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Sequence-dependent synergism between the new generation platinum agent ZD0473 and paclitaxel in cisplatin-sensitive and -resistant human ovarian carcinoma cell lines

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Abstract

ZD0473 is a new generation hindered platinum agent currently undergoing worldwide Phase II clinical studies. The *in vitro* cytotoxicity of ZD0473 either alone or in combination with the anticancer drugs paclitaxel, gemcitabine, vinorelbine, topotecan and doxorubicin was determined using four human ovarian carcinoma cell lines and by the sulphorhodamine B assay (SRB). The lines included one model of acquired cisplatin resistance and one isogenic pair differing only in their p53 status. Notably, the simultaneous exposure to ZD0473 and paclitaxel for 96 h resulted in synergy (as defined by a median effect analysis) in all four cell lines (i.e. independent of cisplatin resistance and p53 status). In addition, synergy was observed in 3/4 lines and 2/4 lines following concomitant exposure to topotecan or gemcitabine, respectively. Sequencing studies with ZD0473 and paclitaxel revealed that, for three of the four cell lines, the combination of ZD0473 administered for 24 h prior to paclitaxel for 24 h conferred a greater growth inhibitory effect than the reverse sequential combination. This scheduling effect was particularly marked for the acquired cisplatin-resistant A2780CisR cell line; synergy being observed with ZD0473/paclitaxel, but antagonism with paclitaxel/ZD0473. This effect did not appear to be correlated with changes in drug-induced cell cycle checkpoints. These data suggest that ZD0473 may be usefully combined with various cytotoxics in the clinic, including paclitaxel, topotecan and gemcitabine.

Keywords: ZD0473; Platinum; Paclitaxel; Synergism

1. Introduction

Platinum compounds such as cisplatin, carboplatin and oxaliplatin currently play a significant role in cancer therapy, for a review see Ref. [1]. Cis-amminedichloro [2-methylpyridine] platinum (II) (ZD0473—formerly known as JM473, AMD473) is a sterically hindered new generation platinum agent that overcomes platinum resistance mechanisms. In preclinical studies, ZD0473 was shown to possess less susceptibility than cisplatin to inactivation by thiol-containing species associated with cisplatin resistance, namely glutathione [2] and metallothioneins [3]. Furthermore, ZD0473 retained activity against a variety of tumour cell lines possessing acquired resistance to cisplatin, including in lines where resistance was due to impaired drug transport and enhanced DNA repair [2]. ZD0473 also showed broad-spectrum activity against several human tumour xenografts *in vivo,* including lines possessing acquired resistance to cisplatin or carboplatin [4]. An initial Phase I intravenous (i.v.) monotherapy clinical trial has now been completed; myelosuppression (thrombocytopenia) was the dose-limiting toxicity at doses of 120–150 mg/m^2 every 3–4 weeks [5–7]. Numerous Phase II studies are now ongoing including in ovarian cancer [8].

The aim of this study was to guide clinical studies of ZD0473 in combination with other cancer cytotoxics through the study of *in vitro* cell line models of human ovarian cancer. The lines included a model of acquired

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cisplatin resistance and an isogenic pair of cell lines differing only in their p53 status. Ovarian cancer is a disease where platinum-based chemotherapy (cisplatin or carboplatin) represents the mainstay of treatment along with paclitaxel [9–12]. For this reason, the initial focus of our combination studies has been on ZD0473 with paclitaxel. Moreover, preclinical studies have shown additive effects or even synergy between cisplatin and paclitaxel with some dependence on the sequence of administration [13–16]. Therefore, we have investigated the growth inhibitory effects of ZD0473 and paclitaxel administered concomitantly or sequentially by median effect analysis as used previously in our studies of cisplatin with the thymidylate synthase inhibitor, raltitrexted (Tomudex) [17]. In addition, we have evaluated the combination of ZD0473 with four other cytotoxics of differing mechanisms of action; the antimetabolite gemcitabine (for which there is also evidence of synergy with cisplatin in cell line models [18]), the vinca alkaloid tubulin-interactive vinorelbine, the topoisomerase I inhibitor topotecan and the widely-used topoisomerase II inhibitor doxorubicin.

