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The ability to accumulate deoxyuridine triphosphate and cellular response to thymidylate synthase (TS) inhibition

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Summary Thymidylate synthase (TS) is an important enzyme catalysing the reductive methylation of dUMP to dTMP that is further metabolized to dTTP for DNA synthesis. Loss of viability following TS inhibition occurs as a consequence of depleted dTTP pools and at least in some cell lines, accumulation of dUTP and subsequent misincorporation of uracil into DNA. The expansion in dUTP pools is largely determined by the expression of the pyrophosphatase, dUTPase. Our previous work has shown that following TS inhibition the ability to accumulate dUTP was associated with an earlier growth inhibitory effect. 3 human lung tumour cell lines and HT29 human colon tumour cells transfected with dUTPase have been used to investigate the relationship between loss of viability following TS inhibition and dUTP accumulation. Cell cycle arrest typical of TS inhibition was an early event in all cell lines and occurred irrespective of the ability to accumulate dUTP or p53 function. However, a large expansion of dUTP pools was associated with mature DNA damage (4 h) and an earlier loss of viability following TS inhibition compared to cells in which dUTP pools were not expanded. In A549 cells damage to mature DNA may have been exacerbated by significantly higher activity of the excision repair enzyme, uracil-DNA glycosylase. Consistent with results using different inhibitors of TS, transfection of dUTPase into HT29 cells significantly reduced the cytotoxicity of a 24 h but not 48 h exposure to ZD9331. Although loss of viability can be mediated through dTTP deprivation alone, the uracil misincorporation pathway resulted in an earlier commitment to cell death. The relevance of this latter pathway in the clinical response to TS inhibitors deserves further investigation. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: dUTPase; thymidylate synthase; ZD9331; dUTP; uracil misincorporation

5-Fluorouracil (5-FU) has been used for some time for the treatment of malignant disease and mechanism by which the drug is thought to act is through inhibition of thymidylate synthase (TS). In addition, several antifolate TS inhibitors have recently been investigated for their clinical use as chemotherapeutic agents (Jackman and Judson, 1996). TS is a rate-limiting enzyme catalysing the reductive methylation of dUMP to dTMP which is further metabolized to dTTP for DNA synthesis. Inhibition of TS results in depletion of intracellular dTTP pools and an elevation in dUMP pools (Jackson et al, 1983; Aherne et al, 1996). In some cell lines (but not all) expanded dUTP pools are also a consequence of TS inhibition.

It is not entirely clear how dNTP perturbations following TS inhibition result in cell death, but this is often assumed to occur through uracil misincorporation. When significantly elevated, dUTP can be misincorporated into DNA in place of dTTP by DNA polymerase (Brynolf et al, 1978). Incorrectly inserted dUTP coupled with the action of uracil-DNA glycosylase (UDG) results in a futile cycle of misrepair leading eventually to DNA strand breaks and cell death (Goulian et al, 1986). Although this concept is widely supported by several studies (reviewed in Aherne and Brown, 1998), dUTP accumulation may not be a critical event accompanying TS inhibition in all cells. dTTP deprivation itself, if it occurs for a long enough period of time (greater than a generation time), induces cell death (Houghton et al, 1994; Matsui et al, 1996). dATP has also been reported to be elevated following TS inhibition and it has been proposed that this triggers cellular events which result in the cleavage of DNA (Chong and Tattersall, 1995; Houghton et al, 1995; Sundseth et al, 1997).

dNTP perturbations during TS inhibition result in DNA damage (Curtin et al, 1991; Canman et al, 1994; Matsui et al, 1996), the response to which may essentially be governed by key downstream proteins such as the tumour suppressor p53. Activation of p53 results in the transactivation of several proteins involved in G1 cell cycle arrest, apoptosis and DNA repair (O'Connor, 1987; Zambetti and Levine, 1993). The specific TS inhibitor raltitrexed (RTX; Tomudex™; ZD1694) has been shown to induce DNA damage at both the single- and double-stranded level (Yin et al, 1992; Matsui et al, 1996) and p53 has been reported to be responsive to the effects of this drug (Arrendondo et al, 1994). Another extensively studied function of p53 is as a G1 cell cycle checkpoint, where the protein is elevated in response to DNA damage (Hartwell and Kastan 1994; Nelson and Kastan, 1994). Cell cycle alterations typical of G1/S phase arrest appear to be a universal consequence of TS inhibition (Matsui et al, 1996; Tonkinson et al, 1996; Skelton et al, 1998).

