

5-Bromo-2-deoxyuridine activates DNA damage signalling responses and induces a senescence-like phenotype in p16-null lung cancer cells

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5-Bromo-2-deoxyuridine (BrdU) is a thymidine analogue that is incorporated into replicating DNA. Although originally designed as a chemotherapeutic agent, sublethal concentrations of BrdU have long been known to alter the growth and phenotype of a wide range of cell types. Mechanisms underlying these BrdU-mediated effects remain unknown, however. We have characterized the effects of BrdU on A549 lung cancer cells by examining DNA damage responses, cell cycle effects and phenotypic changes. A549 cells express wild-type p53, but are p16-null. Sublethal concentrations of BrdU evoke a DNA damage response in these cells that involves the activation of Chk1, Chk2 and p53. Increased numbers of enlarged nuclei and multinucleated cells are evident in the treated populations. Cell cycle inhibition occurs, resulting in reduced proliferation and accumulation of cells in the S, G₂/M and G₀ phases. BrdU induces an early inhibition of p21 expression that coincides with nuclear localization of proliferating cell nuclear antigen. Subsequently, p21 levels increase, whereas proliferating cell nuclear antigen levels decrease compared with control cells. Upregulation of p27

and p57 expression also occurs. By day 7 of exposure to BrdU, treated cells acquire a senescent-like phenotype with an increase in cell size, granularity and β -galactosidase activity. We conclude that BrdU induces a DNA damage response in A549 cells, which results in reduced proliferation mitotic exit and phenotypic changes that resemble senescence. *Anti-Cancer Drugs* 18:1053–1068 © 2007 Lippincott Williams & Wilkins.

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Introduction

Although currently widely used to measure DNA synthesis in proliferating cells, the halogenated pyrimidine, 5-bromo-2-deoxyuridine (BrdU), was originally rationally designed in the 1950s as a thymidine analogue to perturb DNA synthesis in cancer cells. BrdU was subsequently found to be a potent radiosensitizer and photosensitizer [1–3], and trials using combined chemotherapies that include BrdU as a radiosensitizer continue today [4]. BrdU incorporation into DNA results in several types of DNA lesions, including mutations [5], fragile sites [6], chromatid breaks [7] and sister chromatid exchanges [8]. In addition, BrdU induces micronucleation [9], hypermethylation of DNA [10] and polyploidization [11]. Remarkably, investigations into the DNA damage responses elicited by BrdU incorporation into cellular DNA are limited. The mismatch-repair system is activated in BrdU-containing cells [12,13]. Other than this, little is known regarding the damage pathways that are activated by BrdU, and their consequences for cell cycle and cell fate.

In addition to its DNA-damaging effects, early studies with BrdU revealed significant effects on cell morphology

and phenotype in various cell types [14]. Subsequently, BrdU became known as a differentiating agent owing to its effects on the morphology and phenotype of a wide range of cell types *in vitro* and *in vivo*, including normal chondrocytes, muscle, molar, brain, pancreatic and blood cells [15–20], and leukaemic and lung cancer cells [21–24]. More recently, the Ayusawa group [25,26] has reported that BrdU induces a senescent-like phenotype in both normal and transformed cells. Senescence, particularly in transformed cells, remains relatively poorly defined; it is now unclear whether the phenotypic changes observed in BrdU-treated cells in earlier studies were, in fact, senescence-related events rather than true differentiation effects. The Ayusawa group in their reports did not address this question. The ability of a chemotherapeutic agent, such as BrdU, to induce differentiation or senescence is potentially a desirable side effect that can enhance its antitumour activity. Moreover, BrdU might have clinical potential as a differentiation therapeutic, distinct from its chemotherapeutic role. Conversely, it remains to be proved whether the induction of senescence in tumours is, in fact, advantageous. Evidence exists that senescent cells can alter the microenvironment surrounding preneoplastic

cells and may encourage tumour growth [27]. The cellular effects and the mechanisms of action of BrdU, therefore, require clarification in this context.

Suggested mechanisms underlying the effects of BrdU on gene expression and cell phenotype include DNA mutations, alterations in DNA methylation, altered interactions with DNA-binding proteins and reversal of the remodelling of regulatory DNA into specific chromatin architecture that occurs during cancer. BrdU-substituted DNA has an increased capacity to bind histones [28], nonhistone proteins [29] and chromosomal proteins [30], and might become concentrated in repetitive DNA nucleotide sequences [31]; such observations have, however, not yet been linked to downstream effects. The Ayusawa group speculates that BrdU incorporation into AT-rich inactive chromatins, such as in scaffold/nuclear matrix attachment region (S/MAR) sequences, results in altered interactions with AT-binding ligands and leads to changes in gene expression consistent with senescence [32,33]. The mechanisms underlying the subsequent halting of the cell cycle and the induction of a senescence-like phenotype, however, remain unexamined and unexplained.

In this study, we have characterized the DNA damage response and cell cycle effects of BrdU in A549 lung cancer cells. The A549 cell line is widely used for lung studies. These adenocarcinoma-derived cells contain wild-type p53 and pRb but do not express p16, a protein usually required for senescence [34]. The cell line is also aneuploid and contains chromosomal abnormalities [35], and heterogenous subpopulations [36]. We report that the DNA damage response to BrdU in these cells involves the activation of Chk1, Chk2 and p53. Subsequent cell cycle effects include halts at G₀, S and G₂/M phases, and morphological changes consistent with the induction of a senescent-like phenotype. It has recently been proposed that the induction and maintenance of senescence requires the efficient activation of the DNA damage response pathways [37,38]. Our data are consistent with this and indicate that DNA damage responses in BrdU-treated cancer cells lead to the inhibition of cell cycles and to the establishment of a senescence-like phenotype.

Materials and methods

Cell culture and 5-bromo-2-deoxyuridine treatment

The A549 lung adenocarcinoma cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were routinely cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Hams F12 (Gibco, Paisley, UK), supplemented with 5% fetal bovine serum (Gibco) and 2 mmol/l of L-glutamine (Gibco). Cells were maintained in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. Stock solutions of 10 mmol/l of BrdU (Sigma, Poole, UK) were prepared

in sterile water and stored at -20°C. Cells were seeded at $0.3 \times 10^3/\text{cm}^2$ in tissue-culture vessels, appropriate to each assay. After 24 h BrdU was added at a final concentration of 10 $\mu\text{mol/l}$ in a volume of 200 $\mu\text{l}/\text{cm}^2$ (day 0). The medium was replaced with fresh BrdU-containing medium on day 3 and cells were harvested for assays as appropriate.

Cell proliferation

Cells were seeded into 24-well plates and BrdU was added after 24 h. At each time point, both suspension cells and adherent cells were harvested. Adherent cells were harvested by trypsinization. Viability counts were carried out using ethidium bromide/acridine orange staining and an ultraviolet microscope. Total cell counts were obtained by combining the suspension and adherent cell counts.

Immunofluorescence

Cells were seeded into eight-well chamber slides (Nalge Nunc, Naperville, Illinois, USA) and BrdU was added after 24 h. At appropriate time points, cells were fixed in ice-cold methanol for 5 min and blocked in 20% serum. Cells were then incubated with antiphosphorylated Chk1 (Ser 345), antiphosphorylated Chk2 (Thr68), anti-p53, antiphosphorylated-p53 (Ser 15, 37 or 46), antiphosphorylated Rb (Ser 807/811) (Cell Signaling, New England Biolabs, Hitchin, UK), antiproliferating cell nuclear antigen (PCNA), anti-p27 (Sigma), anticyclin D1, anti-p57, anti-p21 (Santa Cruz Biotechnology, Heidelberg, Germany) or anti-actin (Sigma). Alexa 488 secondary antibodies (Molecular Probes, Invitrogen, Paisley, UK) were used for visualization. Nuclei were counterstained with DAPI (Sigma). Slides were mounted in fluorescent aqueous mounting media (Dako Cytomation, Galway, Ireland) and photomicrographs were acquired using a fluorescent microscope or confocal microscope, as indicated.

Fluorescence flow cytometry

Cells were seeded into 75-cm² tissue-culture flasks and BrdU was added after 24 h. On day 7 of treatment, cells were harvested by trypsinization and washed twice in phosphate-buffered saline (PBS) before being fixed in 70% ice-cold ethanol. Cells were washed twice in PBS/1% fetal bovine serum to remove ethanol and blocked with neat rabbit serum. For detection of p21, cells were incubated with anti-p21 antibody (Santa Cruz Biotechnology) followed by a secondary rabbit antimouse antibody conjugated to Alexa 488 fluorescent dye (Molecular Probes). For detection of cyclin B1, Ki-67 (BD Biosciences, Oxford, UK) and pan-keratin (Sigma), cells were incubated with primary antibodies conjugated to fluorescein isothiocyanate fluorescent dye. Fluorescence was detected using a FACScan flow cytometer (Becton Dickinson, BD Biosciences) and analysed using Cell-Quest software (Becton Dickinson, BD Biosciences).

