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Expression of uracil DNA glycosylase (UDG) does not affect cellular sensitivity to thymidylate synthase (TS) inhibition

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Abstract

Uracil DNA glycosylase (UDG) is a base excision repair enzyme responsible for the removal of uracil present in DNA after cytosine deamination or misincorporation during replication. Inhibition of thymidylate synthase (TS), an important target for cancer chemotherapy, leads to deoxythymidine triphosphate (dTTP) pool depletion and elevation of deoxyuridine monophosphate (dUMP) pools which may also result in the accumulation of deoxyuridine triphosphate (dUTP). Large quantities of dUTP are believed to overwhelm the pyrophosphatase dUTPase, leading to misincorporation of uracil into DNA. Uracil is removed from DNA by uracil DNA glycosylase (UDG) resulting in an abasic site, but since the ratio dUTP:dTTP may remain high during continuing TS inhibition uracil can become re-incorporated into DNA causing a futile cycle eventually leading to DNA damage and cell death. This study has used isogenic cell lines differing in their expression of UDG to investigate the role of this enzyme in sensitivity to the specific TS inhibitors, ZD9331 and raltitrexed. The study showed that although increased expression and activity of UDG may lead to increased cell growth inhibition after TS inhibition over the first 24 h of treatment (measured using 3-(4,5-dimethyl (thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), probably due to increased damage to single-stranded DNA, the level of enzyme expression does not affect cell viability or cell death (measured using clonogenic assay, cell counting of attached/detached cells and cleavage of both poly ADP-ribose polymerase (PARP) and caspase 3). Increased expression and activity of UDG did not affect sensitivity to TS inhibition at later time points (up to 72 h treatment). Therefore UDG does not appear to play a major role in the response to TS inhibition, at least in the model used, and the results suggest that other determinants of response previously investigated, such as TS and dUTPase, may be more important for the response to TS inhibition.

Keywords: Thymidylate synthase; Uracil DNA glycosylase; ZD9331; Raltitrexed

1. Introduction

Thymidylate synthase (TS) catalyses the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to form 2′deoxythymidine-5′monophosphate (dTMP). TS is an important target for cancer chemotherapy as it provides the sole *de novo* source of thymidylate (dTMP) which is essential for DNA synthesis and repair [1]. TS is an important target for cancer chemotherapy.

The importance of TS as a chemotherapeutic target is now well established. 5-fluorouracil (5-FU), a fluoropyrimidine drug, is widely used in the treatment of breast, gastrointestinal, and head and neck cancers [1]. Several specific TS inhibitors have recently been clinically evaluated. These include the quinazoline antifolates, raltitrexed (Tomudex, ZD1694 [2] and ZD9331 [3,4]).

However, the mechanisms of cell death following inhibition of TS are not clearly defined. Following TS inhibition, deoxythymidine triphosphate (dTTP) pools become depleted and dUMP pools increase which may also result in the accumulation of 2′-deoxyuridine-5′triphosphate (dUTP) [5–7]. Since DNA polymerase recognises dUTP and dTTP with equal efficiency [8], uracil may become misincorporated into DNA during periods of TS inhibition if the pyrophospatase dUTPase

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is overwhelmed, when the ratio of dUTP:dTTP is high [6]. Uracil is not normally found in DNA due to the activity of dUTPase and the base-excision repair enzyme uracil DNA glycosylase (UDG) [9]. If incorporated into DNA, uracil is excised by UDG, but the resulting abasic site is likely to be refilled by uracil due to the continuing high dUTP:dTTP ratio. This may cause a futile cycle of misincorporation, excision, repair and further misincorporation resulting in DNA strand breaks and cell death (reviewed in Ref. [10]).

Studies in prokaryotic and eukaryotic systems have shown that uracil misincorporation may occur after inhibition of TS (reviewed in Ref. [10]). In addition, the extensive misincorporation of uracil into DNA is lethal in bacteria [11] and yeast [12] and likely all cellular systems. Although the reason for this is not completely known, it is thought that UDG-mediated DNA repair is central to the process [13–18]. Ultimately these events may inhibit daughter strand synthesis or result in the induction of extensive single strand breaks leading to hyper-recombination, DNA fragmentation and cell death [15–17].

Several studies, using isogenic cell line models, have shown that dUTPase plays an important role in the sensitivity to TS inhibition [19–22] especially during the first 24 h of treatment [6,22]. However, relatively little attention has been paid to the role of UDG in determining response to TS inhibition. In addition, a recent report showed high variability in tumour UDG, elevated tumour: normal tissue ratio, and an association with proliferation for UDG in human colorectal cancer [23].

This study has used isogenic cell lines differing in their expression of UDG to assess the role of UDG in determining response to the specific TS inhibitors ZD9331 and raltitrexed (ZD1694).