The purpose of this study was to further investigate the importance of uracil misincorporation in cell death following inhibition of TS. ZD9331 (Jackman et al, 1997) is a specific non-polyglutamatable antifolate TS inhibitor which is currently being clinically evaluated. Unlike other TS inhibitors this compound does not require metabolic activation and the duration of TS inhibition can be more readily controlled. Earlier studies using this agent have shown that TS activity or protein expression, TS inhi-

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bition as determined by dTTP depletion or intracellular ZD9331 levels could not account for differences in sensitivity between 4 human lung tumour cell lines (Webley et al, 2000). A significant difference in the extent of dUTP accumulation following ZD9331 was observed. For example in the A549 cell line, increased pools of dUTP was an early event and over 24 h considerable amounts of dUTP accumulated. In contrast, in MOR cells dUTP pools were only marginally increased over those of untreated cells, even when the dose and duration of drug exposure were increased several-fold. dUTP accumulation was negatively associated with the expression of dUTPase, the pyrophosphatase responsible for maintaining low intracellular levels of dUTP. These results were consistent with reports on the role of dUTPase in mediating the effects of TS inhibition (Canman et al, 1993, 1994). In the current study, the relative roles of dUTP accumulation and dTTP depletion in cell cycle arrest and cell death following exposure of cells to ZD9331 in 3 of these cell lines has been addressed. The study has also briefly assessed the roles of UDG and p53 in downstream events following TS inhibition. The mutant p53 HT29 human colon carcinoma cell line transfected with *E. coli* dUTPase or vector control (Canman et al, 1994) were used as a paired isogenic model.

MATERIALS AND METHODS

Materials

All standard laboratory chemicals used in this study were of AnalaR® grade purchased either from British Drug Houses (BDH) (Poole, Dorset UK) or from Sigma (Poole, Dorset UK). ZD9331 was synthesized at Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK), dissolved in 0.15 M sodium bicarbonate ($NaHCO_3$) (pH 8.3) and filter sterilized. [^{14}C]-thymidine (specific activity 51.4 Ci mMol^{-1}) and [methyl-^{3}H] thymidine (specific activity 5.0 Ci mMol^{-1}) were purchased from Amershan International plc.

Cell culture

The human lung carcinoma cell lines, A549 (squamous), MOR (adenocarcinoma) and HX147 (large cell) were maintained at 37 ˚C in air containing 5% CO_2 in DMEM tissue culture medium (Gibco, Paisley, Scotland) and 10% heat inactivated dialysed fetal bovine serum (FBS) supplemented with 2 mM glutamine, 50 μg ml^{-1} gentamycin, 2.5 μg ml^{-1} fungizone, 10 μg ml^{-1} insulin and 0.5 μg ml^{-1} hydrocortisone. The human HT29 colon adenocarcinoma *E. coli* dUTPase transfected cell line dutE7 and its neotransfected control CON were kindly provided by Dr Jonathan Maybaum. These cells were maintained using the above conditions. All cells were free of mycoplasma during experimentation. Growth inhibition was measured using MTT assay as previously described (Webley et al, 2000).

Flow cytometry

Following ZD9331 treatment (1 μM for 4 or 24 h), adherent cells (1×10^6) were harvested by gentle scraping in 10 ml of ice cold PBS and pelleted by refrigerated centrifugation for 5 min at 2500 rpm (600 ***g***). Cell pellets were resuspended in 200 μl PBS and 2 ml of ice-cold 70% ethanol was added to the cell suspension (whilst being vortexed) and left for at least 30 min at 4˚C. The cells were centrifuged at 2500 rpm (600 ***g***) for 5 min (4˚C), resuspended in 800 μl of PBS into which 100 μl RNase (1 mg ml^{-1}) and 100 μl propidium iodide (400 μg ml^{-1}) were added. After a 30 min incubation period at 37˚C, cell cycle analysis was performed using an argon-ion laser (Coulter Ltd, Luton UK) operated at 488 nm. Following pulse shape analysis and gating on a cytogram of orthogonal *vs* forward light scatter, a DNA histogram of cell number against red (DNA-PI) fluorescence was recorded.

Colony-forming assays

Cells were plated in a T-25 tissue culture flask (2×10^5 cells/5 ml) and exposed to ZD9331 1 μM) once exponential growth had been reached. After 4, 24 or 48 h drug exposure, cells were harvested using cell dissociation solution (Sigma) and suspended in 8.5 ml of culture medium containing non-dialysed FBS. After centrifugation at 1500 rpm (300 ***g***) for 5 min (room temperature), cells were resuspended in 2 ml of the same medium and then diluted to between 400–1000 cells/10 ml and plated onto 10 cm Petri dishes. The cells were left to form colonies for 14 days, after which the colonies were stained with 0.2% crystal violet in PBS containing 10% formalin and those with greater than 50 cells counted. The average plating efficiency for each cell line was approximately 40%. The loss of colony formation was expressed as the percentage plating efficiency of the test cells compared to the plating efficiency of the control cells.