Western blot analysis

Cells were lysed in radioimmunoprecipitation (RIPA) buffer for total protein extracts. Ten micrograms of protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Blots were incubated with primary antibodies directed against either p53 (Cell Signaling), PCNA (Sigma), antiphosphorylated cdc-2 (Tyr15) (Cell Signaling) or anti-actin (Sigma), followed by incubation with horseradish peroxidase-labelled secondary antibody. Chemiluminescence detection was then carried out. Equal loading was confirmed by immunostaining for actin (Sigma).

Cell cycle analysis

Subsequent cell cycle analysis was determined using propidium iodide DNA stain. After incubation with fluorescein isothiocyanate-labelled cyclin B1, Ki-67 and pan-keratin antibodies, cell cycle analysis was carried out using propidium iodide (Sigma) and 100 µg/ml RNase (Sigma) solution diluted in PBS. Fluorescence was detected using a FACScan flow cytometer and analysed using CellQuest software.

Assay for metabolic activity

Cell metabolic activity was measured using CellTitre 96 Aqueous One Solution Cell Proliferation Assay (Promega, Southampton, UK). The kit was used according to the manufacturer's instructions and adapted for use in a 24-well tissue-culture plate. Briefly, cells were seeded into 24-well plates and BrdU was added after 24 h. On day 7 of treatment, the supernatant was removed and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) salt reagent was added to the cells in fresh culture media and incubated for 1 h at 37°C, in 5% CO₂. Absorbance was measured at 450 nm using a Labsystems Multiscan microplate reader (Labsystems, Helsinki, Finland).

Senescence assays

For lysosomal content and senescence-associated β-galactosidase (SA-β-gal) activity, cells were seeded into 24-well plates and treated with BrdU as before. Lysosomal content of the cells was determined using the lysosomotropic acridine orange stain (Sigma). Cells were washed with PBS and incubated for 10 min with 5 µg/ml acridine orange in PBS. After four washes with PBS, cells were harvested by trypsinization and fluorescence was quantified by flow cytometry. SA-β-gal activity was determined using the ImaGene Green C₁₂FDG lacZ detection kit (Molecular Probes) [39] according to the manufacturer's instructions. Briefly, cells were incubated for 90 min with 300 µmol/l chloroquine in culture medium to inhibit endogenous β-galactosidase activity. After washing with PBS, cells were incubated for 3 h with 33 µmol/l C₁₂FDG, a fluorogenic substrate of β-galactosidase. After two washes with PBS, cells were resus-

ended by trypsinization and fluorescence was quantified immediately by flow cytometry as above. SA-β-gal was also detected using an immunohistochemical X-gal stain (Sigma). Briefly, cells were seeded in an eight-well chamber slide and treated with BrdU as before. After 7 days, cells were fixed and stained using the kit according to the manufacturer's instructions.

Statistical analysis

All experiments were performed three times, unless otherwise stated, and the results are presented as the mean of these experiments with 95% confidence interval of the mean. Wherever indicated, significance was obtained using a two-sided Student's *t*-test.

Results

5-Bromo-2-deoxyuridine activates DNA damage response pathways in A549 cells

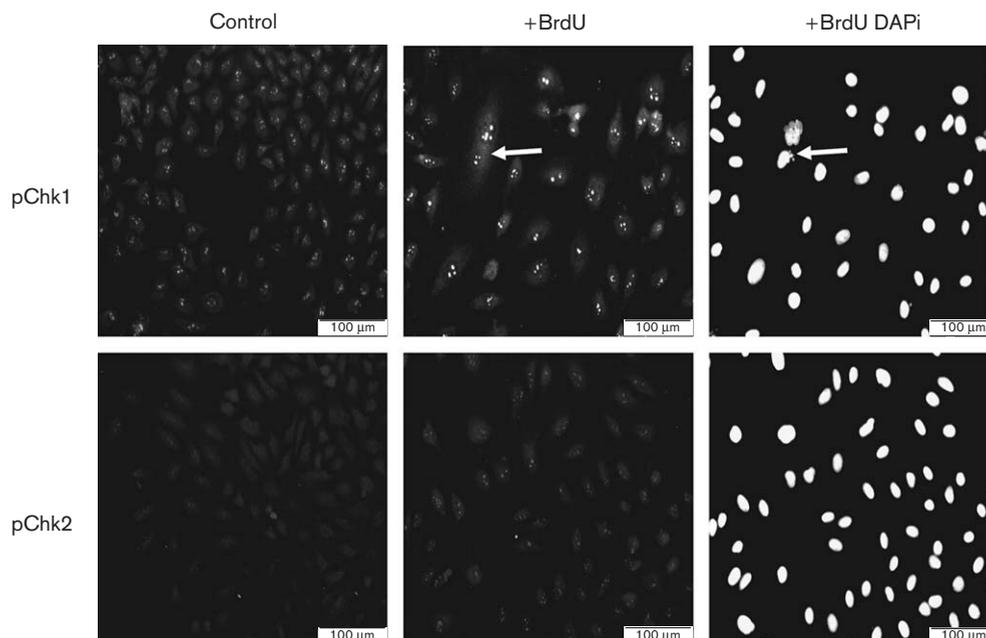
Both Chk1 and Chk2 proteins are transducers of DNA damage responses and lie downstream of the ATM and the ATR DNA damage sensors [40]. The ATR/Chk1 pathway has been implicated in responses to single-strand breaks, whereas the ATM/Chk2 pathway is involved in responses to double-strand breaks, although there can be an overlap between the two pathways. Both pathways are involved in mismatch-repair responses. p53 is activated in response to various cellular stresses by phosphorylation at specific sites, depending on the upstream signal [41]. We began by examining the DNA damage response induced in BrdU-treated A549 cells. Phospho-Chk1 and phospho-p53 (Ser 15) were detected in control cells at culture day 7 (Figs 1 and 2a), indicating background DNA damage response activity, which is predictable in an aneuploid tumour cell line such as A549. Cells cultured for 7 days in BrdU-containing medium displayed increased DNA damage response activity, with increased levels of phospho-Chk1 and the induction of phospho-Chk2 (Fig. 1). Increased levels and nuclear localization of total p53, increased levels of phospho-p53 (Ser 15) and induction of phospho-p53 (Ser 37, 46) were also evident in response to BrdU treatment (Fig. 2a). An increase in the levels of total p53 was confirmed by Western blotting (Fig. 2b). Thus, several DNA damage response pathways are activated in A549 cells in response to BrdU exposure.

Altered morphology of 5-bromo-2-deoxyuridine-treated cells

BrdU induced substantial changes in the morphology of A549 cells. The incidence of binucleation, multinucleation, micronucleation and enlarged nuclei was increased in A549 cells cultured with BrdU, compared with control cells (Fig. 3a).

After 7 days of culturing, the BrdU-treated cells were enlarged and flattened and resembled senescent-like cells (Fig. 3a). Similar cells were present sporadically in

Fig. 1



DNA damage-signalling responses in BrdU-treated cells. Phospho-Chk1 was detected in control cells (control) at day 7 of culture. Increased levels of phospho-Chk1 and induction of phospho-Chk2 occurred in response to 7 days of BrdU treatment (+ BrdU). Nuclei were visualized using a DAPI counterstain (+ BrdU DAPI). Abnormal nuclear morphology is indicated with an arrow. Results represent three experimental repeats. BrdU, 5-bromo-2-deoxyuridine.

control populations, but their incidence, estimated microscopically to be approximately 1%, was significantly lower than in treated populations. We were interested in comparing enlarged BrdU-treated cells (BrdU^L) with large cells in control populations (Cntl^L) and with control and treated cells that appeared to have more normal morphologies (Cntl^N or BrdU^N, respectively). We therefore defined the Cntl^L population as the largest 1% of the total control population, according to their forward-scatter properties by flow cytometry. We then used the same gate to analyse the treated population (Fig. 3b). Using this method, it was confirmed that the larger morphology was approximately 10 times more prevalent in treated populations ($P < 0.01$). In addition, the average size of the BrdU^N cells was significantly greater ($P < 0.001$) than that of the Cntl^N cells, as indicated by a shift in the forward scatter of the BrdU-treated population (Fig. 3b and c).

