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DNA interstrand cross-linking and *TP53* status as determinants of tumour cell sensitivity *in vitro* to the antibody-directed enzyme prodrug therapy ZD2767

Noel R. Monks[a], David C. Blakey[b], Simon J. East[b], Robert I. Dowell[b], Joanne A. Calvete[a], Nicola J. Curtin[a], Christine E. Arris[a], David R. Newell[a,*]

[a]*Cancer Research Unit, University of Newcastle, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK*
[b]*Cancer and Infection Research Department, AstraZeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK*

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Abstract

Cellular determinants of sensitivity to the bifunctional alkylating agent 4-[*N*,*N*-bis(2-iodoethyl)amino]phenol (ZD2767D), the active drug produced by ZD2767 antibody-directed enzyme prodrug therapy (ADEPT), were studied. The prodrug 4-[*N*,*N*-bis(2-iodoethyl)amino]phenoxycarbonyl L-glutamic acid (ZD2767P) + activating enzyme carboxypeptidase G2 (CPG2) displayed growth inhibitory activity (IC_{50} 0.04–2.2 μM) in colorectal tumour and non-small cell lung cancer (NSCLC) cell lines, and was more potent than a monofunctional ZD2767D analogue (colorectal cell lines—IC_{50} 18–38 μM), synthesised for the first time. ZD2767P + CPG2 rapidly formed DNA–DNA interstrand cross-links (maximal at 10 min), and semi-quantitative analyses indicate that levels were similar in 3 of 4 cell lines studied (25–75 rad equivalents) at equitoxic ($10\times IC_{50}/LC_{50}$) concentrations. In matched HCT116 *TP53* functional/non-functional cell lines, there was no significant difference in the sensitivity to ZD2767P + CPG2. Together, these results suggest that cellular sensitivity to ZD2767P + CPG2 is, in part, related to the levels of interstrand crosslinks, but that *TP53* status does not markedly effect chemosensitivity.

Keywords: ZD2767; ADEPT; Alkylating agent; Sensitivity; DNA cross-linking; *TP53*

1. Introduction

A major limiting factor in cancer chemotherapy is the toxicity of cytotoxic drugs to normal tissues. Attempts to circumvent this problem have led to the tumour targeting approach, and antibodies to tumour-associated proteins have been used in the development of a number of targeted anti-tumour therapies including: immunotherapy, radioimmunotherapy and immunotoxin therapy [1,2]. Disadvantages identified with these first generation antibody-based therapies, for example poor tissue penetration and cellular heterogeneity in tumour antigen expression, led to the development of antibody-directed enzyme prodrug therapy (ADEPT) [3]. In ADEPT, an inactive prodrug is converted to a highly cytotoxic agent at the tumour site by the action of an enzyme linked to an antibody directed at a tumour-associated antigen.

The ZD2767 ADEPT system utilises the A5B7 $F(ab)_2$-carboxypeptidase G2 (CPG2) antibody-enzyme conjugate (ZD2767C) which is targeted to carcinoembryonic antigen (CEA) -expressing tumours. The enzyme, CPG2, activates a di-iodophenol mustard glutamate prodrug (ZD2767P) to the potent di-iodophenol mustard (ZD2767D) *via* the cleavage of the carbamate bond (Fig. 1) [4,5]. There are also a number of other enzyme-drug combinations which are being developed as potential ADEPT systems [6,7]; however, the ZD2767 ADEPT system is one of the most advanced and is undergoing phase 1 clinical evaluation.

Structurally, ZD2767D is similar to the classical nitrogen mustards melphalan and chlorambucil (Fig. 1), all 3 agents having a bifunctional bis-2-haloethyl aniline moiety. It is therefore possible that cellular factors which are known to be determinants of nitrogen mustard activity may also be important in the cellular

* Corresponding author. Tel.: +44-191-222-8233; fax: +44-191-222-7556.
E-mail address: herbie.newell@newcastle.ac.uk (D.R. Newell).

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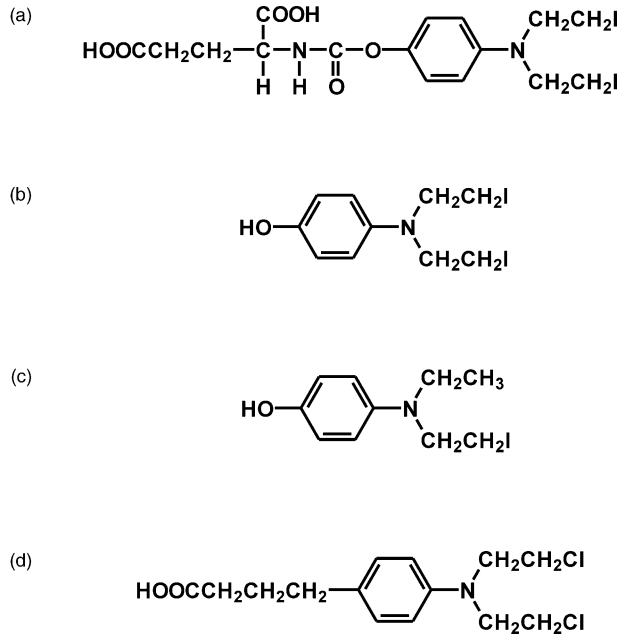

Fig. 1. Structures of the compounds investigated. (a) 4-[*N*,*N*-bis(2-Iodoethyl)amino]phenoxycarbonyl L-glutamic acid prodrug (ZD2767P), (b) 4-[*N*,*N*-bis(2-iodoethyl)amino]phenol drug (ZD2767D), (c) 4-[*N*-(iodoethyl)-*N*-ethyl-amino]phenol drug (monofunctional ZD2767D) and (d) 4-[*p*-bis(2-chloroethyl)aminophenyl]butyric acid (chlorambucil).

pharmacology of ZD2767D. Such mechanisms include: membrane transport, intracellular detoxification (both chemical and enzymatic) and repair of drug-induced damage [8,9]. To understand determinants of ZD2767D activity, the sensitivity of a panel of colorectal and non-small cell lung cancer (NSCLC) cell lines to ZD2767 was investigated, cell lines representative of tumour types known to express CEA in patients [10,11]. Chlorambucil was used as a comparator and the importance of bifunctionality was investigated by examining the activity of a monofunctional ZD2767D analogue (Fig. 1).