2. Materials and methods

2.1. Cell lines and chemicals

Two parental human ovarian carcinoma cell lines were used (A2780 and CH1) and a subline possessing approximately 15-fold acquired resistance to cisplatin (A2780CisR). These lines were chosen on the basis that they featured prominently in our previous preclinical studies of ZD0473 [2,3]. Furthermore, the possible impact of p53 status on the cellular response to these drugs was addressed by inclusion of a subline of A2780 (which possesses wild-type p53) stably transfected with the p53-inactivating human papillomavirus *E6* gene (A2780E6, as previously described in Ref. [19]). Cells were grown as monolayers cultured in Dulbecco's Modified Eagle's Medium (Life Technologies, Paisley, UK) supplemented with 10% heat inactivated fetal bovine serum (Life Technologies), 0.5 μg/ml hydrocortisone, 2 mM L-glutamine and 1% MEM non-essential amino acids in a humidified 6% CO_2/94% air atmosphere.

ZD0473 was obtained from AstraZeneca Pharmaceuticals and prepared as a stock solution of 1 mM in isotonic saline immediately prior to use. Paclitaxel (from Calbiochem, Nottingham, UK) was prepared as a stock solution of 2 mM in absolute ethanol and diluted at least 1:100 into growth medium as previously described to prevent precipitation [20]. Other drugs were pharmacy preparations (obtained from the Royal Marsden Hospital NHS Trust). Remaining chemicals were from Sigma Chemicals (Poole, UK) unless otherwise stated.

2.2. Growth inhibition assay

Cytotoxicity was assessed using the sulphorhodamine B assay (SRB) as previously described by us in Refs. [2,20]. Briefly, between 3000 and 6000 cells/well were seeded into 96-well microtitre plates and allowed to attach overnight. For single-agent studies, drugs were then added at a range of concentrations to quadruplicate wells and left in contact for 96 h. For combination studies, drugs were either added concomitantly for 96 h or sequentially by adding each drug in turn for 24 h, aspirating and washing with phosphate-buffered saline (PBS) after each 24-h drug incubation, followed by 48 h in drug-free growth medium. Control wells were treated in the same way with aspiration at each 24-h period. For combination experiments, drugs were added at a constant ratio of concentrations based on their respective individual IC_{50} values. These ratios are shown in the results tables.

At the completion of the 96-h incubation period, cell numbers were compared in treated versus control wells by fixing in ice-cold 10% w/v trichloroacetic acid (TCA) (30 min) and staining with 0.4% SRB in 1% v/v acetic acid (15 min). The mean absorbance at 540 nm for each drug concentration was expressed as a percentage of the control untreated well absorbance.

The effect of combining drugs was studied using a median effect analysis whereby a combination index (CI) is calculated from pooled data from 3–5 individual experiments each comprising at least five data points for each drug alone and for the combination [17,21]. The CI represents the degree of synergy, additivity or antagonism occurring for any given drug combination. As before [17], we have determined this with respect to the fraction-affected (Fa) values (typically 0.5; CI_{50}), but also at CI_{75} and CI_{90}, which reflect the percentage of cells killed/inhibited by the drug. For the purposes of comparing drug combinations, we subjectively assigned the following interpretation of CI_{50} values: 0.30–0.69 synergism; 0.70–0.84 moderate synergism; 0.85–0.89 mild synergism; 0.90–10.09 additive; 1.10–1.19 slight antagonism; 1.20–1.44 antagonism; >1.45 moderate-strong antagonism.