Inhibition of cell proliferation by 5-bromo-2-deoxyuridine

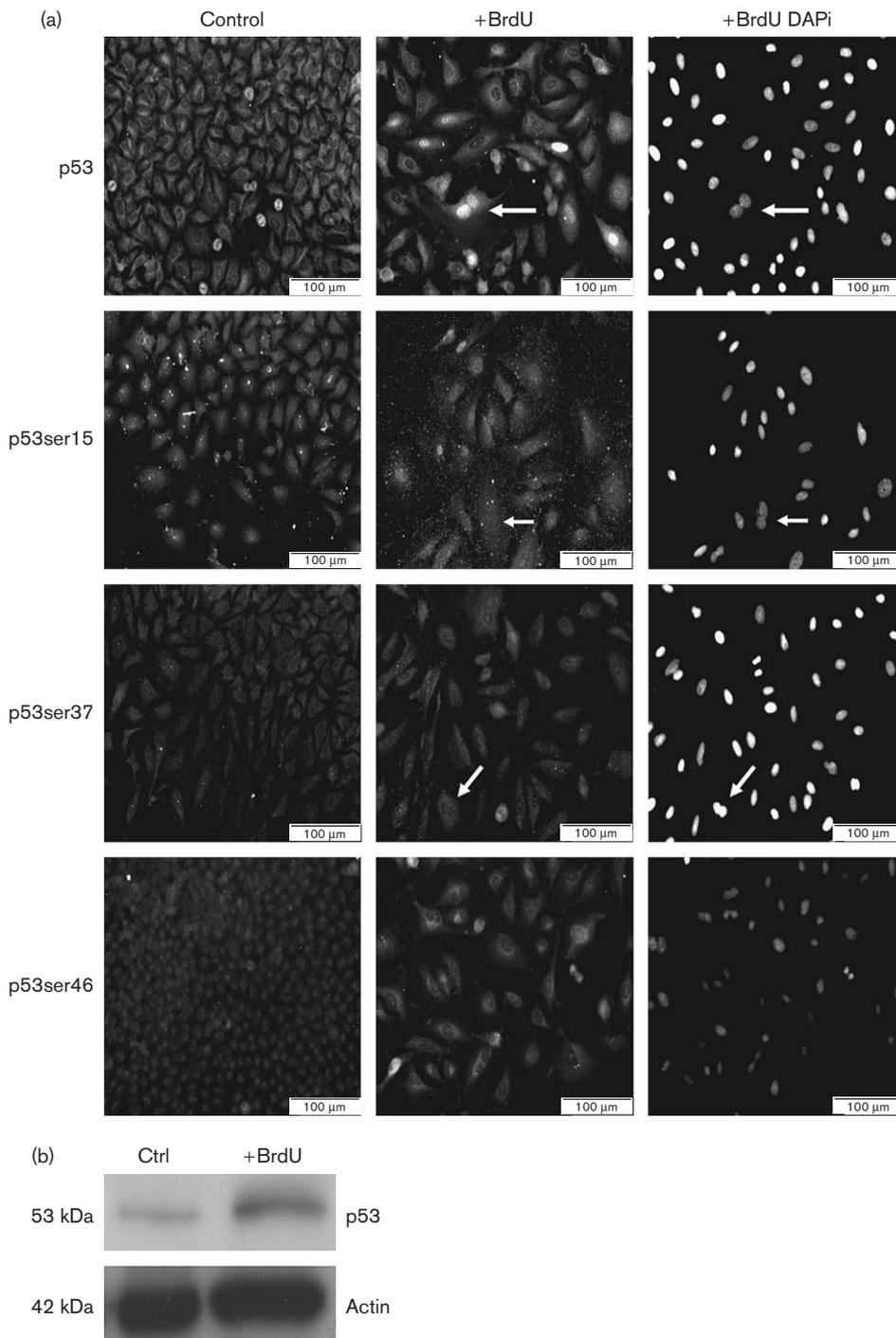
BrdU significantly inhibited proliferation of A549 cells (Fig. 3d). By day 7 of treatment, there was a 75% reduction in cell number in BrdU-treated populations compared with controls ($P < 0.05$). This reduction in cell number was not due to toxicity as the number of nonviable cells remained low during culture in both control and treated populations.

Modulation of cell cycle regulatory proteins in response to 5-bromo-2-deoxyuridine

We proceeded to determine the effects of BrdU on cell cycle regulatory proteins and to investigate the extent to which cell cycle arrest, senescence and differentiation were occurring in response to BrdU. PCNA plays an essential role both in cell proliferation and in DNA repair processes [42]. At day 1, BrdU-treated cells had increased levels of nuclear PCNA, compared with control cells (Fig. 4a). At day 7, however, BrdU-treated cells had reduced levels of PCNA, compared with control populations, with some cells also exhibiting nuclear exclusion. This might reflect initial attempts at PCNA-mediated DNA repair, followed by downregulation of PCNA during cell cycle inhibition. The reduction in PCNA expression at day 7 was confirmed by Western blotting (Fig. 4b). Cyclin D1, the G₁ initiating protein, was virtually absent in treated cells at day 7 (Fig. 5a). This, along with a reduction in phosphorylated Rb protein (Fig. 5b), indicates inhibition at the G₁ and G₁/S phase transition point of the cell cycle [40].

The cyclin-dependent kinase inhibitors (CDKIs), p21, p27 and p57, function primarily as negative regulators of the cell cycle [43]. p21 plays a central role in cell cycle regulation, apoptosis and differentiation [44], and has inhibitory effects on cyclins at all phases. In control cells, the number of p21-positive cells and the levels of p21

Fig. 2

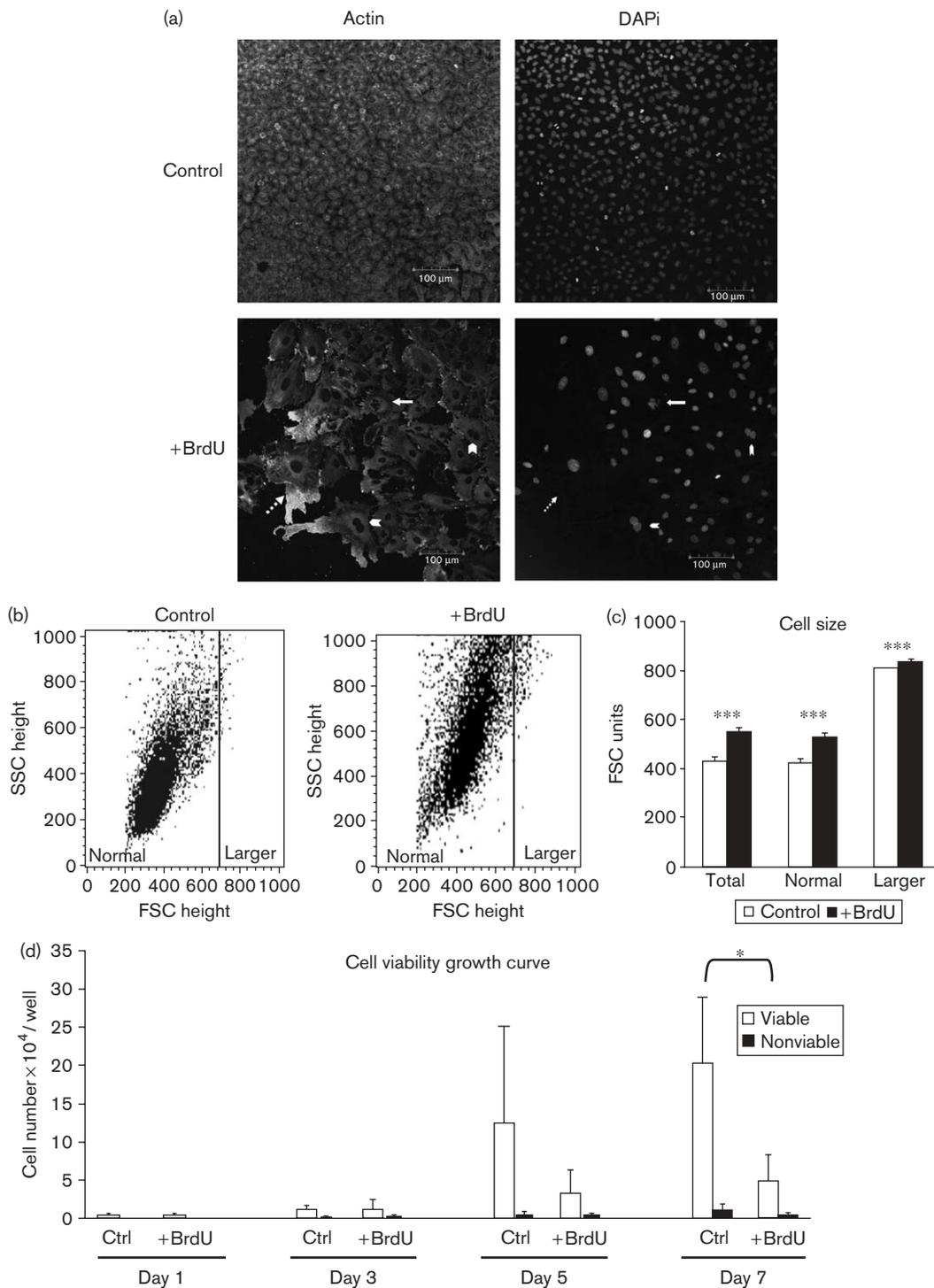


p53-signalling responses in BrdU-treated cells. (a) p53 and phospho-p53 (Ser 15) were detected in control cells (control) at day 7 of culture. Increased levels of and nuclear localization of p53 and increased levels of phospho-p53 (Ser15, 37 and 46) occurred in response to 7 days of BrdU treatment (+BrdU). (b) Increased total p53 protein levels, as detected by Western blot. Nuclei were visualized using a DAPI counterstain (+BrdU DAPI). Binucleation is indicated with an arrow. Results represent three experimental repeats. BrdU, 5-bromo-2-deoxyuridine; Ctrl, control.