The most important cytotoxic lesion formed by the nitrogen mustards is generally considered to be the DNA–DNA interstrand cross-link [12,13]. DNA–DNA interstrand cross-links are thought to exert their cytotoxic effects by inhibiting DNA duplex strand separation, progression of the replication fork and DNA transcription [14,15], and hence the relationship between DNA–DNA interstrand cross-links and the activity of ZD2767D was studied.

In the 60 cell line panel used in the National Cancer Institute (NCI) drug screening programme, cell lines containing mutated *TP53* are less sensitive overall to the majority of clinical anti-cancer agents, including the alkylating agents [16]. To investigate the impact of *TP53* function on the sensitivity to ZD2767P + CPG2, ZD2767P and chlorambucil, human colorectal and NSCLC cell lines with defined *TP53* status were used. In addition, matched HCT116 human colon cancer cell lines with wild-type *TP53* (HCT116) and disrupted *TP53* function (due to transfection with the human papillomavirus type-16 *E6* gene — HCT116-N7 cells) were investigated.

2. Materials and methods

2.1. Chemicals

The prodrug ZD2767P was synthesised as previously described in Ref. [4]. The monofunctional ZD2767D analogue was synthesised as described below. CPG2 was provided by CAMR (Salisbury, Wiltshire, UK). All other reagents were supplied by Sigma Chemical Co. (Poole, Dorset, UK), unless otherwise stated.

2.2. Cell lines

The colorectal tumour cell lines HT29, LoVo, LS174T, HCT116, HCT15 and SW620 were obtained from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, UK). The NSCLC cell lines A459, EKVX, NCI-H522, NCI-H322m and NCI-H460 were kindly supplied by the National Cancer Institute (NCI, Bethesda, MD USA). The matched wild-type and N7 HCT116 cell lines were available within the authors' laboratory. All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 (complete) medium containing 2 mM L-glutamine (Gibco-BRL Life Technologies Ltd., Paisley, UK), supplemented with 10% (v/v) fetal calf serum (FCS) (Globerpharm Ltd., Esher, Surrey, UK) and incubated at 37 °C, 5% CO_2. The HCT116-N7 cells were maintained in RPMI 1640 medium containing 400 μg/ml of geneticin (G418). All cell lines were routinely tested to exclude mycoplasma infection [17].

2.3. Monofunctional ZD2767D synthesis

In the following procedure, all evaporations were done under reduced pressure and chromatography was performed using the 'flash' technique. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 300 instrument at 300 MHz.

To a solution of 4-benzyloxyaniline (Aldrich, Poole, Dorset, UK) (10 g) in acetonitrile (200 ml) di-*tert*-butyl dicarbonate (15 g) was added in portions at ambient temperature. The mixture was stirred at ambient temperature for 3 h, poured into water (500 ml) and extracted with ethyl acetate (2× 500 ml). The combined extracts were washed with water, dried over magnesium sulphate and evaporated to dryness. Trituration with ether/hexane (1:1) gave *N*-Boc-4-benzyloxyaniline as a white solid (7.5 g): NMR $CDCl_3$. δ 7.4 (m, 7H); 6.9 (d 2H); 6.9 (d 2H); 5.0 (s 2H); 1.4 (s 9H).

N-Boc-4-benzyloxyaniline (7.3 g) in dry DMF (70 ml) was added to a suspension of NaH (1.3 g 50% oil dispersion) in dry dimethyl formamide (DMF) (10 ml) under an argon atmosphere at 5–10 °C. The mixture was allowed to warm to ambient temperature over 2 h and a solution of ethyl iodide (2.9 ml) in dry DMF (3 ml) was added. After stirring for 18 h at ambient temperature, the mixture was poured into water (700 ml) and extracted with ethyl acetate (2×500 ml). The combined extracts were washed with water, dried over magnesium sulphate and evaporated to dryness. Trituration with ether/hexane (1:1) gave *N*-ethyl-*N*-Boc-4-benzyloxyaniline as a white solid (5.7 g): NMR $CDCl_3$. δ 7.4 (m, 5H); 7.1 (d 2H); 6.9 (d 2H); 5.1 (s 2H); 3.6 (t 2H); 1.4 (s 9H); 1.1 (t 3H).

N-Ethyl-*N*-Boc-4-benzyloxyaniline (3.5 g) was dissolved in 4 M HCl in ethyl acetate (40 ml) and stirred at ambient temperature for 4 h. The mixture was evaporated to dryness and the residue partitioned between dichloromethane and sodium bicarbonate solution. The dichloromethane was dried over magnesium sulphate and evaporated to dryness to give *N*-ethyl-4-benzyloxyaniline as an off-white solid (1.98 g): NMR $CDCl_3$. δ 7.4 (m, 5H); 6.9 (d 2H); 6.6 (d 2H); 5.0 (s 2H); 3.1 (t 2H); 1.1 (t 3H).