2. Materials and methods

All standard laboratory chemicals used in this study were commercial products of AnalaR® grade purchased from either British Drug Houses (BDH) (Poole, Dorset, UK) or from Sigma (Poole, Dorset, UK). ZD9331 and raltitrexed (ZD1694) were synthesised at Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK) and the procedures for their synthesis have been previously described in Refs. [24,25], respectively. Both compounds were dissolved at 10 mM in 0.1 M $NaHCO_3$ (pH 8.3). The dissolved compounds were then passed through a 0.22 μm filter and stored at −20 °C for a maximum of 3 months.

2.1. Cell lines

HX147 human lung carcinoma and A549 human lung carcinoma cell lines were obtained from ATCC (American Tissue Culture Collection, Manassas, VA, USA). All adherent cell lines were maintained in Dulbeccos Modified Eagle (DMEM) tissue culture medium (Gibco, Paisley, Scotland, UK), supplemented with 10% heat inactivated (56 °C, 30 min) dialysed fetal calf serum (dFCS) (ICN Flow, Thames, Oxfordshire, UK), 2 mM L-glutamine, 50 μg/ml gentamycin and 2.5 μg/ml fungizone (all from Gibco). All cells were Mycoplasma-negative, as tested using the polymerase chain reaction (PCR) (Stratagene, UK) at the time of this study.

For determination of cell doubling times, 10^5 cells were seeded in 25 cm^2 tissue culture flasks. Cells were trypsinised off 24, 48, 72, 96 and 120 h following the initial seeding and counted using a haemocytometer. Doubling times were determined as the time taken for the cell number to double over the linear range of growth.

2.2. Transfection

The cDNAs for the nuclear form of UDG were kindly provided by Dr Rein Aasland (pUNG15H) [26] and Dr Robert Ladner (UDG1A) [27]. pUNG15H is a derivitive of the original pUNG15 cDNA from which the 3′ untranslated region has been removed by cutting pUNG15 (in pGEM7ZF(+)) with *HindIII* and religating into pGEM7ZF(+). The pUNG15H and UDG1A coding regions were obtained in plasmids pGEM7ZF(+) and pGEM3Z, respectively, which are designed for expression in bacteria. pUNG15H and UDG1A cDNAs were therefore transferred into pEFIRES-P (also known as F373) using standard techniques. This is a plasmid vector designed for expression of protein in eukaryotic cells in which the gene of interest and the puromycin resistance gene (*pac*) and the gene of interest are translated from a single bicistronic message [28]. The final vectors containing pUNG15H and UDG1A cDNA were designated F504 and F502, respectively. HX147 cells were transfected using Lipo-TAXI transfection reagents (Stratagene) according to the manufacturer's instructions.

2.3. Drug exposure

To measure the response of cells to ZD9331 and raltitrexed, cell lines were exposed to the drugs for 0, 24, 48 or 72 h at doses standardised to IC_{50} values. At the end of drug exposure, cells were harvested using trypsinisation and washed with phosphate-buffered saline (PBS). IC_{50} values (the concentration causing 50% cell growth inhibition) were determined using 24 or 72 h 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as previously described in Ref. [6]. After drug exposure for the required time, the medium of the cells treated with raltitrexed was replaced every 48 h with fresh DMEM containing 10 μM thymidine to prevent further inhibition of TS.

2.4. Cell viability (clonogenic) assays

Cells were plated at a density of 500–1000 cells/well in six-well plates (Falcon) and allowed to adhere overnight. Drugs were diluted in un-supplemented DMEM and added at the required concentration to each well. DMEM was added to control wells. Following exposure to drug for 24 or 72 h, the wells were washed three times with DMEM then fresh DMEM was added and colonies were allowed to form for 10–14 days. Colonies were then stained with 0.5% methylene blue and those of >50 cells counted. Experiments were repeated three times, with six replicates in each experiment. Plating efficiency was determined as the percentage of untreated cells able to form colonies.

2.5. Measurement of UDG activity

UDG activity was measured as previously described [7,29]. Briefly, 5×10^5 cells were lysed in 0.5 ml lysis buffer (50 mM Tris–HCl (pH 7.4), 0.25 M NaCl, 0.1% Nonidet P40 (NP40), 50 mM NaF and 5 mM ethylene diamine tetra-acetic acid (EDTA) and were freeze/thawed and sonicated twice (10 amplitude microns, Soniprep 150 MSE). Supernatants were then collected by centrifugation at 18 000 rpm for 10 min in a MSE Microcentaur centrifuge at 4 °C.

To prepare ^{3}H-labelled uracil-containing DNA for use as the substrate in the assay, 15 μl of 1 mg/ml calf thymus DNA was labelled with ^{3}H-dUTP (Amersham) using random priming (Random Primers DNA Labelling System, Gibco). The percent incorporation of label was determined using a glass fibre filter disk assay (as described in the Random Primer DNA Labelling Kit). The sample was then applied to a G-50 Sephadex spin column to remove any unincorporated nucleotides.