Measurement of DNA damage by alkaline elution

The method used to measure DNA single-strand breaks following TS inhibition has been described previously (Curtin et al, 1991). Irradiated internal standard cells were co-eluted with experimental cells to allow standardization of elution rate. For studies measuring mature DNA damage, experimental cells were incubated with 0.4 μCi [^{14}C] Thd and internal standards were incubated with 1 μCi [methyl-^{3}H] Thd for 24 h. After a 4–24 h post-labelling chase period in fresh medium, the medium was replaced with ZD9331-containing medium for 4, 24 or 48 h. For measurements of nascent DNA damage, radiolabelled Thd was present for only the last 4 h of drug treatment prior to harvesting. For quantitation, the retention of sample cell DNA (^{14}C) was evaluated at the point when the retention of internal standard (^{3}H) was 0.5. The rad equivalents were determined in each experiment as previously described (Kohn, 1981).

UDG assay

5×10^5 cells were lysed (50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 0.1% NP40, 50 mM NaF and 5 mM EDTA) frozen at –80˚C, thawed, and sonicated twice for 30 s (10 amplitude microns, Soniprep 150 MSE). Following centrifugation at 9000 ***g*** for 10 min at 4˚C the supernatant was used to determine UDG activity. The UDG assay (Caradonna and Cheng, 1980) is based on the release of ^{3}H-uracil from a [^{3}H] uracil-containing calf thymus DNA preparation that was kindly prepared by Dr Salvatore Caradonna. The assay was performed using the procedures described previously (Muller-Weeks and Caradonna, 1996). Briefly, reactions took place at 37˚C in a volume of 100 μl containing 45 μl 100 mM Tris HCI pH 7.5, 4 mM DTT, 10 mM EDTA, 0.2 mg ml^{-1} BSA, 2 μl calf thymus [^{3}H]uracil-DNA substrate (8.35 nmoles ml^{-1}) and 3 μl water. The reaction was

initiated by the addition of 50 µl of diluted cell extract and left for 5 to 10 min. 25 µl of salmon sperm (DNA 1 mg ml^{-1}) (Sigma) and 25 µl 4M PCA were used to terminate the reaction. After 10 min on ice, the samples were centrifuged at 18 000 rpm (9000 ***g***) in a Micro Centaur (MSE) centrifuge for 10 min and the amount of radioactivity in the supernatant counted. A unit of UDG activity was expressed as the amount of glycosylase required to release 1 pmol of uracil min^{-1} mg^{-1} of protein at 37°C.

Western immunoblot analysis

Following exposure of 1×10^6 cells to 1 µM ZD9331 for 4, 24 or 48 h, Western blot analysis was performed according to standard protocol, and the protein bands of interest were probed using either a mouse monoclonal antibody to p53 (D01) (Autogen Bioclear, UK, Calne, Wiltshire) or p21 (Santa Cruz, clone 19). The expression of tubulin was also determined using a mouse monoclonal anti-α-tubulin (Clone B-5-1-2, Sigma) to provide evidence of equal protein loading. In irradiation studies, cells were exposed to 5 Gy delivered using a ^{137}Cs source-to-flask distance of 40 cm and a dose rate of 1.36 Gy min^{-1} then left for 4 h before harvesting. Visualization of the protein bands of interest was performed using enhanced chemiluminescence (ECL) reagents (Amersham International).

Protein estimation

Protein concentrations were measured in cell lysates using the BCA assay (Perbio) according to the manufacturer's instructions.

RESULTS

Changes in the cell cycle following ZD9331

Representative DNA histograms generated following ZD9331 (1 µM) treatment of A549, HX147 and MOR cells for 4 and 24 h are shown in Figure 1. Prior to ZD9331 exposure, all cell lines showed a cell cycle profile typical of cells in log phase growth, with most cells in G1 and the remaining cells distributed between S and G2/M. A 4 h exposure to ZD9331 resulted in the disappearance of a discernible G2/M peak in all cell lines. A longer drug exposure (24 h) caused an increase in the percentage of cells in G1 and a decrease in the percentage of cells in S phase. In A549 cells, there was evidence of a sub G1 peak that may represent cell debris following a 24 h exposure to ZD9331. By 48 h, this peak had broadened and a sub-G1 peak was clearly discernible (data not shown). Profiles of this type result from the presence of apoptotic cells (Darzynkiewicz et al, 1992).