N-Ethyl-4-benzyloxyaniline was dissolved in 1:1 acetic acid/water (80 ml) and a slow stream of ethylene oxide was passed in until 3.7 g had been absorbed. This mixture was allowed to stand at ambient temperature for 18 h and evaporated to dryness. The residue was re-dissolved in ethyl acetate and washed with sodium bicarbonate solution, water, dried over magnesium sulphate and evaporated to dryness. The product was chromatographed on Merck silica gel Art 9385 eluting with ethyl acetate/hexane (1:1) to give *N*-ethyl-*N*-2-hydroxyethyl-4-benzyloxyaniline as an oil (1.65 g): NMR $CDCl_3$. δ 7.4 (m, 5H); 6.9 (d 2H); 6.8 (d 2H); 5.0 (s 2H); 3.7 (t 2H); 3.3 (t 4H); 1.1 (t 3H).

To a stirred solution of triphenylphosphine (3.93 g) in dichloromethane (50 ml) imidazole (1.04 g) and iodine (3.81 g) were added followed by a solution of *N*-ethyl-*N*-2-hydroxyethyl-4-benzyloxyaniline (1.6 g) in dichloromethane (5 ml). The mixture was stirred for 18 h, filtered, and the filtrate evaporated to dryness. The residue was re-dissolved in ethyl acetate, washed with water, dried over magnesium sulphate and evaporated to dryness. The residue was chromatographed on Merck silica gel Art 9385 eluting with ethyl acetate/hexane (1:9) to give *N*-ethyl-*N*-2-iodoethyl-4-benzyloxyaniline as a yellow solid (800 mg): NMR $CDCl_3$. δ 7.4 (m, 5H); 6.9 (d 2H); 6.6 (d 2H); 5.0 (s 2H); 3.6 (t 2H); 3.3 (t 2H); 3.2 (t, 2H); 1.1 (t 3H).

N-Ethyl-*N*-2-iodoethyl-4-benzyloxyaniline (382 mg) was dissolved in dichloromethane (5 ml) under an atmosphere of argon. Iodotrimethylsilane (1.4 ml) was added and the mixture stirred at ambient temperature for 18 h. The mixture was slowly poured into 100 ml of 5% acetic acid in diethyl ether with stirring when a yellow solid separated. This was collected by filtration to give 4-[ethyl(2-iodoethyl)amino]phenol hydroiodide (300 mg). m.p. = 142–144 °C. NMR dimethyl sulphoxide (DMSO)d_6 + D4 acetic acid. δ 7.4 (d, 2H); 6.8 (d 2H); 3.9 (t 2H); 3.5 (t br 2H); 3.0 (br, 2H); 0.95 (t 3H).

2.4. Growth inhibition studies

The tumour cell lines were incubated for 1 h with ZD2767P, ZD2767P + CPG2 (0.1 unit/well), monofunctional ZD2767D analogue or chlorambucil, in 96-well microtitre plates (200 μl, 1×10^3–1×10^4 cells/well). One unit of CPG2 is defined as the amount of enzyme required to hydrolyse 1 μmol of methotrexate/min/ml of reaction mixture at 37 °C [18]. The medium was subsequently removed and the cells incubated in 200 μl of drug-free medium for a further 96 h. Cell growth was determined using the sulphorhodamine B (SRB) dye assay as previously described by Skehan and colleagues in Ref. [19]. The growth inhibitory activity of the compounds was expressed as the concentration required to inhibit cell growth by 50% (IC_{50}).

2.5. Cytotoxicity studies

HT29 and LS174T colorectal tumour cells were incubated with ZD2767P + CPG2 (1 unit/ml) for 1 h. The cells were then washed in phosphate-buffered solution (PBS) and seeded into Petri dishes at a cell density that resulted in the subsequent formation of 50–100 colonies. The cells were then allowed to grow for 14–21 days, until visible colonies were observed (> 50 cells/colony). The colonies were then fixed using Carnoy's fixative (1:3 acetic acid:methanol v/v) and stained with 0.4% (w/v) crystal violet. The number of colonies on each plate was counted and the cytotoxicity expressed as the concentration of drug required to reduce cell survival to 50% of control untreated colony formation (LC_{50}). The control plating efficiencies were: HT29 — 24±18% and LS174T — 32±6%.

2.6. DNA interstrand cross-linking studies

DNA interstrand cross-links formed by ZD2767P + CPG2 were determined using the methods described by Kohn and colleagues [20]. To summarise the data obtained, the results are expressed as DNA interstrand cross-link frequency in rad equivalents (ISC-rad eq) [20]. Monofunctional ZD2767D treated cells were also subjected to elution without irradiation to investigate the formation of direct drug-induced DNA strand-breaks.

2.7. Statistics

All statistical analyses were performed using Instat software, Version 2.0 (Graphpad Software, Inc., San

Table 1
IC_{50} values for growth inhibition in colorectal and NSCLC[b] cell lines treated with ZD2767P + CPG2 or ZD2767P alone[a]

Cell line	ZD2767P + CPG IC_{50}[c] (μM)	ZD2767P IC_{50} (μM)	Difference (fold)
HCT116	1.10 ± 0.47	179 ± 106	163
HT29	0.82 ± 0.62	154 ± 35	188
HCT15	0.78 ± 0.07	150 ± 14	192
LoVo	0.41 ± 0.14	132 ± 24	322
SW620	0.25 ± 0.01	16 ± 9	64
LS174T	0.04 ± 0.03	8 ± 2	200
EKVX	2.2 ± 0.9	807 ± 179	367
NCI-H522	0.9 ± 0.2	423 ± 313	470
NCI-H322m	0.9 ± 0.3	352 ± 296	391
A549	0.15 ± 0.06	22 ± 15	147
NCI-H460	0.05 ± 0.02	14 ± 10	280