The UDG assay was carried out at 37 °C in a volume of 100 μL containing 45 μl 100 mM Tris–HCl pH 7.5, 4 mM dithiothreitol (DTT), 10 mM EDTA, 0.2 mg/ml bovine serum albumin (BSA), 2 μl calf thymus [^{3}H] uracil-DNA substrate (8.35 nmol/ml) and 3 μl water. The reaction was initiated by adding 50 μl of diluted cell preparation (1:25 dilution in dH_2O) and left to proceed for 10 min. The reaction was terminated by adding 25 μl salmon sperm DNA (1 mg/ml) (Sigma) and 25 μl 4M perchloric acid. After 10 min on ice, samples were centrifuged at 9000*g* for 10 min and the amount of radioactivity in the supernatant was counted. Protein concentrations in the extracts were determined using the Pierce BCA assay kit (Pierce, UK). A unit of UDG activity was expressed as the amount of glycosylase required to release 1 pmol of uracil/min/mg protein at 37 °C.

2.6. Immunoblotting

Western blotting was carried out as previously described in Ref. [7]. Total cellular protein was isolated from cells in lysis buffer containing proteinase inhibitors, and equal amounts (50 μg) of protein were separated by sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) (8–16% tris/glycine gels purchased from Novex, Germany) and electroblotted onto nitrocellulose membrane. Blots were probed with the following antibodies: rabbit anti-TS (1:1000) [5]; rabbit anti dUTPase (1:1000) [30]; rabbit anti-UDG (1:350) [31]; rabbit anti-caspase 8 (1:2000, Pharmingen, San Diego, CA, USA); mouse anti-poly ADP-ribose polymerase (PARP) (1:10,000; Clontech, Palo Alto, CA, USA) and mouse anti-actin (1:1000, Sigma).

2.7. Single-cell gel electrophoresis (the 'Comet' assay)

Glass slides were coated with 20 μl low gelling temperature agarose (Sigma) in boiled PBS and were allowed to dry for 1 h at 37 °C. A single-cell suspension was prepared by gentle trypsinisation of appropriately treated cells and dilution at 1.6×10^5 cells/ml. 200 μl of cell suspension was mixed with an equal volume of warmed agarose in a pre-warmed universal to give a solution of 0.5% agarose with cells. 150 μl of this solution was layered onto a pre-warmed, pre-coated glass slide (giving 12 000 cells per slide), creating a thin monolayer. The agarose was allowed to set by placing slides at 4 °C for 1 h in the dark. Cells were then lysed in the dark in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 1% SDS, 1% Triton X-100, 10% dimethyl sulphoxide (DMSO), 10 mM Tris, pH 10) for 1 h at 4 °C. Slides were rinsed in electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 12) and incubated in the same buffer for 20 min to allow unwinding of the DNA.

Electrophoresis was carried out for 25 min at 1 V/cm. The 'comets' were then neutralised with 2 ml 0.4 M Tris (pH 7.5) before staining with 5μg/ml ethidium bromide. The comets were viewed under green light (515–560 nm) using a fluorescence microscope (Leica), and stored and analysed using a Bioscan Optimas image analysis program (Bioscan, Washington, USA). Twenty-five random comets at each time point were analysed, avoiding comets within 5 mm of the edge of the agar.

2.8. Statistical analysis

All statistical analysis was performed using Graphpad Prism software. For statistical hypothesis testing, *P* values were determined using an unpaired *t*-test (parametric) and the level of confidence was set at 95%.

3. Results

3.1. Characterisation of UDG-transfected cell lines

Human lung carcinoma HX147 cells possess low levels of UDG activity (approximately 6-fold less

activity than human lung carcinoma A549 cells) [7]. Fig. 1 confirms this finding as HX147 cells showed significantly lower UDG activity than A549 cells (approximately 4-fold greater activity in A549 cells). This value is slightly lower than that previously reported, but this may reflect differences in the cell lines obtained from different sources and that a different substrate was used in each of the studies. Since it has been previously suggested that higher levels of UDG activity may cause an increased sensitivity to TS inhibition [7,22] HX147 cells were transfected with nuclear UDG to investigate the effect.

HX147 cells were transfected with two different cDNAs encoding the nuclear form of UDG (UDG1A and pUNG15H in expression vectors F502 and F504, respectively). Twelve clones were isolated following transfection of HX147 cells with both the F502 and F504 expression vectors. Six clones were isolated following transfection with the empty vector (F373). A range of levels of expression of UDG protein was observed between clones by immunoblotting (1- to 40-fold increased compared with controls) (data not shown). Three UDG over-expressing clones were selected for further characterisation (F502-10, F504-6 and F504-10) and one empty-vector control clone (F373-A). These clones were selected as F504-10 over-expressed UDG protein to the highest extent (>40-fold compared with the parental cell line) and F502-10 and F504-6 clones expressed an intermediate level of UDG protein (5-fold compared with the parental cell line), measured by western blotting. F373-A was used as a control cell line since it expressed UDG protein at a similar level as the parental cell line (data not shown).