Reversal of ZD9331 cell cycle effects following re-suspension in DFM

The HT29 dutE7 and CON cell lines showed similar cell cycle profiles following a 24 h exposure to 1 µM ZD9331 i.e. loss in the G2/M peak (Figure 1). To establish whether the drug-treated cells retain their ability to synthesize DNA and re-enter the G2/M phase once extracellular drug is removed, dutE7 and CON cells were treated with 1 µM ZD9331 (24 h) and then resuspended in non-dialysed DFM for a further 24 h before cell cycle analysis. Once the drug was removed from the medium, the G2/M peak in both cell lines re-

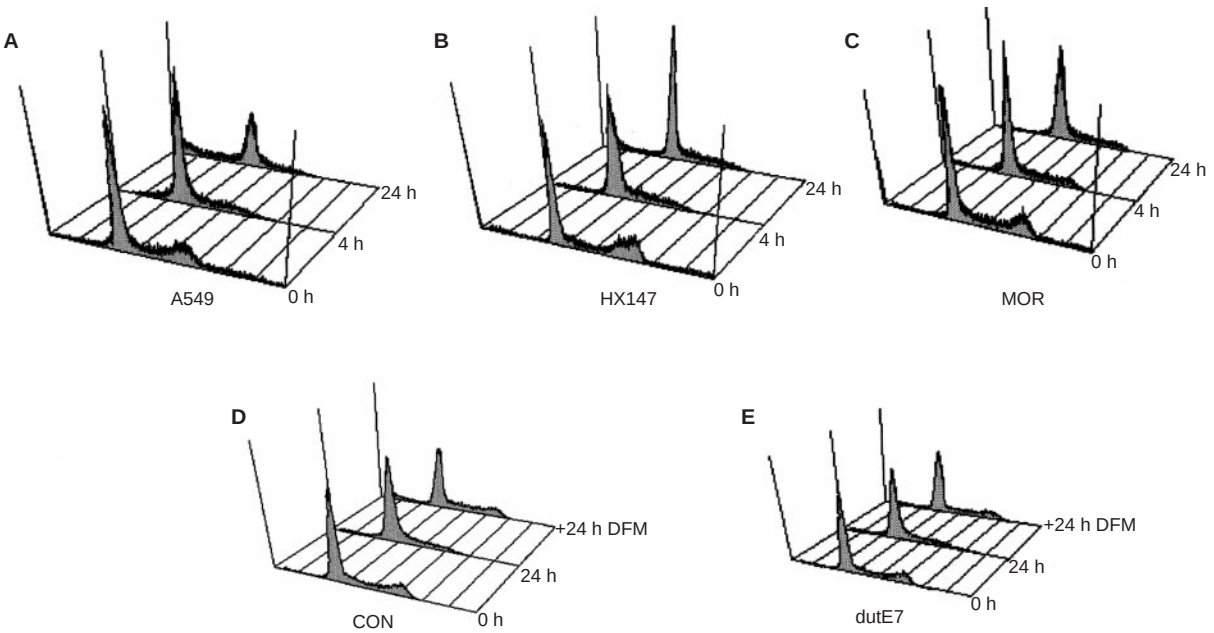

Figure 1 DNA histograms of cell cycle changes following 1 µM ZD9331 in A549 (**A**), HX147 (**B**) and MOR (**C**) cells at 0, 4 and 24 h after ZD9331 (1 µM) and in CON (**D**) and dutE7 (**E**) cells 0 h and 24 h after ZD9331 (1 µM) and after a further 24 h period in drug-free medium (DFM). At the indicated times after dosing, cells were fixed with 70% ethanol and stained with propidium iodide as described in the Materials and Methods. Histograms are representative of the results of 2 experiments

emerged indicating that some cells at this point were capable of leaving S phase and could re-enter the G2/M phase of the cell cycle.

ZD9331 induced cytotoxicity

The 24 h growth inhibitory IC_{50} concentrations previously determined by MTT assay for these cell lines were 0.11, 0.17 and 0.28 μM for A549, HX147 and MOR respectively (Webley et al, 2000). A 4 h exposure to ZD9331 (1 μM) had no effect on viability in any of the cell lines. However, by 24 h, A549 cells showed a significant ($P < 0.0001$) loss of viability (7.7% of control) which was further reduced to 0.96% of control after 48 h in drug (Figure 2). The viability of HX147 cells was also significantly reduced to 36.5 and 2.9% survival at 24 h ($P = 0.01$) and 48 h ($P < 0.001$) respectively. In contrast, MOR cells were more resistant to the cytotoxic effect of ZD9331 at 24 h compared to both HX147 and A549 cells, and did not begin to significantly lose the ability to form colonies until 24–48 h in drug ($P < 0.0001$). Indeed MOR cells were significantly ($P < 0.05$) more resistant to a 24 h exposure to ZD9331 than A549 cells.