[a] Values are the mean ± S.D. (standard deviation) of ⩾three experiments.
[b] NSCLC, non-small cell lung cancer.
[c] IC_{50}, the growth inhibitory activity of the compounds expressed as

Table 2
IC_{50} values for growth inhibition in colorectal cell lines treated with chlorambucil and the monofunctional ZD2767D analogue[a]

Cell line	Chlorambucil IC_{50} (μM)	Monofunctional ZD2767D IC_{50} (μM)
HCT116	277 ± 106	ND[b]
HT29	201 ± 61	38 ± 4
LoVo	79 ± 5	18 ± 5
HCT15	76 ± 11	ND[b]
SW620	66 ± 5	ND[b]
LS174T	26 ± 4	18 ± 6

[a] Values are the mean ± S.D. of ⩾three experiments.
[b] ND—not determined.

Diego, California, USA). Linear relationships were determined using Graphpad Prism software, Version 2.01 (Graphpad Software, Inc.). Throughout the text and in the tables, data are given as the mean ± standard deviation (S.D.) of $n \geqslant 3$ independent experiments.

3. Results

3.1. Growth inhibition studies

The panel of colorectal cell lines showed differential sensitivity to both ZD2767P + CPG2 (one way analysis of variance (ANOVA) $P < 0.001$, IC_{50} values ranging from 0.04 μM (LS174T) to 1.1 μM (HCT116)), and ZD2767P alone (one way ANOVA, $P < 0.01$, IC_{50} values ranging from 8 μM (LS174T) to 179 μM (HCT116)). A similar rank order of sensitivity (Spearman non-parametric correlation, $r = 1$, $P < 0.01$) was seen for both treatments within the panel of colorectal cell lines. The ratios of the potencies of ZD2767P + CPG2 and ZD2767P alone ranged from 63 to 322 (Table 1), and there was a highly significant linear relationship ($r^2 = 0.86$, $P < 0.001$) between the sensitivity of the cell lines (IC_{50} values) to ZD2767 + CPG2 and ZD2767P alone.

Similar observations were made with the panel of NSCLC cell lines (Table 1). IC_{50} concentrations for ZD2767P + CPG2 ranged from 0.05 μM (NCI-H460) to 2.2 μM (EKVX), and the differential sensitivities of the cell lines were again significant (one way ANOVA, $P < 0.001$). A significant differential in sensitivity was also detected in the NSCLC cell line panel with ZD2767P alone (one way ANOVA, $P < 0.001$), with IC_{50} values ranging from 14 μM (NCI-H460) to 807 μM (EKVX). ZD2767P alone was again found to be far less potent than the prodrug plus enzyme, with ratios of IC_{50} values ranging from 147 to 470 (Table 1). Regression analysis identified a significant linear relationship between ZD2767P + CPG2 and ZD2767P sensitivity (IC_{50} values) in the panel of five NSCLC cell lines ($r^2 = 0.98$, $P < 0.01$), which was confirmed by a rank order analysis (Spearman non-parametric correlation, $r = 0.97$, $P < 0.05$).

Differential sensitivity was also seen across the panel of colorectal cell lines following a 1 h treatment with chlorambucil (one way ANOVA, $P < 0.001$). HCT116 (IC_{50} 277 μM) and HT29 cells (IC_{50} 201 μM) were the least sensitive, and LS174T cells (IC_{50} 26 μM) the most sensitive (Table 2). A comparison of the sensitivity of the colorectal cell lines to ZD2767P + CPG2 and chlorambucil (IC_{50} values) also demonstrated a significant linear correlation ($r^2 = 0.76$, $P < 0.05$), which was again confirmed by a rank order analysis (Spearman non-parametric correlation $r = 0.94$, $P < 0.05$).

To determine whether the activity of ZD2767P + CPG2 is related to the bifunctionality of ZD2767D, a monofunctional ZD2767D analogue was synthesised and its activity assessed in three colorectal cell lines (HT29, LoVo and LS174T), selected for their range of sensitivities to ZD2767P + CPG2. As shown in Table 2, the sensitivity of the three cell lines to the monofunctional ZD2767D analogue was found to differ significantly (one way ANOVA, $P < 0.01$), the HT29 cell line being the least sensitive ($IC_{50} = 38$ μM). However, in contrast to the data generated for ZD2767P, ZD2767P + CPG2 and chlorambucil, the LoVo and the LS174T cell line were equally sensitive to the monofunctional ZD2767D analogue (IC_{50} values = 18 μM). Together, the growth inhibition studies demonstrated that ZD2767P + CPG2 was far more potent than either chlorambucil or the monofunctional ZD2767D analogue (Fig. 2).

3.2. Cytotoxicity studies

To confirm that the differential sensitivity of the colorectal tumour cell lines observed in the growth inhibition

assay reflected differential sensitivity to drug-induced cytotoxicity, clonogenic assays were performed with the HT29 and LS174T cells. Following a 1 h exposure to increasing concentrations of ZD2767P + CPG2, there was concentration-dependent cytotoxicity (data not shown). For HT29 cells, the ZD2767P + CPG2 concentration required to inhibit colony formation by 50% (LC_{50}) was 0.16 ± 0.08 μM, whereas the LS174T cells were found to be more sensitive (LC_{50} = 0.02 ± 0.01 μM). These cytotoxicity data are consistent with the SRB growth inhibition studies, confirming that in both assays LS174T cells were more sensitive than HT29 cells to ZD2767P + CPG2.

