc2 kinase activity after 24 h and increased tyrosine 15 phosphorylation on cdc2. The tyrosine 15 phosphorylation peaks by 24 h, corresponding with the G2/M arrest seen at 24 h by flow cytometry. These findings are consistent with numerous reports that demonstrate G2/M arrest due to increased inhibitory phosphorylation of cdc2 and a decreased kinase activity [8–10,24]. DLKP-resistant variants, in contrast, had a somewhat higher kinase activity after doxorubicin treatment, with the possible exception of DLKP-SQ A250 10p#7. This higher kinase activity may allow the cells to bypass the G2/M checkpoint. Furthermore, tyrosine 15 phosphorylation levels did not change following drug treatment in the resistant variants, even at time-points where temporary G2/M arrest had occurred. These findings suggest the presence of a mechanism in resistant cells, possibly mediated through cdc2 kinase, allowing them to overcome the G2/M arrest induced by the DNA damage checkpoint.

Cdc25 and wee1 are two key controllers of Tyr15 phosphorylation levels, and consequently cdc2 kinase activity and entry to mitosis. Cdc25B is involved in promoting entry to mitosis [25]. Cdc25C is responsible for removal of the inhibitory phosphates on cdc2 and promotes entry to mitosis [26]. G2 arrest has been shown to be accompanied by a decrease in cdc25C phosphatase activity [6,12]. No alterations in levels of cdc25B or cdc25C proteins were observed in our experiments in sensitive versus resistant cells, or in untreated versus doxorubicin-treated cells. There may be alterations in cdc25C phosphorylation levels which were not detected in our asynchronous cultures, or there may be an alternative mechanism present in resistant variants that allows them to override the doxorubicin-induced arrest seen. For example, entry to mitosis can be regulated by subcellular localisation of cyclin B due to the presence of a cytoplasmic retention sequence (CRS) which contains a nuclear export signal (NES) [27,28]. Alterations in the NES of cyclin B result in abrogation of G2 arrest [28]. Wee1 is also closely involved in the control of cdc2 kinase activity [29].

The G2 checkpoint is particularly important due to the segregation of the genome in mitosis. Progression past M phase in the presence of incompletely repaired DNA would result in the acquisition of damaged genomic material in the progenitor cells. Cells without an intact checkpoint will continue to divide with their DNA damaged or incorrectly repaired and therefore possibly containing chromosomal deletions, rearrangements and multiplications, perhaps further advancing their genomic instability. Kaufmann [30] investigated the possibility that attenuation of the G2 checkpoint may contribute to the genomic instability of immortal human cell lines and concluded that G2 checkpoint attenuation preceded immortalisation and plays a role in immortalisation of human fibroblasts. Defective G2/M checkpoints occur in many familial cancer syndromes such as Li-Fraumeni syndrome and Ataxia Telangiectasia [31], perhaps resulting in a more unstable genome.

In conclusion, the results presented here demonstrate an altered cell cycle response to chemotherapeutic drug exposure in drug-resistant human lung carcinoma cells. The role of these changes in drug resistance and the possible relevance to drug resistance in cancer patients requires further elucidation.

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