protein per cell decreased during the culture period (Fig. 6a). This very likely reflects an initial increase in p21 activity to prevent the apoptosis following trypsinization and a subsequent decrease on reattachment to

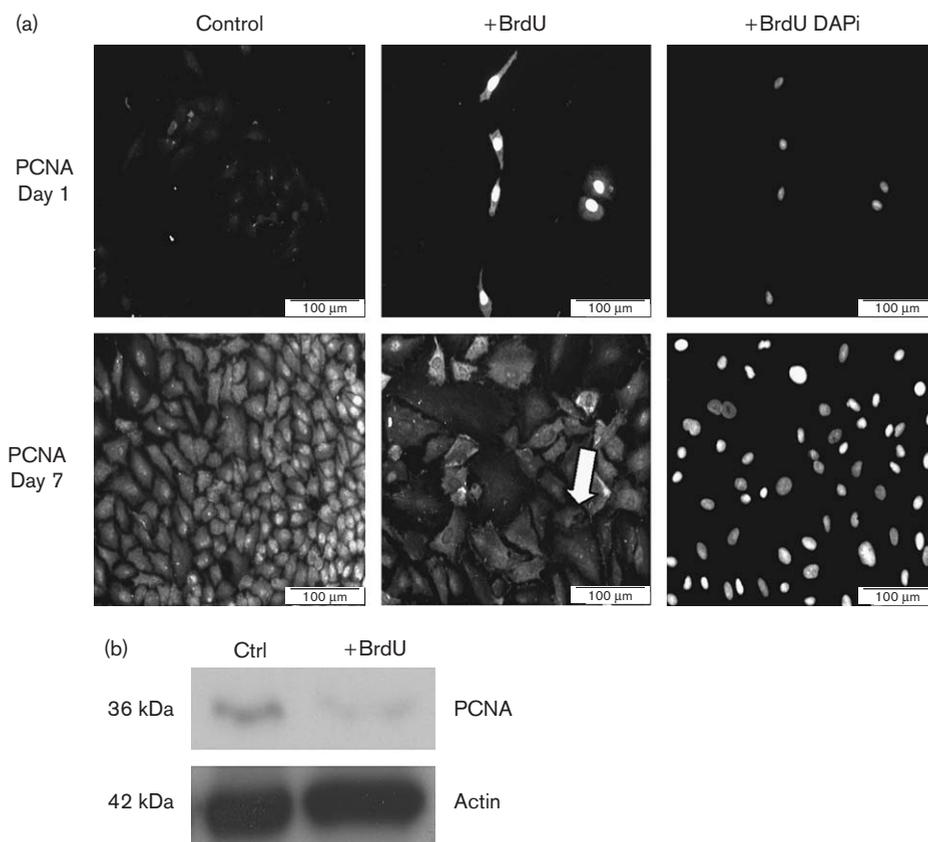
permit growth in culture. In contrast, p21 activity was almost completely inhibited in BrdU-treated cells at day 1; thereafter, the levels increased and exceeded the levels in the control cells by day 7 (Fig. 6a and b). Even at day 7,

Fig. 3



Effects of BrdU on cell proliferation and morphology. (a) Actin immunofluorescence with DAPI counterstaining, which was visualized using confocal microscopy, indicated increased cell size (dashed line arrow), binucleation (arrowhead) and micronucleation (arrow) in populations after 7 days of BrdU treatment. (b) An overall increase in cell size was apparent in the BrdU-treated population by day 7, as indicated by a shift in forward scatter (FSC) by flow cytometry. BrdU^L cells were approximately 10 times more prevalent than Cntl^L cells. (c) The increase in cell size was significant in the total populations and also when 'normal' sized (Cntl^N and BrdU^N) and 'large' cells (Cntl^L and BrdU^L) were compared, $P < 0.01$ and $P < 0.001$, respectively. (d) BrdU induced a significant reduction of proliferation in A549 cells by day 7 of treatment ($P < 0.05$). Results represent at least three experimental repeats. BrdU, 5-bromo-2-deoxyuridine; Ctrl, control.

Fig. 4



Effects of BrdU on PCNA expression. (a) Nuclear PCNA was induced at day 1 of BrdU treatment. In contrast, PCNA was absent or at low levels in control cells at this time point. Subsequently, PCNA levels increased in the control cells and decreased in the BrdU-treated cells after 7 days' treatment with BrdU. Nuclear exclusion is indicated by an arrow. (b) Reduction in PCNA protein levels following 7 days of BrdU treatment, as detected by Western blot. Results represent at least three experimental repeats. BrdU, 5-bromo-2-deoxyuridine; Ctrl, control; PCNA, proliferating cell nuclear antigen.

p21 was present in only approximately 10% of the treated cells (Fig. 6a). In contrast to p21, cells treated for 7 days with BrdU also had increased levels of nuclear p27 (Fig. 6c) and p57 (Fig. 6d), compared with control cells.

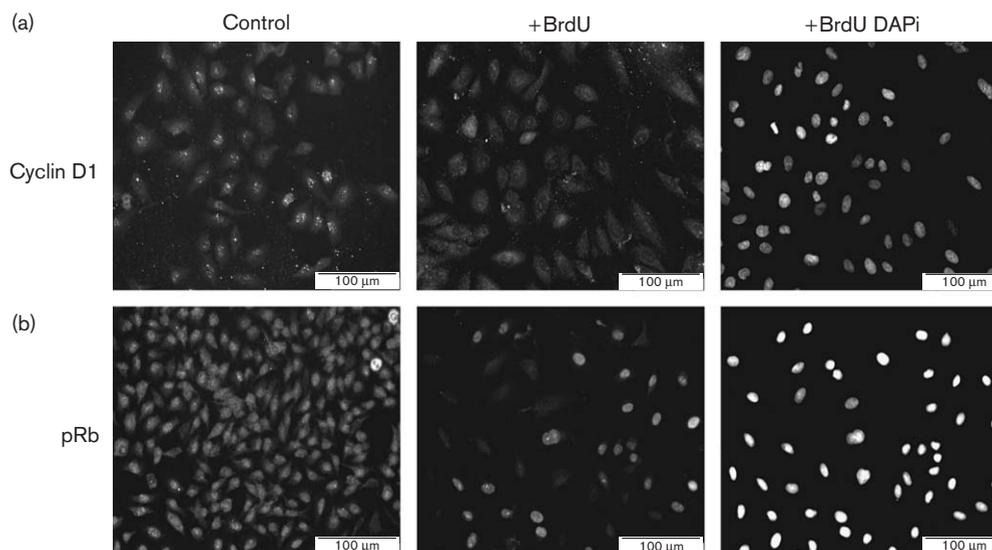
5-Bromo-2-deoxyuridine activates cell cycle checkpoints

The effects of BrdU on cell cycle progression were analysed. Ki-67 was used as a marker of actively cycling cells to allow separation of 2C populations into G_0 and G_1 phases. When total populations were examined at day 7, control cells displayed a normal cell cycle distribution. They also displayed a larger 4C population, accounting for approximately 8% of the total population, which presumably reflected the aneuploid nature of A549 cells (Fig. 7a). In contrast, BrdU induced a significant increase in the number of cells accumulated in G_0 and S phase with approximately 17 and 16% of the total BrdU population in each respective phase, compared with 2 and 7% of total control populations. A corresponding significant reduction in 2C/ G_1 phase BrdU-treated cells

was evident. This indicates that BrdU induced a prolonged or halted S phase, which was probably due to futile cycles of repair; it also caused cells to undergo mitotic exit and to withdraw from the cell cycle into G_0 . When BrdU^N and BrdU^L, as previously defined in Fig. 3, were analysed separately, it was found that BrdU^N cells predominantly accounted for the halt at G_0 ($P < 0.01$) (Fig. 7b). In contrast, approximately 40% of the BrdU^L cells were evenly divided between the S and the G_2/M phases; the latter distribution had not been apparent in the analysis of the total population (Fig. 7c). Significantly more BrdU^L than Cntl^L cells were present in the S ($P < 0.0001$) and the G_2/M phases ($P < 0.01$). In contrast, over 90% of Cntl^L cells were halted with a greater 4C DNA content, significantly more than in corresponding BrdU^L cells ($P < 0.00001$).