The 24 h growth inhibition IC_{50} concentrations determined by MTT assay for CON and dutE7 were 0.22 μM ± 0.05 and 15.6 μM ± 3 respectively. The 120 h IC_{50} values were 0.04 μM ± 0.01 and 0.152 μM ± 0.04 for CON and dutE7 respectively (Brown et al, 1997). By 24 h ZD9331 was significantly more cytotoxic in the neotransfected controls than in the dUTPase transfected cells (Figure 3). By 48 h however both cell lines showed a similar loss of viability (1.27% and 5.3% for CON and E7 cells respectively) although the protective effect of dUTPase was still evident.

Effect of ZD9331 on DNA integrity

Nascent DNA

A 4 h exposure to ZD9331 (1 μM) caused no measurable effect on nascent DNA in A549 cells but by 24 h substantial DNA damage was measured (Table 1). In contrast, MOR cells showed an earlier and greater amount of damage to nascent DNA. HX147 cells showed a similar pattern of DNA damage in response to ZD9331 as A549 cells.

Table 1 Nascent and mature DNA damage rad equivalents.

Nascent DNA damage			
	A549	**MOR**	**HX147**
4 h	0	117 ± 5.4	0
24 h	264 ± 9.8	500: 608	134 ± 153.4
Mature DNA damage			
	A549	**MOR**	**HX147**
4 h	0	0	ND
24 h	471 ± 95	134 ± 28	153 ± 21
48 h	268: 279	281: 295	ND

Data represents the mean of 2–3 experiments conducted in duplicate (± SD). ND=not determined.

Mature DNA

In A549 cells no damage to mature DNA was measured following a 4 h exposure to ZD9331. However by 24 h, damage to mature DNA was substantial (471 ± 95 rad equivalents) (Table 1). In contrast 3–4-fold less damage was observed in MOR cells following a 24 h exposure to ZD9331. A 48 h exposure to drug resulted in a similar extent of damage to mature DNA in A549 and MOR cells. HX147 cells had less mature DNA damage compared to A549 cells.

Uracil DNA glycosylase (UDG) activity

A549 cells had significantly ($P > 0.05$) higher UDG activity (932 ± 248 pmole uracil min^{-1} mg^{-1}) compared to MOR and HX147 cells (201 ± 30.6 and 166.9 ± 16.4 pmole uracil min^{-1} mg^{-1} respectively) (Figure 4). UDG activities in CON and dutE7 were not significantly different (404 ± 88 and 454 ± 238 pmole uracil min^{-1} mg^{-1} respectively).

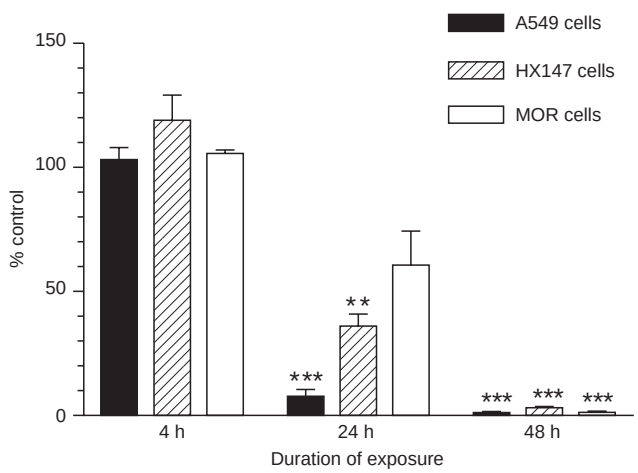

Figure 2 Effect of ZD9331 on colony formation in 3 human lung tumour cell lines. Cells were treated with 1 μM ZD9331 for the indicated times before plating out in DFM. The data represents the results of 3 experiments carried out in duplicate. Bars represent SD.*** and ** denotes a significant ($P < 0.0001$ and $P < 0.01$ respectively) loss of viability

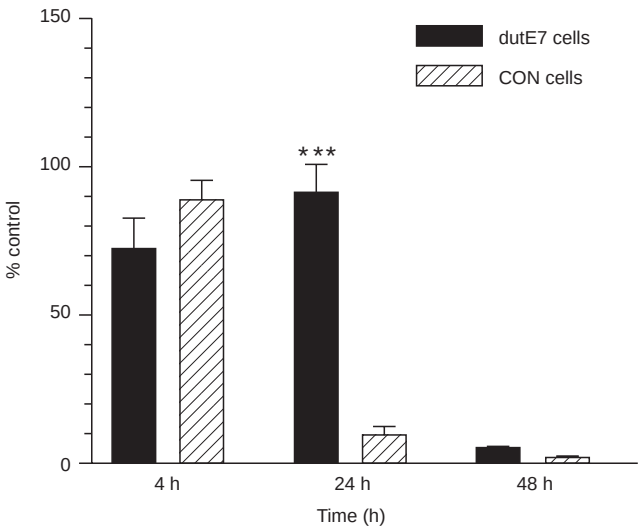

Figure 3 Effect of ZD9331 on colony formation in HT29 dutE7 and CON cell lines. Cells were treated with 1 μM ZD9331 for the indicated times before plating out in DFM. The data represents the results of 3 experiments carried out in duplicate. Bars represent SD. *** and ** denotes a significant ($P < 0.0001$ and $P < 0.01$ respectively) loss of viability