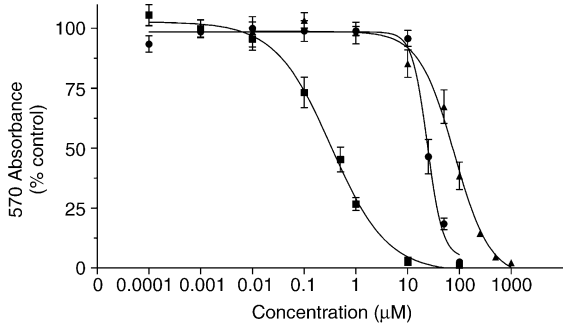

Fig. 2. Growth inhibitory activity of ZD2767P + CPG2 (■), chlorambucil (▲) and the monofunctional ZD2767D analogue (●) in LoVo colorectal tumour cells. The data shown are for a single representative experiment, with each data-point representing the mean ± S.D. of ⩾ three intra-experimental replicates.

3.3. DNA cross-linking studies

ZD2767P + CPG2 caused a concentration-related retardation of the rate of DNA elution in the alkaline elution assay, indicative of cross-link formation (Fig. 3). Both HT29 and LS174T cells were found to display significant levels of cross-links at drug concentrations higher than the LC_{50} concentration (Fig. 4), whereas relatively low levels of cross-links were detected following treatment with ZD2767P + CPG2 at their respective LC_{50} concentrations (LS174T = 0.02 μM and HT29 = 0.2 μM). At ZD2767P + CPG2 concentrations 10× the LC_{50}, both cell lines had similar levels of DNA interstrand cross-links, suggesting a semi-quantitative relationship between the level of DNA interstrand cross-links formed after a 1 h exposure in these two cell lines and their sensitivity to ZD2767P + CPG2 treatment. No strand breaks were seen following ZD2767P + CPG2 treatment in un-irradiated cells.

A concentration-dependent increase in the levels of cross-links (Fig. 5) was also seen in the two NSCLC cell lines studied following treatment with ZD2767P + CPG2. In the NCI-H460 cell line there were again only low levels of cross-links following treatment with an IC_{50} concentration (0.05 μM) of ZD2767P + CPG2, but readily detectable following exposure to 10× the IC_{50} concentration (0.5 μM). In contrast to the other three cell lines, in the EKVX cells substantial levels of cross-links were observed following treatment with an IC_{50} concentration (2 μM) of ZD2767P + CPG2 and detectable levels were present at 0.2 μM.

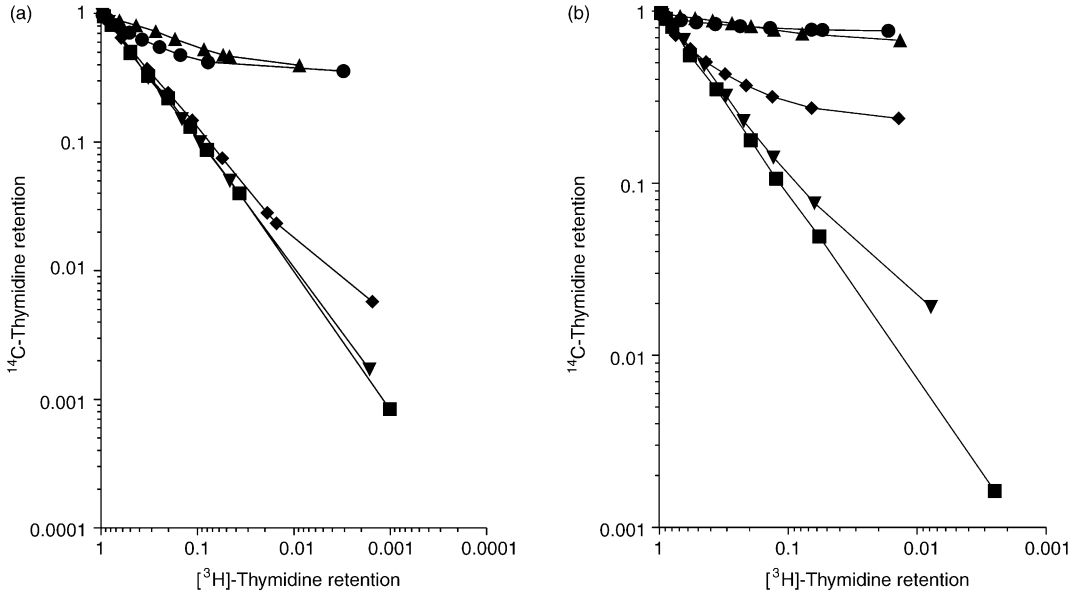

Fig. 3. Alkaline elution profiles for colorectal tumour cell lines following treatment with ZD2767P + CPG2. HT29 (a) and LS174T (b) cells were exposed for 1 h to (▼) 0.02 μM, (◆) 0.2 μM or (●) 2 μM ZD2767P + 1 unit/ml CPG2. (■) Control cells irradiated with 200 rads γ-radiation, (▲) un-irradiated control cells. The data shown are from a single representative experiment, with each data-point representing a single replicate.

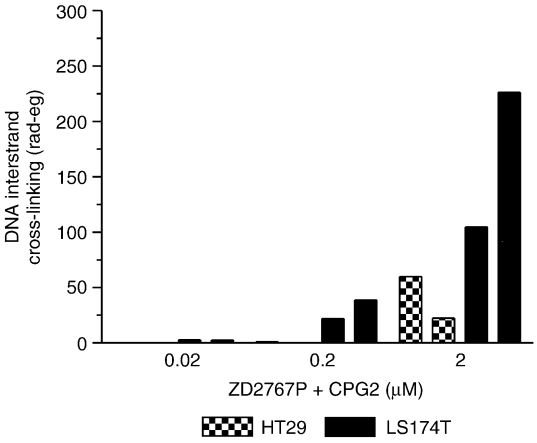

Fig. 4. DNA interstrand cross-link levels measured by alkaline elution analyses of HT29 and LS174T colorectal tumour cell lines treated with ZD2767P + CPG2 for 1 h. Each bar represents the result of a single independent experiment.