The cdc-2/cyclin B complex is pivotal in regulating the G_2/M checkpoint [40]. Phosphorylation of cdc-2 renders it inactive. Downregulation of active cdc-2/cyclin B

Fig. 5



Effects of BrdU on cell cycle activators. (a) Cyclin D1 and (b) phospho-Rb expression was reduced in BrdU-treated cells by day 7 compared with control cells. Results represent at least three experimental repeats. BrdU, 5-bromo-2-deoxyuridine.

complexes is required for cytokinesis. Delayed degradation of cyclin B can lead to cytokinesis failure and mitotic catastrophe with multinucleation and multimicronucleation, and can halt it at the G_2/M checkpoint [45,46]. Although the percentage of control and treated cells expressing cyclin B was similar during all the phases of the cell cycle (data not shown), the amount of cyclin B per cell was higher in treated cells, compared with control, by day 7 (Fig. 8a). Increased cyclin B expression was also associated with abnormal mitosis, both as multinucleation and enlarged nuclei, in treated cells (Fig. 8b). A slight increase in the activity of *cdc-2* was also suggested by a moderate decrease in its phosphorylation levels (Fig. 8b and c). This suggests that cyclin B activity is elevated in response to BrdU-mediated DNA damage, leading to cell cycle arrest at the G_2/M checkpoint. Although this would lead to cell death in normal cells, the dysregulated cell cycle machinery in A549 cells can result in the G_2/M halt and the mitotic catastrophe observed in a subpopulation of these cells.

Induction of senescence-related markers in 5-bromo-2-deoxyuridine-treated cells

A range of senescence-related markers was used to evaluate the induction of a senescence-like phenotype in A549 cells in response to BrdU.

As shown above, increased numbers of large cells with a senescent-like morphology were present in BrdU-treated A549 populations (Fig. 3b and c). Moreover, there was also an increase in average cell size in the BrdU^N population, compared with Cntl^N cells (Fig. 3c). When

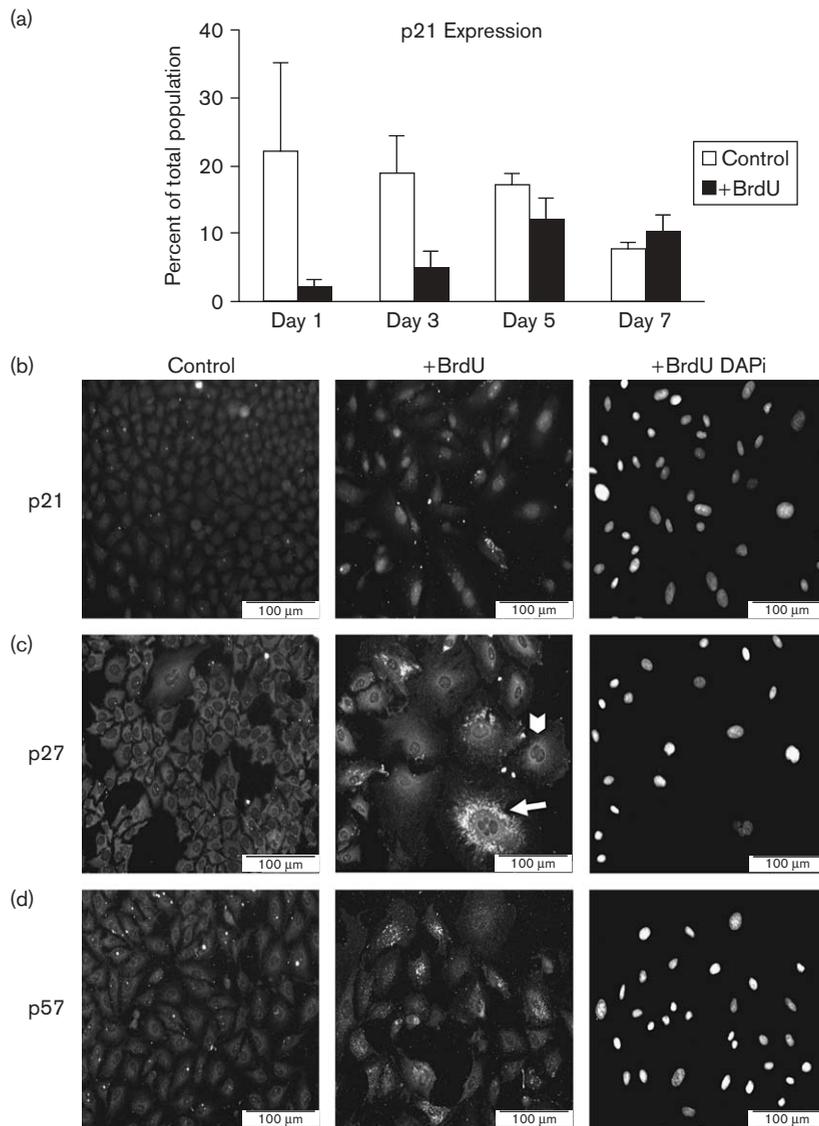
cell size was compared with cell cycle phase in total populations, it was found that BrdU-treated cells were larger than control cells at all stages of the cell cycle. Therefore, despite their increased size, BrdU-treated cells remained capable of progressing through the cell cycle and did not accumulate in any particular phase (Fig. 9a).

Analysis of side scatter revealed a significant increase in cell granularity in response to BrdU ($P < 0.01$), which again was evident at all cell cycle phases (Fig. 9b). Granularity is considered to reflect an increased organelle content in senescent cells [39]. Increased lysosomal content is also associated with cellular senescence [39]. Lysosomal content was slightly increased in BrdU-treated cells, compared with control cells, at day 7 as determined by uptake of the lysosomotropic fluorescent dye, acridine orange (Fig. 9c). Separation of Cntl^N and BrdU^N populations from the Cntl^L and BrdU^L populations predictably showed that increased lysosomal content correlated with increased cell size in both control and treated cells.

BrdU-treated cells had a higher rate of metabolic activity per cell than control cells ($P < 0.01$) (Fig. 9d). This is consistent with a senescence-like phenotype.

SA- β -gal activity was measured using two different assays. Cleavage of the β -galactosidase substrate $C_{12}FDG$, measured by flow cytometry, revealed a significant increase in SA- β -gal activity in BrdU-treated cells

Fig. 6



Effects of BrdU on p21, p27 and p57. (a) The number of control cells expressing p21 declined from approximately 20 to 7% during the culture period. In contrast, p21 expression was inhibited by BrdU on days 1 and 3 of the culture. Although levels rose thereafter, no more than 10% of the treated cells expressed p21. Results represent two experimental repeats. (b) p21 was weakly detectable by immunofluorescence in control cells at day 7. In contrast, nuclear localization of p21 was detected in BrdU-treated cells. (c) Cytoplasmic p27 was present in control cells throughout the culture period. Cytoplasmic localization was evident and levels of expression were higher than in control populations, particularly in cells with abnormal nuclei, by day 7. Binucleate cells are indicated by arrowheads and micronucleation indicated by arrows. (d) Low levels of cytoplasmic p57 were present in control cells throughout the culture period. p57 localization became cytoplasmic and a 'speckled' immunostaining pattern (arrow) over time in culture with BrdU was evident after 7 days. Results represent three experimental repeats. BrdU, 5-bromo-2-deoxyuridine.