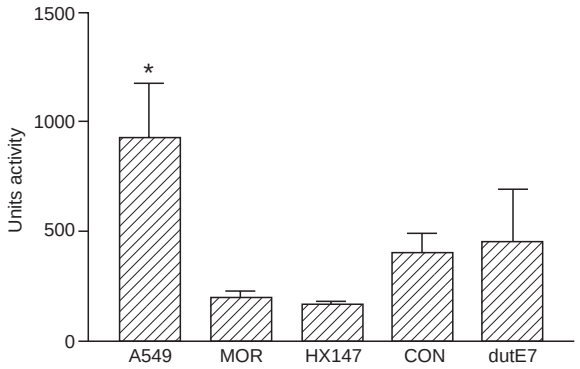

Figure 4 UDG activity. Cells in exponential growth were harvested and assayed for UDG activity as detailed in Materials and Methods. UDG activity is expressed as pmole uracil released min^{-1} mg^{-1} protein. The data represents the results of 3–4 experiments carried out in duplicate. Bars represent SD. * denotes a significantly ($P < 0.05$) higher UDG activity in A549 cells than MOR and HX147 cells

Changes in the expression of p53 and p21 following exposure to ZD9331

Using Western blot analysis, levels of p53 were undetectable in all 3 untreated human tumour cell lines. The functional p53 status in these cell lines was confirmed by irradiation. Following irradiation (5 Gy), only A549 cells showed increased p53 expression. Low expression of p21 protein was detected in A549 control cells and was markedly elevated following irradiation. However, p21 was not detectable in either MOR or HX147 before or after irradiation (Figure 5A). Treatment with ZD9331 caused a time-related increase in p53 protein in A549 cells; p53 was clearly detected by 24 h and rose considerably by 48 h (Figure 5B). Expression of p21 was also increased in response to ZD9331. These proteins were not detected in MOR or HX147 cells following ZD9331 exposure.

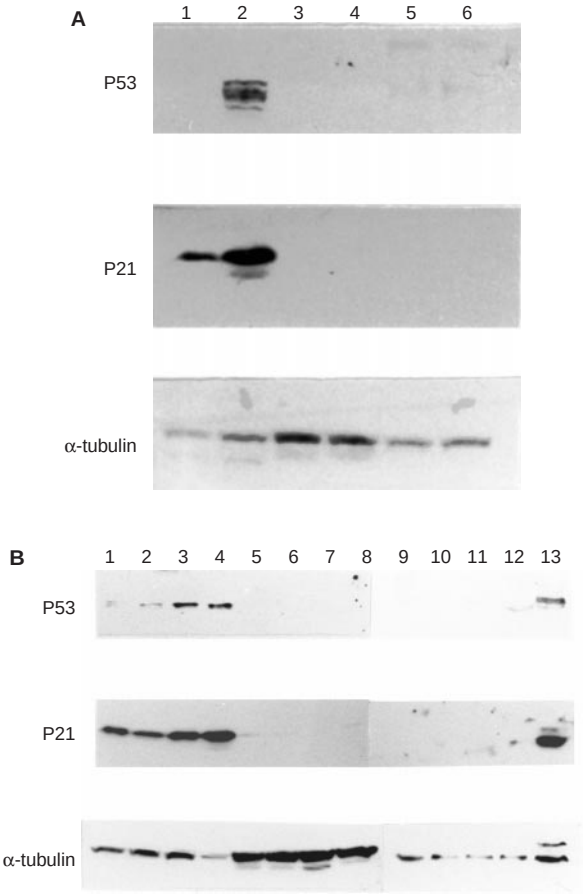

Figure 5 Changes in the level of p53 and p21 following (**A**) irradiation and (**B**) 1 μM ZD9331. (**A**) Lanes: (1) A549 control; (2) A549 5 Gy; (3) MOR control; (4) MOR 5 Gy; (5) HX147 control; (6) HX147 5 Gy. (**B**) Lanes: (1) A549 control; (2) A549 4 h; (3) A549 24 h; (4) A549 48 h; (5) MOR control; (6) MOR 4 h; (7) MOR 24 h: (8) MOR 48 h; (9) HX147 control; (10) HX147 4 h; (11) HX147 24 h; (12) HX147 48 h; (13) 5 Gy A549 positive control. Tubulin was used to confirm equal protein loading