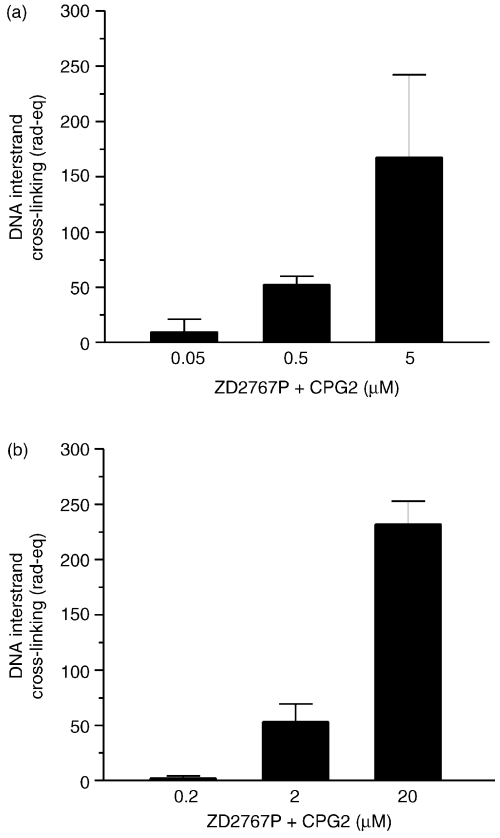

Fig. 5. DNA interstrand cross-link levels measured by alkaline elution analyses of NCI-H460 (a) and EKVX (b) NSCLC cell lines treated with ZD2767P + CPG2 for 1 h. Each bar represents the mean ± S.D. of three replicate experiments.

To understand further ZD2767P + CPG2 induced cross-linking, studies were performed in HT29 and LS174T cells to determine the time course of DNA interstrand cross-link formation. The formation of DNA interstrand cross-links in both cell lines were found to be maximal after a 10 min exposure at each of the drug concentrations studied, with no increase being seen over the subsequent 50 min (Fig. 6). Furthermore, there was no evidence of a decrease in interstrand cross-link levels over the 60 min studied, suggesting that either no repair occurred during this time period or that there was a balance between cross-link formation and removal.

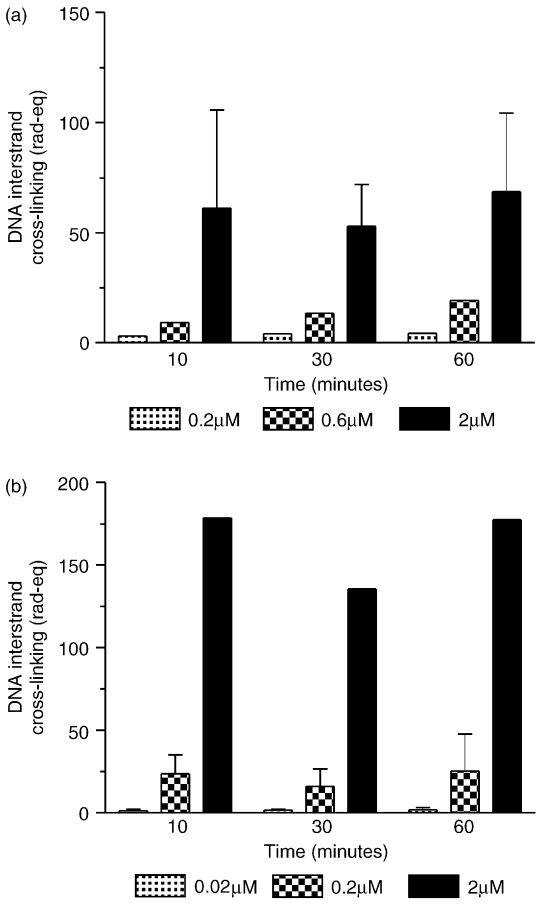

Fig. 6. Time course of DNA interstrand cross-link formation induced by ZD2767P + CPG2 in HT29 (a) and LS174T (b) colorectal tumour cell lines. Each bar represents the mean of $n \geqslant 2$ experiments (±S.D. for $n = 3$) with the exception of the two μM ZD2767P + CPG2 treatment of LS174T cells which was $n = 1$.

Alkaline elution experiments were also performed with the monofunctional ZD2767D analogue to confirm that, as would be expected from its chemical structure, the monofunctional ZD2767D analogue did not generate DNA interstrand cross-links. Following monofunctional ZD2767D analogue treatment, DNA from the LS174T cell line (Fig. 7a) showed a faster rate of elution than the DNA of the HT29 cells (data not shown), a result which is consistent with the greater sensitivity of the LS174T cell line to the compound (Table 2). In both cell lines, DNA eluted more rapidly after drug treatment, regardless of whether or not cells were subjected to γ-irradiation (Fig. 7 and data