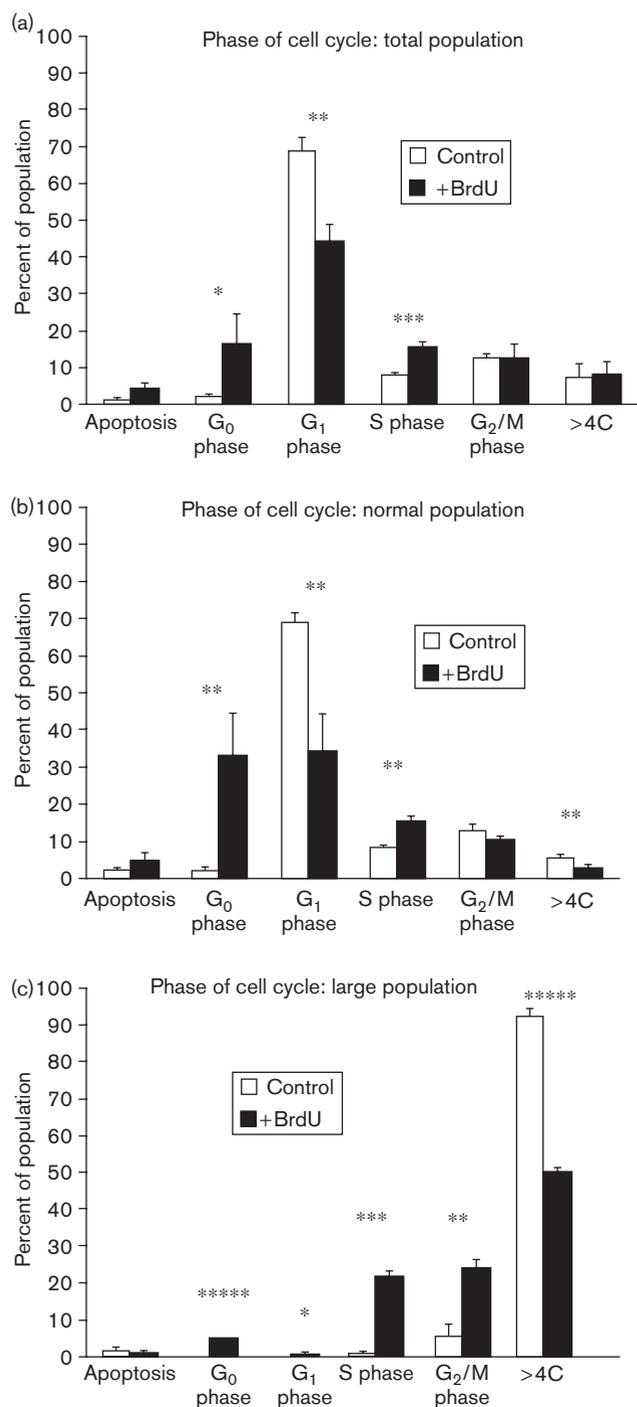
($P < 0.01$) (Fig. 10a). Staining of cells for SA- β -gal using an X-gal substrate confirmed the increase in SA- β -gal activity in response to BrdU (Fig. 10b).

Thus, per cell, BrdU-treated cells were larger, more granular, had increased lysosomal content, were more metabolically active and displayed more SA- β -gal activity than their control counterparts; all these features indicate the induction of a senescence-like phenotype.

Effects of 5-bromo-2-deoxyuridine on cell differentiation

It has previously been reported that BrdU modulates the expression of differentiation-related proteins such as cytokeratins, which can be induced or upregulated in lung cancer cell lines, depending on the endogenous expression levels [23]. We investigated whether BrdU-mediated upregulation of cytokeratin expression in A549 cells was cell cycle dependent. If so, this might reflect a

Fig. 7



Activation of cell cycle checkpoints. (a) Analysis of total populations showed that control cells had a normal cell cycle distribution, whereas BrdU treatment induced accumulations of cells at G₀ and S phases, and a reduction of cells in G₁. Separate analysis of (b) Cntl^N and BrdU^N and (c) Cntl^L and BrdU^L populations revealed that BrdU^N cells accounted for most of the BrdU-treated cells in G₀; BrdU^L cells were halted at S and G₂/M phases and had a greater 4C DNA content, whereas most control cells were halted with greater 4C DNA content. Results represent at least three experimental repeats. BrdU, 5-bromo-2-deoxyuridine.

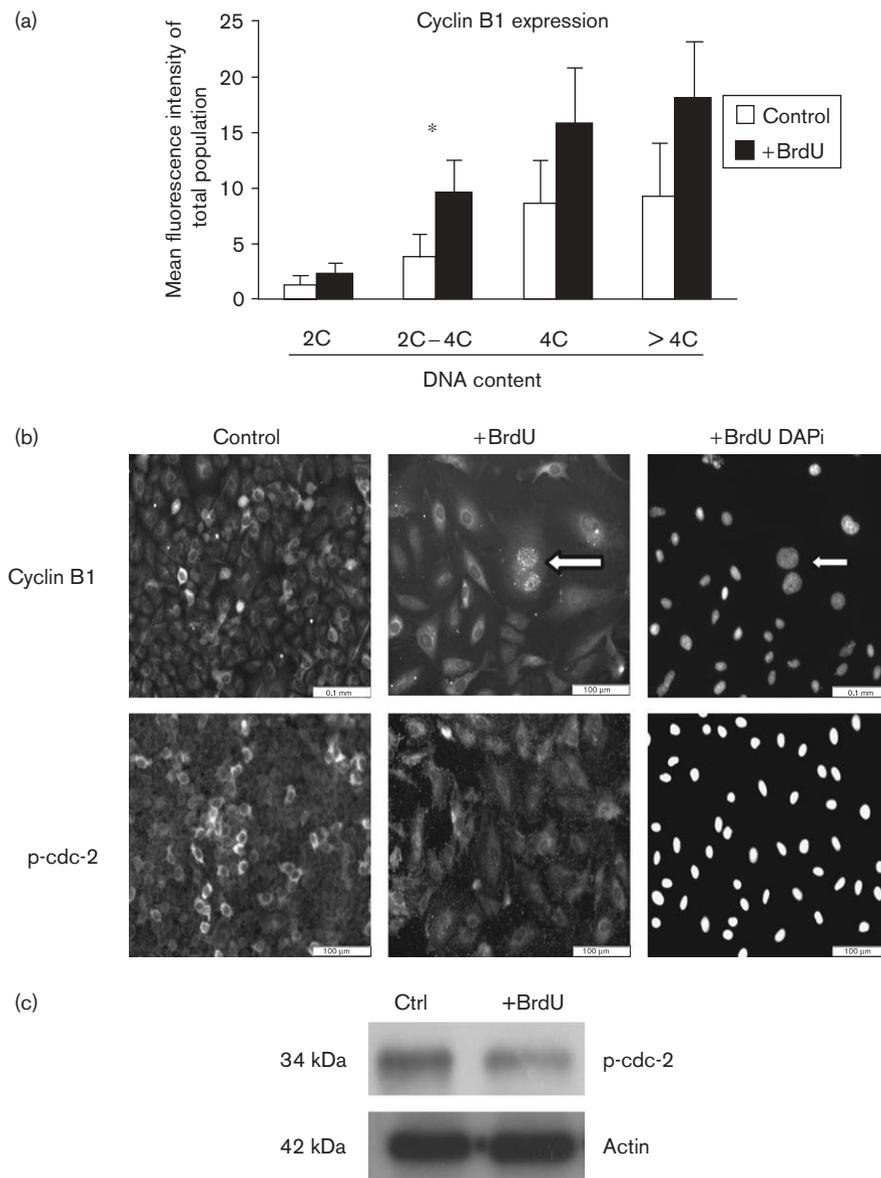
differentiation-related halt in the cell cycle. One hundred percent of the control and the BrdU-treated cells expressed cytokeratins (results not shown). Cytokeratin expression, however, was significantly upregulated in all treated cells during all the phases of the cell cycle ($P < 0.01$), with no accumulation occurring in any one phase (Fig. 11).

Discussion

The ability of BrdU to both induce DNA damage and modulate the cell phenotype has long been recognized. The molecular mechanisms associated with these effects, however, are unknown and potential links between the two processes have not been explored. Furthermore, the question of whether BrdU induces differentiation or senescence requires clarification. We have examined the effects of BrdU on DNA damage response and cell cycle checkpoint pathways, and the related impacts on cell cycle progression and cell phenotype in a lung cancer cell line. BrdU-induced DNA damage occurs during several cell cycle phases. The 'multihit' nature of BrdU-induced DNA damage led us to examine unsynchronized cell populations, thus enabling us to examine the ensemble of BrdU effects. Our studies show that A549 cells respond to BrdU-induced DNA damage by upregulating background Chk1 responses, activating Chk2 and phosphorylating p53. Activation of the DNA-repair function of PCNA is also suggested by the early upregulation of this protein after exposure to BrdU. A decoupling of normal DNA damage signalling and cell cycle checkpoint activation in these transformed cells leads to a deregulated mitosis with an increase in the incidence of abnormal nuclei and accumulation of cells in G₀, S and G₂/M phases of the cell cycle. An increase in average cell size occurs and phenotypic changes consistent with senescence are apparent (summarized in Fig. 12).