DISCUSSION

The main aim of this study was to further investigate the relative roles of dTTP and uracil misincorporation in cellular sensitivity to TS inhibition. 3 human tumour cell lines and a pair of isogenic cell lines differing only in the expression of dUTPase have been used. Against a background of dTTP depletion (>80% compared to untreated controls), A549, HX147 and MOR cells accumulate markedly different amounts of dUTP (57, 17 and 1.2 pmole/10^6 cells respectively) following a 24 h exposure to 1 μM ZD9331 (Webley et al, 2000). A longer duration of exposure to the drug served to further expand the dUTP pools in A549 and HX147 cells but had little effect on MOR cells. This difference in dUTP accumulation broadly related to dUTPase activity and protein expression. The cell lines also displayed differences in sensitivity to the growth inhibitory effects of ZD9331 depending on the duration of exposure; A549 cells were equally sensitive to a short and long exposure, whereas MOR and HX147 cells were more sensitive to a long drug exposure. The current study has addressed whether the extent of dUTP accumulated is related to changes in the cell cycle, loss of viability and DNA damage following treatment with ZD9331. In addition, the roles of p53 and p21 in mediating cell cycle and cytotoxic events following TS inhibition have also been addressed.

The viability of the cell lines after ZD9331 exposure depended on the duration of exposure; A549 cells showed an earlier (24 h) cytotoxic response compared to MOR cells which did not significantly lose clonogenicity until 48 h. ZD9331 was less cytotoxic to HX147 cells than A549 cells. A direct association therefore exists between the amount of dUTP formed and the time required to significantly lose viability. This observation is similar to that previously reported with fluoropyrimidines (Canman et al, 1993). Following exposure to FdUrd, loss of viability was later (24–32 h) in SW620 cells which accumulated low levels of dUTP than in HT29 cells (10–16 h) that accumulated 45-fold more dUTP. The neotransfected HT29 cells (CON) (mutant p53) accumulate a similar level of dUTP as A549 cells (wild-type p53) following exposure to ZD9331 (Brown et al, 1997). The former also showed an early loss in clonogenicity compared to the dUTPase transfected dutE7 HT29 cell line although this difference was lost at later time points. This observation has been reported with other TS inhibitors (Canman et al, 1994; Parsels et al, 1998). In addition, a greater difference in the level of growth inhibition was observed between CON and dutE7 at 24 h (71 fold), than at 120 h (3.8 fold). Therefore, against the same genetic background, reduced dUTP pools (and presumably DNA damage) can decrease the cytotoxic response to TS inhibition but this effect is largely time dependent.

Alterations in the cell cycle in response to antifolates such as methotrexate (Taylor et al, 1982; Savage and Prasad, 1988) RTX (Matsui et al, 1996; Tonkinson et al, 1997) and other TS inhibitors (Skelton et al, 1998) have previously been reported. The cell cycle effects of TS inhibition (S-phase arrest resulting in reduced numbers of cells in G2/M and increased numbers of cells in G1) appears to be a universal consequence of TS inhibition and also occur following ZD9331 exposure. The cell cycle pattern typical of TS inhibition was observed by 4 h following drug treatment in all the cell lines studied. Thus in contrast to cytotoxicity, cell cycle arrest does not appear to depend on variations in dUTP accumulation (or as discussed later, p53 function). Also, the differential cytotoxicity observed is occurring during a cell cycle arrested state.

Re-suspension of ZD9331-treated cells (HT29 CON and dutE7) in drug-free medium restored normal S phase progression for up to 24 h at least in some cells. Thus arrest at the G1/S border may be a consequence of incomplete DNA synthesis due to reduced dTTP pools and not the irreversible inactivation of the DNA synthesis machinery. The RTX induced S-phase arrest in HCT-8 and SW620 cells was also shown to be due to the block in DNA synthesis as result of dTTP depletion (Matsui et al, 1996) and the addition of exogenous thymidine to the arrested cells restored normal S-phase progress.

The formation of DNA single strand breaks has widely been reported following treatment with TS inhibitors (Curtin et al, 1991; Yin et al, 1992; Canman et al, 1993). Nascent DNA damage in response to ZD9331 was observed in MOR cells as early as 4 h and by 24 h a significant extent of damage was measured. In contrast, A549 cells were less sensitive to this type of damage. However, cytotoxicity in MOR cells was significantly less than in A549 cells although it is thought that the inhibition of nascent DNA synthesis for an extended period of time would eventually result in commitment to cell death (Houghton et al, 1994).