The UDG enzyme activity of each of the selected clones was also examined (Fig. 2). HX147 and F373-A showed a similar level of UDG activity, as expected. F502-10 and F504-6 show significantly greater UDG activity than HX147 and F373-A (3.25 ± 1.6 and 2.8 ± 0.7-fold, respectively, ($P\leqslant0.001$). F504-10 cells showed significantly greater UDG activity compared with any of the other cell lines (17.5 ± 3.8-fold) ($P\leqslant0.001$). F502-10 and F504-6 cells expressed levels of UDG within the physiological range observed in a panel of cell lines previously examined. F504-10 cells express higher levels of UDG than any of the cell lines examined previously. These figures are less than those obtained following quantitation of the data obtained using western blotting. This finding may be due to regulation of UDG activity within the cell, as previously described in Ref. [32].

No significant differences were observed in the doubling times of each cell line ($P\geqslant0.05$) (22 ± 7.5, 28.6 ± 5.8, 24 ± 9.5, 22 ± 8.2 and 22 ± 9.0 h for HX147, F373-A, F502-10, F504-6 and F504-10, respectively). In addition, no significant differences were observed in dTTP, 'dUMP' or dUTP pools following 0 or 24 h treatment with 1 μM ZD9331 (Table 1), nor in the levels of TS or dUTPase (data not shown) ($P\geqslant0.05$). A 17-fold variation in dUTPase protein, correlating with dUTPase activity, has previously been reported in a lung tumour cell line panel [6]. HX147 cells showed the lowest dUTPase activity and the second highest accumulation of dUTP after exposure to 1 μM ZD9331 for 24 h in this cell line panel.

3.2. Effect of UDG on sensitivity to TS inhibitors

The specific TS inhibitors ZD9331 and raltitrexed were used to investigate the effect of UDG on the sensitivity to TS inhibition. F504-10 cells with a very high expression of the UDG protein and activity) were significantly more sensitive to ZD9331 and raltitrexed

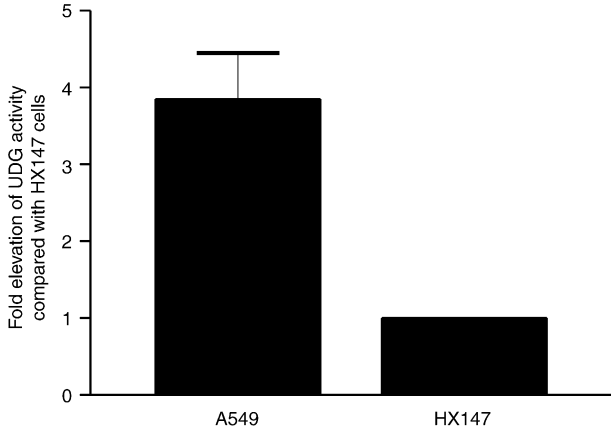

Fig. 1. Uracil DNA glycosylase (UDG) activity in HX147 and A549 cells. Cells were grown to 60% confluency and the basal UDG activity was measured by monitoring the release of ^{3}H-uracil from a ^{3}H-uracil-containing DNA substrate. Data represent the mean±standard deviation S.D. of three experiments carried out in duplicate.

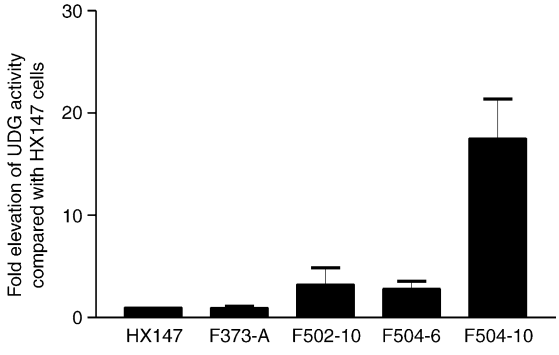

Fig. 2. UDG activity in HX147 UDG transfected cell lines. Basal UDG activity was measured in cell extracts from HX147, F373-A, F502-10, F504-6 and F504-10 cells grown to 60% confluency. The release of ^{3}H-uracil-from a ^{3}H-uracil containing DNA substrate was used to determine the activity of the extracts. Data represent the mean ±S.D. of three experiments carried out in duplicate.

measured using growth inhibition (MTT) assays (Table 2). However, this effect was only observed following 24 h treatment as no statistically significant differences were observed between the cell lines following 72 h treatment with the inhibitors (Table 2).

In contrast to the results seen with growth inhibition assays, no significant differences in cell survival were seen after 24 h treatment between any of the cell lines with either ZD9331 or raltitrexed (1, 4, 7, or 10×24 h IC_{50}) using clonogenic assays (Figs. 3a and b, respectively). No cell survival was observed in any cell line after 72 h treatment with ZD9331 or raltitrexed (10×72 h IC_{50}) (data not shown).

3.3. Effect of UDG on cell death kinetics of TS inhibited cells

The cell death kinetics of each of the cell lines was initially investigated by counting the numbers of cells detaching from the substratum over 72 h treatment with ZD9331 (10×IC_{50}). In agreement with the results from the clonogenic assays, no significant differences were observed between the cell lines at any time point after treatment with ZD9331 (Fig. 4).