Our findings thus support the contention that BrdU induces a senescence-like phenotype in cancer cells. Senescence is a mechanism to suppress inappropriate proliferation, e.g. that follows DNA damage; it is defined as an irreversible halt in cell cycle when cells become unresponsive to mitogenic or apoptotic signals [27]. Senescent cells are enlarged and have increased granularity; nevertheless, they remain viable and metabolically active [47]. In this study, the A549 populations that appeared morphologically normal were termed Cntl^N and BrdU^N. The majority of both of these populations had a 2C DNA content. Unlike the Cntl^N population, however, approximately one-third of the BrdU^N population had withdrawn from the cell cycle and was halted at G₀ (Fig. 7b). The BrdU^N cells were also slightly larger than their Cntl^N counterparts (Fig. 3d) and senescence-associated features were increased in the BrdU-treated 2C population (Fig. 9). Despite their more 'normal'

Fig. 8



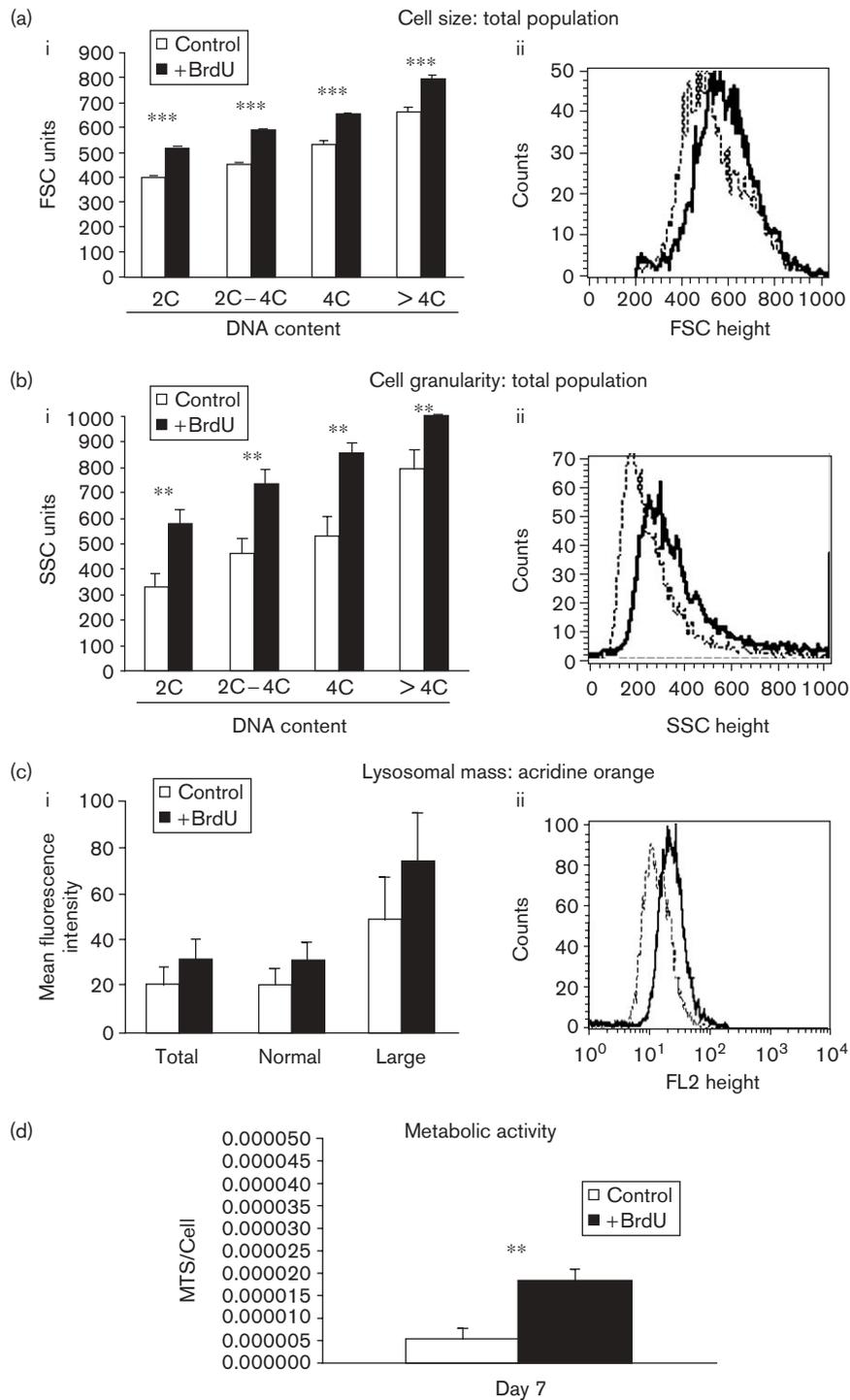
(a) Cyclin B1 levels were increased in BrdU-treated cells at each phase of the cell cycle compared with control populations. (b) Increased levels of nuclear cyclin B1 were frequently evident in BrdU-treated cells containing enlarged or binuclei (arrow) (day 7). A slight decrease in the levels of phosphorylated cdc-2 was evident in BrdU-treated cells (day 7). (c) Western blot confirmation of decreased levels of phospho-cdc-2. Results represent three experimental repeats. BrdU, 5-bromo-2-deoxyuridine; Ctrl, control.

morphological appearance, therefore, the BrdU^N population had several senescent-like characteristics. It seems likely that BrdU-induced DNA damage caused these cells to arrest, in this case mainly at G₀. The result of this arrest was the induction of a senescence-like phenotype rather than apoptosis.

Unlike the Cntl^N population, the enlarged Cntl^L population, by definition, possessed senescent-like features. The Cntl^L population, which represented only 1% of the total control population, had increased cell sizes

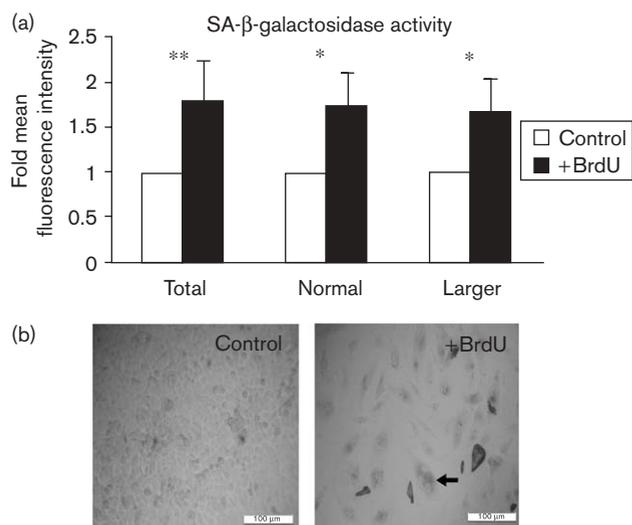
and almost exclusively a greater 4C DNA content. These cells probably reflected ongoing, spontaneous senescence due to aneuploidy and in-vitro culture conditions in the control population. In contrast, the incidence of enlarged senescent-like cells was increased 10-fold in the presence of BrdU. Unlike the Cntl^L population, approximately 50% of BrdU^L cells were accumulated in the S and G₂/M phases, indicating that they were halted at the DNA damage checkpoints. Originally defined by their regulatory role in cell cycle progression, it is now clear that DNA damage checkpoints are also involved in the

Fig. 9



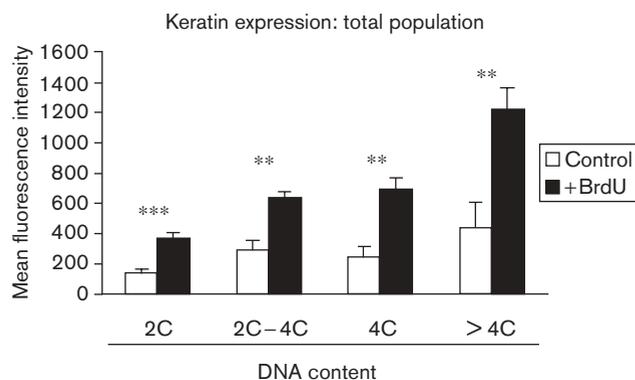
Induction of a senescence-like morphology in BrdU-treated cells at day 7. (a) (i) BrdU-treated cells were significantly larger than control counterparts during each phase of the cell cycle. (ii) A representative flow-cytometry histogram of increased cell size, as detected by forward scatter (FSC) units. (b) (i) Treated cells were also more granular than control cells at each cell cycle phase. (ii) A representative flow cytometry histogram of increased cell granularity as detected by side scatter (SSC) units. (c) (i) BrdU treatment induced increased lysosomal content in cells with both normal and large morphology. (ii) a representative flow-cytometry histogram of increased lysosomal mass. (d) BrdU-treated cells were more metabolically active than control cells. Control population: dotted line; BrdU-treated population: full line. Results represent three experimental repeats. BrdU, 5-bromo-2-deoxyuridine.