2.3. Flow cytometry

In accordance with the above growth inhibition experiments, A2780CisR cells were exposed to ZD0473 alone (24 h), paclitaxel alone (24 h) or to sequential 24 h drug exposure and remaining adherent cells either collected immediately or following a further 24 h in drug-free growth medium, by trypsinisation. Harvested cells were resuspended in PBS and ice-cold 70% ethanol and stored at 4 °C until analysis. Following centrifugation (15 000 rotations per minute (rpm), 5 min) cell pellets were resuspended in 100 mg/ml RNase A and 40 μg/ml

propidium iodide and incubated at 37 °C for 30 min. Samples were then analysed using a Coulter Elite flow cytometer (Beckman Coulter, Buckinghamshire, UK) equipped with a Spectra-Physics argon-ion laser with an output of 200 mW at 488 nm. Typically, data from at least 1×10^4 cells were analysed for forward and orthogonally scattered light together with red fluorescence (peak and integrated area) and using WinMD12.8 Windows Multiple Document Interface Flow Cytometry Application (Cylchred Windows 95 Version 1.02; http://archive.uwcm.ac.uk/uwcm/hg/hoy/software.html).

2.4. Statistical analyses

Typically, all growth inhibition experiments were repeated 3–5 times.

3. Results

3.1. Concomitant 96-h exposure; ZD0473 and other cytotoxics

The combination of ZD0473 with paclitaxel, or gemcitabine, or vinorelbine, or topotecan or doxorubicin was studied using individual and simultaneous exposure in all four cell lines. The individual cell line sensitivities to individual drugs (ZD0473, paclitaxel, gemcitabine, topotecan, vinorelbine and doxorubicin) are shown in Table 1. As expected A2780CisR was slightly more resistant (4.9-fold) to the growth inhibitory effects of the platinum drug ZD0473 in comparison to the parent cell line. The remaining drugs all produced cell kill at nanomolar concentrations. The ratio of drug concentrations used in the simultaneous exposure combination experiments is indicated in Table 1 and was kept constant across all of the four cell lines and was broadly based on individual drug IC_{50} values (1000:1 for paclitaxel; 1000:1 for gemcitabine; 250:1 for topotecan; 200:1 for vinorelbine and 200:1 for doxorubicin). CI_{50} values were calculated from individual full dose–response curves containing at least five data points and pooled from 3 to 5 independent experiments (curves not shown although goodness of fit *r* values are shown) (Table 1). In addition, CI_{75} and CI_{90} values are shown. The most striking and consistent pattern of effect was that of synergism between ZD0473 and paclitaxel. This was observed across a wide range of cell kill/inhibition (Fa), although was lost at very low Fa values (Fig. 1). CI_{50} values ranged from 0.31 in A2780CisR cells, to 0.61 in the parent cell line. The inactivation of p53 in this cell line (A2780E6) resulted in a similar degree of synergism (CI_{50} of 0.54) to that observed in the p53 wild-type parent line.

With the remaining drugs, there was greater cell-line dependency and therefore less consistency in the effect. With the antimetabolite, gemcitabine, synergism was observed in two of the four cell lines (A2780CisR and CH1 cells), while additive to strong antagonistic effects were observed in the remaining two cell lines. For the mitosis-blocking antitubulin drug, vinorelbine, generally antagonistic effects were observed, except in CH1 cells. Notably, synergism was observed for 4/5 agents with the CH1 cell line. For the topoisomerase I inhibitor, topotecan, an effect relating to p53 status was observed that was unique among the drugs studied. Synergism occurred in A2780E6 cells, but antagonism occurred in the parent A2780 cell line. The cell lines differ only in their p53 status. With doxorubicin, antagonist effects were observed in all four cell lines.

3.2. Sequential drug exposure: ZD0473 and paclitaxel

As from the initial concomitant exposure experiments the combination of ZD0473 and paclitaxel showed the greatest degree of synergism, we then evaluated this drug combination sequentially to address issues of possible sequence dependency. Results are shown, in terms of CI_{50}, CI_{75} and CI_{90} values, in Table 2. For three of the four cell lines, at the CI_{50} level the combination of ZD0473 administered for 24 h prior to paclitaxel for 24 h conferred a greater inhibitory effect than the reverse sequential combination. This sequence-dependent effect was particularly marked for the acquired cisplatin-resistant A2780CisR cell line; synergism being observed with ZD0473 followed by paclitaxel, but antagonism with the reverse sequence. Full dose–response curves are shown in Fig. 2 and show the enhanced growth inhibition of ZD0473 followed by paclitaxel compared with the reverse sequence apparent at % control growth values of 20% and above. Interestingly, the sequence-dependent effect was lost at high drug concentrations/high growth inhibition.