3.4. Cleavage of PARP and caspase-3 following treatment with ZD9331

Complete cleavage of PARP was observed after 72 h treatment with ZD9331 in all cell lines (Fig. 5). However, cleavage of caspase-3 was not seen at any point up to 72 h in any of the cell lines (data not shown).

3.5. Nuclear morphology of UDG over-expressing clones following treatment with ZD9331

Fig. 6 shows the nuclear morphology, visualised by confocal microscopy following PI staining, in F504-10 cells and F373-A cells remaining attached to the flasks after treatment with ZD9331 for up to 72 h (10×72 h IC_{50}). Only F373-A cells are shown since they were representative of the parental, F502-10 and F504-6 cell lines in other assays and an identical response was also observed in this assay. No differences between the cell lines were observed in the nuclear morphology over the time course. Increased condensation of DNA was observed over the course of treatment in both cell lines. Confocal microscopy of detached cells (data not shown) confirmed that the cells were dying via apoptosis since

Table 1
dTTP, immunoreactive 'dUMP' and dUTP pools in HX147 UDG transfected cells[a]

	Deoxynucleotide pools		
	dTTP (pmol dTTP/10^6 cells)	"dUMP" (pmol dUMP/10^6cells)	dUTP (pmol dUTP/10^6 cells)
HX147	2.0±1.0	1987±299	20.1±4.0
F373-A	2.8±1.4	1788±310	22.3±3.0
F502-10	2.0±0.5	1988±401	19.6±2.2
F504-6	2.1±0.8	1823±311	21.6±3.1
F504-10	3.1±0.8	1901±250	21.6±2.2

S.D., standard deviation; dTTP, deoxythymidine triphosphate; dUTP, deoxyuridine triphosphate; dUMP, deoxyuridine monophosphate.

[a] Cells were treated with 1 μM ZD9331 for 24 h and cell extracts were prepared for measurements of deoxynucleotide pools using radioimmunoassays. Data represent the mean ±S.D. of three experiments carried out in duplicate. The mean untreated values were not significantly different between cell lines. The mean untreated values (of all cell lines) were 30.1, 54.2 and 2.1 pmol dTTP, dUMP or dUTP, respectively/10^6 cells.

Table 2
Summary of growth inhibition assays for HX147 UDG-transfected cell lines[a]

	IC_{50} (μM)			
	ZD9331 24 h	ZD9331 72 h	Raltitrexed 24 h	Raltitrexed 72 h
HX147	0.25±0.10	0.014±0.01	0.095±0.03	0.008±0.002
F373-A	0.27±0.09	0.019±0.01	0.097±0.03	0.007±0.002
F504-6	0.20±0.04	0.016±0.01	0.078±0.04	0.010±0.004
F504-10	0.06±0.02[b]	0.015±0.01	0.01±0.007[b]	0.009±0.003

MTT, 3-(4,5-dimethythazd-2-yl)-2,5-diphanyltetrazolium bromide IC_{50}, concentration causing 50% inhibition.

[a] HX147 UDG-transfected cells were assayed for growth inhibition using MTT assays after treatment for 24 h and 72 h with ZD9331. Data represent the mean ±S.D. of three experiments carried out in quadruplicate.

[b] Denotes significant sensitivity compared with other cell lines at that time point ($P \leqslant 0.001$).

all the cell lines showed characteristic condensation and fragmentation of DNA associated with apoptosis. This observation is in agreement with data from a previous study showing that cells die via an apoptotic pathway after inhibition of TS [33].

3.6. DNA damage following inhibition of TS

TS inhibition has previously been shown to cause single-strand DNA damage [6,7,34]. Single-strand DNA damage was investigated using single-cell gel electrophoresis (the Comet assay) under alkaline conditions. Table 3 shows significantly greater single-strand DNA damage in F504-10 cells compared with parental, F373-A, F502-10 or F504-6 cells following 16, 20 and 24 h exposure to 1 μM ZD9331 ($P \leqslant 0.001$). However, no significant differences were observed between F504-10 cells and the other cells following 0 or 12 h treatment ($P \geqslant 0.05$). Significantly greater DNA damage was also observed in F504-6 and F504-10 cells after 24 h treatment, as well as after 16 h and 20 h in F504-10 cells, compared with that observed at 0 h in the same cells ($P \leqslant 0.001$). No significant differences were observed between different time points in the other cell lines. However, the errors for this assay were large as a differential response was observed between individual cells within the same population. This effect has been previously noted in Ref. [35]. Individual cells may be affected differently within the population depending on their position in the cell cycle. Since TS inhibitors cause DNA damage in S phase, some cells may be spared damage until they reach S phase (up to a doubling time). However, a broad trend was observed in all cell lines showing an increase in damage over the time course of treatment, but this was not statistically significant.