It is known that cells in S-phase are most sensitive to the effects of TS inhibitors (Tang et al, 1996) and it is not clear why a larger degree of nascent DNA damage was not found in the A549 cell line. More damage of this nature may have been expected since dTTP pools were depleted (> 80%) to the same extent as in the other cell lines (Webley et al, 2000), and because of the large accumulation of dUTP that occurs following ZD9331 treatment. Damage to mature DNA is believed to reflect the inability to repair spontaneously occurring damage and in this context, may reflect the effect of the misincorporation process.

The presence of mature DNA single strand breaks is consistent with the corresponding loss of viability following treatment with ZD9331. Previous work found that the extent of mature DNA damage in HT29 and SW620 was proportional to the intracellular dUTP accumulated in these cells following FdUrd exposure (Canman et al, 1993). The extent of dUTP accumulation has also been shown to correlate with the extent of DNA damage (Curtin et al, 1991). It has been demonstrated that by transfecting HT29 cells with the antisense-expressing construct of the DUT-N open reading frame, dUTP accumulation and DNA damage can be increased (Ladner and Caradonna, 1997). As expected from the large difference in dUTP accumulation, A549 cells had higher levels of mature DNA damage than MOR and HX147 cells. UDG is a repair enzyme that rapidly removes misincorporated uracil from DNA creating alkaline labile apyrimidinic sites. The significantly ($P < 0.05$) higher UDG activity in A549 cells compared to HX147 cells, may have also contributed to the formation of mature DNA damage.

The similarity between the 24 and 120 h IC_{50} concentrations for ZD9331 in A549 cells (Webley et al, 2000) suggests that these cells are largely committed to cell death within 24 h of drug exposure. This may be due to dUTP accumulation and the level of mature DNA damage that occur during the first 24 h exposure to ZD9331. Following a 48 h exposure to ZD9331 DNA damage was reduced, presumably because of cell loss due to apoptosis. MOR cells required a longer period of exposure to ZD9331 to show significant mature DNA strand breaks (compared to A459 cells). Other studies, using cells that do not accumulate dUTP during a thymineless state, have noted that the appearance of significant damage to mature DNA is a late event occurring between 24 and 48 h (Houghton et al, 1994; Sundseth et al, 1997).

Exposure of A549, MOR and HX147 cell lines to radiation confirmed that only the A549 cells have functional p53 and it was therefore not surprising that only A549 cells showed an elevation in p53 following ZD9331. However, all cell lines displayed an obvious G1/S arrest after only a 4 h exposure to ZD9331. In addition, this arrest occurred before the large rise in p53 protein expression in A549 cells. The same cell cycle effects following ZD9331 were observed in the p53-mutant HT29 dUTPase transfected cell line (dutE7) and its neotransfected control. p53 has been reported to play a passive role in RTX-induced cell cycle perturbation in HCT-8 (wt53) and SW480 (mutant p53) (Matsui et al, 1996).

Cellular response to TS inhibitors is complex but is probably mediated through a combination of both dTTP depletion and uracil misincorporation. It is difficult to evaluate the relative importance of these concurrent effects as dUTP accumulation will occur (to different extents) only in the presence of dTTP depletion. The expression of enzymes such as dUTPase, repair enzymes such as UDG and downstream factors (Fisher et al, 1993) are also important factors. Nevertheless, these studies have confirmed that the cell cycle events that are typical of TS inhibition occur regardless of p53 function and are independent of dUTP accumulation. However, there is an association between *early* cytotoxicity to TS inhibitors and the extent of dUTP accumulation. Importantly, in some cell lines, (e.g. MOR) the mechanism of cell death following TS inhibition is dUTP independent confirming the importance of dTTP depletion and other dNTP perturbations to the process of eventual cell death. It could be argued that the early loss of viability observed in the high dUTP accumulating A549 cell line, is due to the presence of functional p53 rather than uracil misincorporation per se. However, in the p53 mutant HT29 cell line high accumulation of dUTP appears to confer early cytotoxicity compared to a dUTPase expressing transfect. The use of paired cell lines with known p53 function (e.g. transfected with p53 inducible constructs or p53 'knockouts') would provide further insight into the relative roles of dUTPase and p53 in promoting cell death in TS inhibited cells. In addition, high UDG activity in cell lines that accumulate dUTP may exacerbate the effects of TS inhibitors thereby promoting DNA damage. The therapeutic importance of dUTPase and UDG expression in relation to p53 status remains to be determined and further studies using appropriately transfected (dUTPase and UDG) cell lines are warranted.

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