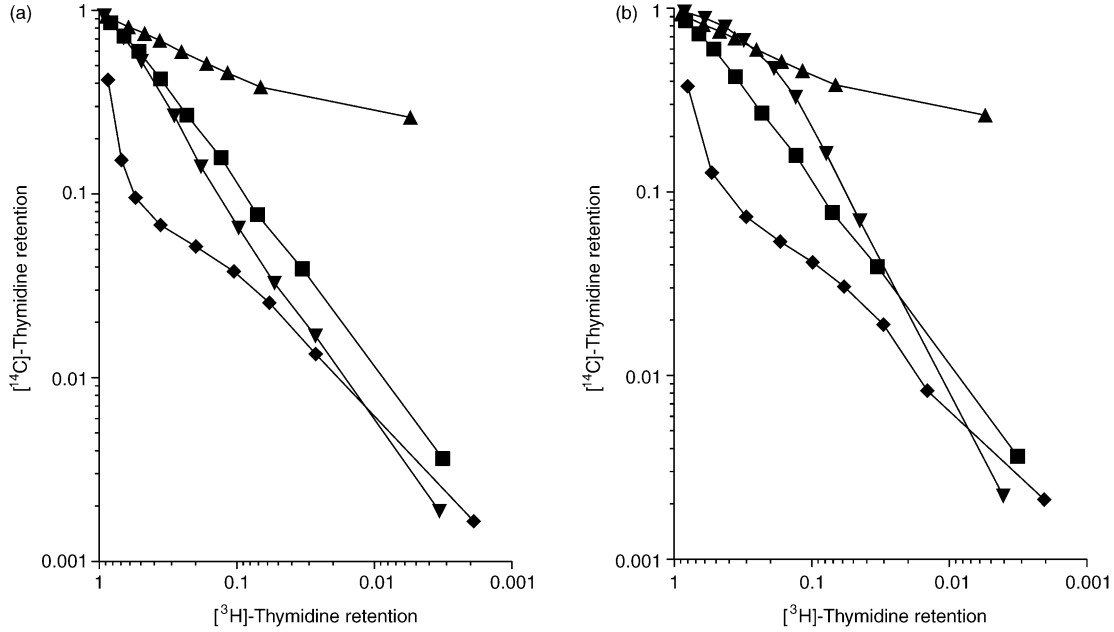

Fig. 7. Alkaline elution profiles for LS174T colorectal tumour cells following treatment with the monofunctional ZD2767D analogue for 1 h. (▼) 20 μM, (◆) 200 μM, (■) control cells irradiated with 200 rads γ-radiation, and (▲) un-irradiated control cells. (a) DNA interstrand cross-links (200 rads γ-radiation), (b) DNA strand-break formation (un-irradiated). The data shown are from single representative experiments, with three experiments being performed each giving consistent data. Each data-point representing a single replicate.

not shown), suggesting that the monofunctional ZD2767D analogue induces DNA strand-breaks, but not DNA–DNA interstrand cross-links. The concave nature of the un-irradiated elution curves following treatment with 20 μM monofunctional ZD2767D in both cell lines (Fig. 7b and data not shown) suggests that strand breaks are being formed during the elution period, causing a change in the rate of DNA elution with time.

Table 3
TP53 status of the colorectal and NSCLC tumour cell lines used in these studies[a]

Tumour type	Cell line	*TP53* status
Colorectal	HCT116	Wild-type
	HT29	Mutant
	HCT15	Mutant/wild-type
	LoVo[b]	Wild-type
	SW620	Mutant
	LS174T[c]	Wild-type
NSCLC	EKVX	Mutant
	NCI-H522	Mutant
	NCI-H322m	Mutant
	A549	Wild-type
	NCI-H460	Wild-type

[a] The cell lines are placed in the rank order of sensitivity to ZD2767P + CPG2 within their cell type. Information taken from Ref. [16].
[b] Information taken from Ref. [29].
[c] Information taken from Ref. [30].

3.4. TP53 *status in relation to sensitivity to ZD2767P + CPG2*

The reported *TP53* genotypes of the cell lines used in these studies are given in Table 3. A comparison of *TP53* status and sensitivity to ZD2767P + CPG2 (Fig. 8) suggests a possible relationship (non-parametric Mann Whitney test, $P = 0.057$) between *TP53* status and sensitivity; cells with wild-type *TP53* being on the whole more sensitive to ZD2767P + CPG2 induced growth inhibition. To address this relationship further, wild-type *TP53* human colon cancer HCT116 cells and

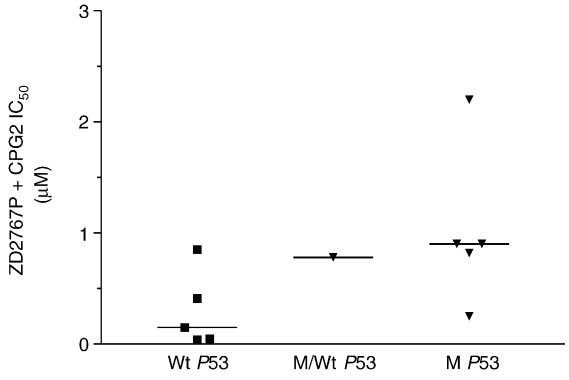

Fig. 8. Relationship between p53 genotype and sensitivity to ZD2767P + CPG2. Wt refers to wild-type p53 genotype, M refers to mutant p53 genotype and Wt/M denotes a heterozygous genotype. The lines designate the median of each group.

Table 4
IC_{50} values for growth inhibition in wild-type HCT116 and HCT116-N7 cells treated with ZD2767P + CPG2, ZD2767P alone and chlorambucil[a]

Drug	HCT116 IC_{50} (μM)	HCT116 N7 IC_{50} (μM)	Significance (paired *t*-test)
ZD2767P + CPG2	1.1±0.47	0.86±0.51	$P=0.31$
ZD2767P	179±106	127±18	$P=0.49$
Chlorambucil	277±70	240±118	$P=0.48$

[a] Values are the mean±S.D. of ⩾three experiments.

HCT116 N7 cells, which lack normal *TP53* function due to the transfection of the human papillomavirus type-16 *E6* gene, were treated with ZD2767P + CPG2, ZD2767P alone and chlorambucil (Table 4). However, disruption of *TP53* function did not markedly alter the sensitivity of the HCT116 cells to the three treatments.