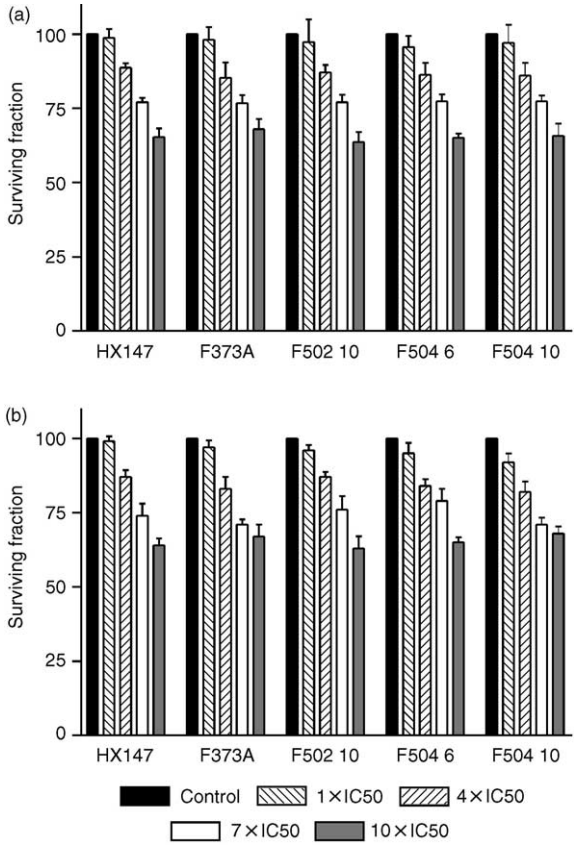

Fig. 3. Survival following treatment of HX147 UDG-transfected cells with TS inhibitors. HX147 UDG-transfected cells were treated with ZD9331 (a) or raltitrexed (b) (1, 4, 7 or 10×24 h IC_{50}) for 24 h then survival was measured using clonogenic assays. Survival was assayed in the presence of 10 μM thymidine after treatment with raltitrexed to ensure rescue from TS inhibition caused by the retention of highly polyglutamated drug. Data represent the mean ±S.D. of three experiments carried out in triplicate. Significant loss of clonogenicity compared with untreated control cells was seen in all cell lines after treatment with 4, 7 or 10×24 h IC_{50} doses of either drug.

4. Discussion

Uracil-DNA glycosylase (UDG) is the base-excision repair enzyme responsible for removing uracil from DNA [36]. TS inhibition has been shown to cause an elevation in dUTP:dTTP ratios which may lead to the misincorporation of uracil into DNA [15,16,18,34,37,38]. In addition to studies in yeast and bacteria showing dUTPase mutants are only lethal in the presence of UDG, several authors have suggested that both UDG and dUTPase may influence the sensitivity of mammalian cells to TS inhibition [7,23,39]. The latter study showed that human lung carcinoma A549 cells were significantly more sensitive to ZD9331 than another cell line with approximately 6-fold less UDG activity (HX147) despite large expansions in dUTP

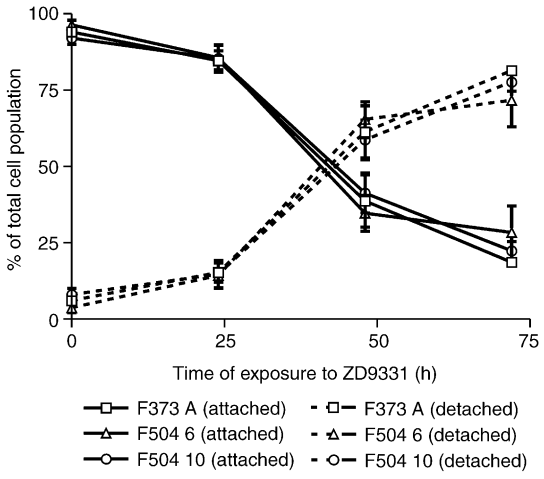

Fig. 4. Cell death kinetics of HX147 UDG-transfected cells following treatment with ZD9331. The numbers of cells attached/detached from the flasks were counted over 72 h treatment with ZD9331 (10×72 h IC_{50}). Data represent the mean ±S.D. of three experiments carried out in duplicate.

pools in both cell lines [7]. In addition, HX147 cells sustained significantly less damage to mature DNA than A549 cells over 24 h treatment with 1 μM ZD9331 and only showed slightly more mature DNA damage than MOR cells (a cell line which does not accumulate dUTP following 24 h exposure to 1 μM ZD9331). However, since these cell lines were not isogenic pairs, the role of UDG was uncertain and the possibility remained that the observed differences in DNA damage were due to factors other than UDG. Dusseau and colleagues [23] also recently reported a high tumour:normal tissue ratio of UDG in colorectal tumours and cell lines.

Isogenic cell lines were therefore produced and characterised as described in this study to determine the importance of UDG expression in determining the sensitivity of cells to TS inhibition. However, UDG appears to only effect drug sensitivity if measured using growth inhibition assays. F504-10 cells with a high expression of the UDG protein and activity compared with control cell lines) were significantly more sensitive over the first 24 h of treatment with ZD9331 and raltitrexed compared with control cells, although no differences were observed after 72 h treatment. Clonogenic and cell counting assays as well as measurements of cell death showed no differences between the cell lines at any time point up to 72 h.