Fig. 10



Induction of senescence-associated (SA)- β -galactosidase activity in BrdU-treated cells. (a) SA- β -galactosidase activity was significantly increased in all BrdU-treated cells, regardless of size, as determined by flow cytometry using a fluorescent β -galactosidase substrate. (b) An X-gal substrate that produces a visible stain confirmed increased levels of SA- β -galactosidase after 7 days of BrdU treatment. Results represent three experimental repeats. BrdU, 5-bromo-2-deoxyuridine.

Fig. 11



Effect of BrdU on cytokeratin expression. Cytokeratin expression was increased in all BrdU-treated cells, regardless of cell size and phase of cell cycle. Results represent three experimental repeats. BrdU, 5-bromo-2-deoxyuridine.

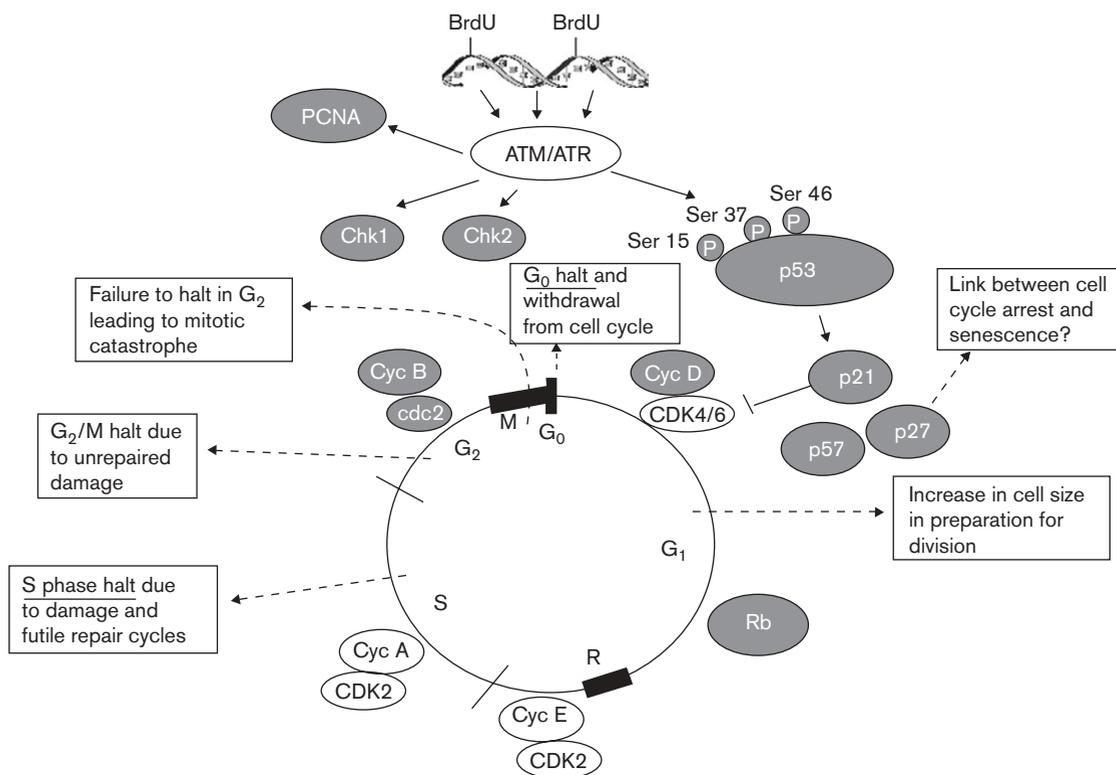
induction and maintenance of senescence [37,38,48]. We therefore believe, first, that the previously reported changes in gene expression and phenotype in BrdU-treated tumour cells now seem to reflect the acquisition of senescent-like phenotypes. Second, the activation of the checkpoints in the A549 senescent-like cells in this study provides evidence that these phenotypic changes are due to BrdU-induced DNA damage responses, rather than to mutations or to alterations in methylation or to altered protein interactions in BrdU-containing DNA.

Although BrdU-induced morphological changes in A549 cells are consistent with the induction of a senescence-like phenotype, the typical gene-expression profile for senescence is not evident. Senescent normal human fibroblasts typically overexpress p21, p16 and cyclin D1. This was not the case in the present study [49]. Treatment-induced senescence of tumour cells can, however, occur in the absence of p53, p21 and p16 [38,50]. The mechanisms underlying senescence in both normal and tumour cells thus remain unclear. p21 is a common mediator of p53-induced cell cycle arrest [43]. Downregulation of p21, however, occurred as an early response to BrdU treatment, probably to avoid the inactivation of the DNA repair function of PCNA [51], indicating that the subsequent G_0 , S and G_2/M checkpoints activated by BrdU might be p21-independent. Downregulation of p21 and the absence of p16 in A549 cells suggest that cell cycle arrest is mediated by p27 and/or p57, both of which are upregulated during BrdU treatment.

The mechanisms by which cell cycle arrest is linked to senescence are also unknown; although, p27 might play a key role in linking the two processes. p27 induces cell cycle arrest during several cell cycle phases usually in response to external signals such as transforming growth factor- β and contact inhibition [44]. Its expression, therefore, correlates with differentiation status in normal and cancer cells [52]. p57 is also involved in differentiation and plays a role in alveolar development [53]. Both p27 and p57 are also involved in senescence [54]. The overexpression of p27 can lead to an increase in cell size, protein content and metabolic activity, similar to the BrdU-mediated effects seen in this study [55,56]. Upregulation of p27 to p57 may explain the appearance of a senescence-like phenotype in BrdU-treated A549 cells in the absence of p21 and p16, which are usually associated with senescence induction and maintenance [47]. p27 and/or p57 could be responsible for most of the cell cycle and phenotypic changes that occur in the A549 cells following BrdU-induced damage. The mitotic exit and accumulation of G_0 A549 cells following treatment could be due to a p27/p57-induced differentiation-related or senescence-related entry directly into G_0 , or to a p27/p57-induced prolonged G_1 halt, which leads to entry into G_0 , or both. Increased cytokeratin protein could result from an altered differentiation programme, terminal differentiation, a senescence-related programme or merely from an increase in cell size and accompanying protein expression: all these can result from increased p27/p57 activity.

It seems most likely that the altered phenotypes present in BrdU-treated populations arise after sublethal DNA damage responses. These responses, rather than leading to apoptosis result in BrdU-specific cell cycle perturbations, and the activation of regulators involved in cell

Fig. 12



Summary of the BrdU mechanism of action in A549 cells. Proteins modulated by BrdU in this study are shown in 'grey fill'. DNA-damage checkpoints activated by BrdU (dashed arrows) and proposed implications for the cell cycle (boxes) are indicated. BrdU, 5-bromo-2-deoxyuridine; PCNA, proliferating cell nuclear antigen; Cyc, cyclin; CDK, cyclin-dependent kinase.

cycle arrest, differentiation and senescence. A precise definition of these phenotypes as being either differentiated or senescent might not be possible. Instead, we suggest that these cells have a BrdU DNA damage-induced altered phenotype that is dependent on their genotype. Rather than a BrdU-specific effect such as altered transcription factor binding to BrdU-substituted DNA, p27-mediated/p57-mediated DNA damage responses (which involve mitotic exit and other cell cycle halts) might, therefore, cause the phenotypic changes observed in BrdU-treated A549 cells. It is possible that the phenotypic changes observed in the other cell types could also be explained by any, or a combination, of these p27-mediated/p57-mediated processes.

The mechanisms by which BrdU induces various types of DNA damage have been studied sporadically since this thymidine analogue had first been designed as an anticancer agent. Similarly, the effects of BrdU on cell morphology and phenotype have been recognized but unexplained for decades. These two aspects of BrdU biology have remained relatively separate till date. We suggest that there is very likely a connection between the two. We provide evidence here that BrdU-mediated effects on cell phenotype might be due, at least in part, to

BrdU-induced DNA damage and to the subsequent perturbation of the cell cycle. As a result, phenotypic response will vary between cell types, depending on their respective DNA damage response and cell cycle machinery. DNA damage-induced cell cycle perturbations can lead to changes in gene expression that might resemble differentiation or senescence. The latter remains poorly understood, particularly in tumour cells [50]. Thus, apparent cell-specific BrdU responses, particularly in tumour cells that have abnormal and varying genotypes, might reflect cell-specific DNA damage responses, rather than BrdU-specific effects *per se*, such as altered transcription factor binding to BrdU-containing DNA. The ability of BrdU to modulate the cell cycle has implications for its use in cell-proliferation assays *in vitro* and *in vivo*. It also remains to be demonstrated whether BrdU induces senescence *in vivo* when used during chemotherapy. If so, is senescence beneficial or detrimental in this situation? Furthermore, other base analogues that are used more commonly than BrdU for cancer treatment might also have similar effects.

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