4. Discussion

The aim of the studies described in this paper was to identify determinants of the sensitivity of human colorectal cancer and NSCLC cell lines to the ZD2767 ADEPT system. In particular, the role of DNA–DNA interstrand cross-linking and *TP53* status were studied. Comparison of the activity of ZD2767P + CPG2, ZD2767P and chlorambucil, in the colorectal cell line panel, demonstrated that ZD2767P and chlorambucil were markedly less active (>64-fold and >97-fold, respectively) than ZD2767P + CPG2. The difference in activity between the ZD2767P and ZD2767D is attributed to the presence of the glutamate moiety, which both reduces the reactivity of the mustard group (5–10-fold, AstraZeneca Pharmaceuticals unpublished observation) and introduces two negative charges on the molecule at physiological pH, thereby impeding uptake into the cell. The differential potency of ZD2767P and ZD2767P + CPG2 has been previously described in Refs. [4,5], and is of a magnitude that satisfies the criteria required for a potential ADEPT system [21]. Despite the greater potency of ZD2767P + CPG2, there was a similar overall rank order of sensitivity to ZD2767P + CPG2, ZD2767P and chlorambucil in the colorectal cell line panel, suggesting that all three compounds share common determinants of sensitivity, which could include a similar final cytotoxic lesion.

The cytotoxicity of nitrogen mustards such as chlorambucil and melphalan is generally attributed to their ability to produce bifunctional DNA adducts, in particular interstrand cross-links [12,13,22]. The monofunctional ZD2767D analogue was not as potent as ZD2767P + CPG2, with the HT29, LoVo and LS174T cell lines being 46-, 44- and 450-fold less sensitive, respectively. Similar reductions in potency have also been previously reported between mechlorethamine (HN2) and 2 monofunctional analogues [23], and between sulphur mustard gas and its corresponding half mustard [24].

Exposure of HT29 and LS174T cells for 1 h to ZD2767P + CPG2 resulted in a ZD2767P concentration-related increase in the formation of DNA interstrand cross-links, an observation which has also been reported with HN2, melphalan, chlorambucil and 4-[bis(chloroethyl)amino] benzoic acid (BAM) [13,25,26]. Similar levels of DNA–DNA interstrand cross-link formation were observed at equitoxic concentrations of ZD2767P + CPG2 in the HT29 and LS174T cell lines, suggesting a semi-quantitative relationship, and Sunters and co workers [13] have demonstrated that the formation of DNA–DNA interstrand cross-links by chlorambucil, melphalan and BAM is related to biological activity in K562 leukaemia cells. Similarly, Gornati and colleagues [27] have demonstrated similar levels of DNA–DNA interstrand cross-links in OAW42 (melphalan-sensitive) and OAW42MER (melphalan-resistant) ovarian cancer cells following treatment with equitoxic melphalan concentrations.

To determine the time course of ZD2767P + CPG2 DNA–DNA interstrand cross-link formation, HT29 and LS174T colorectal tumour cells were exposed to the drug for 10, 30 or 60 min. The levels of DNA interstrand cross-links in HT29 or LS174T cells did not increase with exposure time, suggesting that ZD2767P + CPG2 forms DNA interstrand cross-links rapidly (<10 min). Rapid cross-link formation is consistent with reports that the cytotoxic species produced by ZD2767P + CPG2 has a very short half life (<2 min); a 1 min exposure having a similar IC_{50} (1.57 μM) to a 1 h exposure (0.34 μM) in LoVo cells [5]. Together, these data suggest that ZD2767P + CPG2 treatment generates a short-lived compound which produces cytotoxic DNA lesions very rapidly, consistent with the high chemical reactivity of ZD2767D. HN2 has also been shown to form maximal DNA interstrand cross-link levels rapidly (<1 h) [28], whereas melphalan and chlorambucil are reported to produce the maximal numbers of cross-links 4–8 h after the start of drug treatment [25,28]. The lack of any change in ZD2767 + CPG2-induced cross-link levels over the 60 min period suggests that these adducts are relatively stable, although more extended post-treatment incubation would be needed to define the true stability, and possible repair, of these lesions.

Together, the results of the alkaline elution and cell growth/cytotoxicity data strongly suggest that both the activity and DNA interstrand cross-linking produced by ZD2767P + CPG2 is due to bifunctionality of the active ZD2767D drug that is generated from ZD2767P. The monofunctional ZD2767D analogue is less potent and produces growth inhibition via a mechanism which does not involve DNA interstrand cross-linking, most

probably the formation of DNA single-strand breaks, lesions observed following treatment with monofunctional HN2 analogues [23].

Comparison of the reported *TP53* genotype of the cell lines used here with sensitivity to ZD2767P + CPG2 identified a weakly significant difference between the two groups; cell lines with wild-type *TP53* being more sensitive. However, there were no differences in sensitivity to ZD2767P + CPG2, ZD2767P alone or chlorambucil in a pair of *TP53* functional/non-functional HCT116 cell lines.

In conclusion, these studies have demonstrated that ZD2767P + CPG2 generates a potent bifunctional alkylating agent which rapidly forms cytotoxic DNA interstrand cross-links. Comparisons with the classical nitrogen mustard chlorambucil in two panels of cell lines identified similar patterns of activity, suggesting common determinants of cellular sensitivity. The semiquantitative relationship between the level of cross-links and drug sensitivity in the HT29, LS174T and NCI-H460 cell lines indicates that cross-link levels are a primary determinant of cellular sensitivity. In contrast, *TP53* status did not clearly influence cellular sensitivity.

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