The data observed using growth inhibition (MTT) assays are in agreement with previous studies showing that the effects of dUTP accumulation on the sensitivity of cells to TS inhibitors is most significant during the

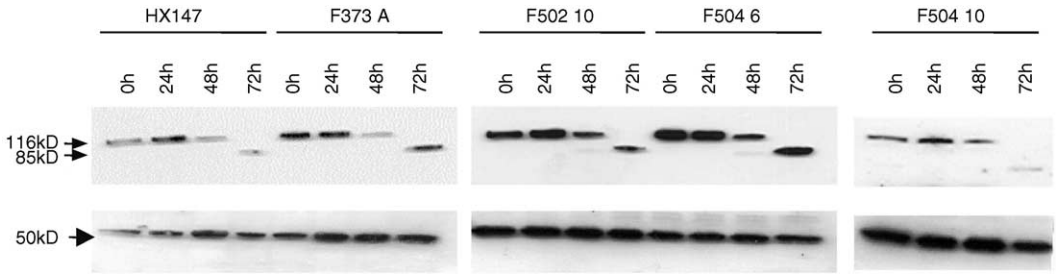

Fig. 5. Cleavage of poly ADP-ribose polymerase (PARP) in HX147 UDG-transfected cell lines following treatment with ZD9331. All selected HX147 UDG-transfected cells were treated for 0, 24, 48 or 72 h with ZD9331 (10×72 h IC_{50}) and were then harvested and lysed. Western blotting was performed to examine the cleavage of PARP (116 kDa) to its 85 kDa cleavage product. The location of the 50 kDa band shows the position of α-tubulin (loading control). Blots are representative of two experiments.

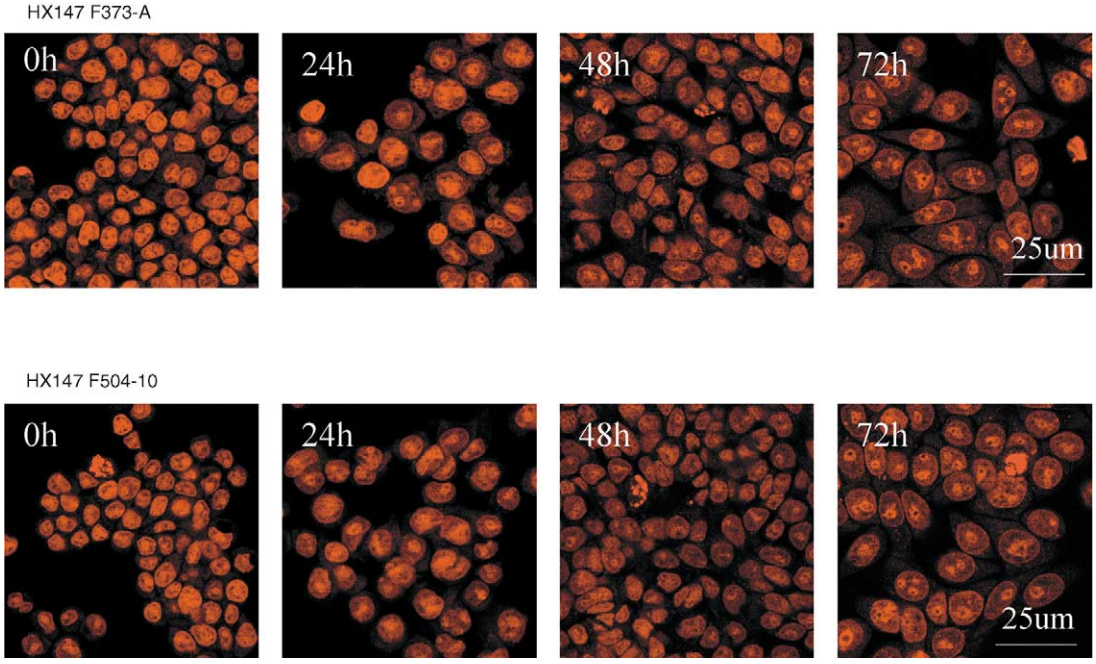

Fig. 6. Effect of UDG on nuclear morphology of HX147 cells transfected with UDG. HX147 F373-A and HX147 F504-10 cells were treated for up to 72 h with ZD9331 (10×72 h IC_{50}). Nuclear morphology in cells remaining attached to the flasks was examined using confocal microscopy following staining with propidium iodide. Photos are representative of three separate experiments.

Table 3
Single-strand DNA damage following treatment with ZD9331. HX147 UDG-transfected cells were treated for 0, 12, 16, 20 or 24 h with 1 μM ZD9331 after which the single strand DNA damage was measured using the Comet assay[a]

	Comet moment ±S.D.				
	0 h	12 h	16 h	20 h	24 h
HX147	0.45±0.1	0.63±0.5	0.91±0.7	0.70±0.7	1.87±1.3
F373-A	0.72±0.5	0.64±0.8	1.18±1.1	1.0±0.5	1.22±2.7
F502-10	0.29±0.3	1.63±1.2	1.3±0.6	2.09±1.2	1.06±1.2
F504-6	0.37±0.1	0.87±0.7	1.45±0.9	1.33±1.0	1.93±0.6[c]
F504-10	0.57±0.3	1.56±1.4	3.82±1.4[b]	4.49±0.7[bc]	4.025±0.6[bc]

[a] The Comet moment was calculated using the Optimas data analysis program. Data represent the mean ±S.D. of three experiments.
[b] Denotes significantly greater DNA damage compared with other cell lines ($P \leqslant 00.001$).
[c] Denotes significantly greater DNA damage compared with 0h sample of the same cell line ($P \leqslant 0.001$).

first 24 h of treatment [6,7,21]. UDG would only be expected to have an effect when dUTP accumulation and uracil misincorporation are relevant. Thus it is not surprising that UDG expression affected sensitivity only during the first 24 h of TS inhibition and as previously shown HX147 cells express low levels of dUTPase and accumulate high levels of dUTP compared with other lung tumour cell lines [6]. UDG would not be expected to play a role in the sensitivity of cells to TS inhibition in the absence of dUTP accumulation since no uracil would be incorporated into DNA and UDG would not be required to maintain DNA integrity, although this has not been addressed in this study.

However, results obtained using growth inhibition assays were not confirmed using clonogenic or cell counting assays, nor the cell death assays. Interestingly, significantly more single-strand breaks were observed in F504-10 cells compared with the other cell lines ($P \leqslant 0.05$). This result may explain the discrepancies observed between the assays since single-strand DNA damage is not thought to result in the death of cells unless the single strand breaks are converted to double-strand breaks leading to genome fragmentation and cell death [39]. Therefore, although a greater amount of damage was observed in the cells over-expressing UDG it seems likely that the single-strand breaks were not converted to double-strand breaks and the cells were able to repair this damage causing no loss of viability compared with the cell lines expressing lower levels of UDG.

The results presented in this study do not support studies carried out in *Escherichia Coli* [12,40,41]. These studies showed that mutations in the *ung* gene could suppress *dut-1*-induced lethality and in *dut*$^-$ *ung*$^-$ double mutants of *E. coli*, up to 20% of the DNA thymine can be replaced by uracil. Therefore, any increase in UDG activity would be expected to cause sensitisation of the cells to TS inhibition as observed in the present study. Although it is important to note that all *ung*$^-$ suppressible *dut*$^-$ alleles of *E. Coli* have been proposed to be leaky since complete inactivation of dUTPase was shown to be lethal even in ung$^-$ strains [11].

Analyses with isogenic UDG (*UNG1*) deficient or proficient strains of *Saccharomyces cerevisiae* indicate that in the absence of dUTPase, cell death results from the incorporation of uracil into DNA and the attempted repair of this damage by UNG1-mediated excision repair [12]. However, in *dut1 ung1* double mutants, starvation for dTMP caused dividing cells to arrest and die in all phases of the cell cycle. The authors suggested that the extensive stable substitution of uracil for thymine in DNA leads to a general failure in macromolecular synthesis. The present study did not address the last point since although HX147 cells accumulate high levels of dUTP following TS inhibition, a previous study has shown that these cells are not null for dUTPase (hydrolyse 1.2±0.15 nmol dUTP/min/mg protein) [6]. A genetic approach would be required to determine if this effect was also seen in mammalian cells.

An alternative explanation for the lack of an increase in sensitivity of HX147 cells transfected with UDG may be that these particular cells have developed an additional pathway for the removal of uracil from U:A mismatches. Several enzymes have been reported to potentially provide back-up activity for UDG. These include SMUG1 [42], T(U):G mismatch DNA glycosylase; TDG [43], MBD4 [44] and a cyclin-like enzyme [45]. The role of these enzymes requires further investigation.

An additional important point is that although the elevation of UDG protein and activity to high levels (>40-fold compared with controls) resulted in an increase in sensitivity to ZD9331 and raltitrexed over the first 24 h of treatment, no differences were observed in cell lines over-expressing lower levels of UDG (5-fold). No previous studies have recorded any cells with such high levels of UDG protein or activity which implies that this level of UDG may not exist physiologically, hence UDG may not be expected to contribute towards response to TS inhibitors within the clinical setting.

In summary, the results presented in this study have shown that although cells over-expressing very high levels of the base excision repair enzyme UDG (>40-fold compared with control cells) show significantly increased growth inhibition on treatment with ZD9331 and raltitrexed over the first 24 h of treatment, UDG does not play a major role in determining sensitivity to these drugs, at least in the HX147 UDG-transfected cell line model. It therefore seems more likely that sensitivity to TS inhibition is determined by the expression of other factors such as TS, dUTPase as well as proteins involved in the cell death response.

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