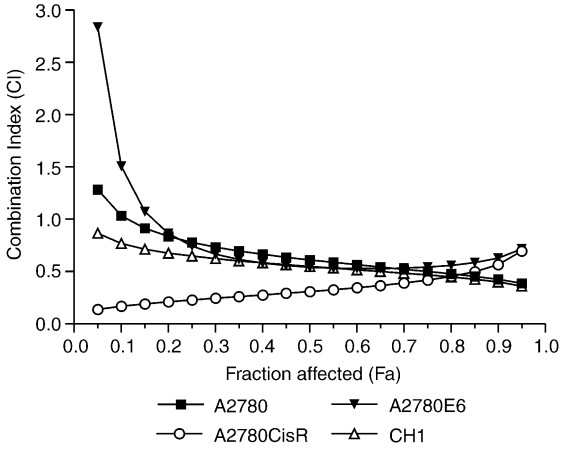

Fig. 1. Combination Index (CI) versus fraction-affected (Fa) curves for 96-h ZD0473, paclitaxel, concomitant exposure for four human ovarian carcinoma cell lines; A2780 (■), A2780CisR (○), A2780E6 (▼) and CH1 (△). Curves generated from pooled data from 3–5 individual repeats.

Table 1
Combination Index at Fa 0.5 (CI_{50}), 0.75 (CI_{75}) and 0.90 (CI_{90}) for 96h simultaneous exposure to ZD0473 and other anticancer agents[a]

Cell Line	Compound	IC_{50}	r	CI_{50}	CI_{75}	CI_{90}
A2780	ZD0473	2.793 μM	0.888			
	Paclitaxel	0.003 μM	0.829			
	ZD0473 + paclitaxel (1000:1)	0.948 μM	0.900	0.611	0.500	0.426
	ZD0473	2.793 μM	0.888			
	Gemcitabine	0.011 μM	0.224			
	ZD0473 + gemcitabine (1000:1)	4.443 μM	0.917	2.003	1.065	0.710
	ZD0473	2.793 μM	0.888			
	Topotecan	0.017 μM	0.818			
	ZD0473 + topotecan (250:1)	4.682 μM	0.898	2.157	1.952	1.768
	ZD0473	2.793 μM	0.888			
	Vinorelbine	0.001 μM	0.495			
	ZD0473 + vinorelbine (200:1)	4.273 μM	0.608	2.107	3.313	5.969
	ZD0473	1.078 μM	0.958			
	Doxorubicin	9.59 μM	0.977			
	ZD0473 + doxorubicin (200:1)	1.009 μM	0.953	1.462	2.288	3.619
A2780CisR	ZD0473	13.738 μM	0.753			
	Paclitaxel	0.004 nM	0.942			
	ZD0473 + paclitaxel (1000:1)	0.949 μM	0.900	0.309	0.418	0.566
	ZD0473	13.738 μM	0.753			
	Gemcitabine	0.014 μM	0.587			
	ZD0473 + gemcitabine (1000:1)	4.443 μM	0.917	0.646	0.482	0.393
	ZD0473	13.738 μM	0.753			
	Topotecan	0.030 μM	0.787			
	ZD0473 + topotecan (250:1)	4.682 μM	0.898	0.616	0.576	0.663
	ZD0473	13.738 μM	0.753			
	Vinorelbine	0.001 μM	0.643			
	ZD0473 + vinorelbine (200:1)	4.273 μM	0.608	1.167	1.744	3.357
	ZD0473	5.49 μM	0.882			
	Doxorubicin	89.07 μM	0.865			
	ZD0473 + doxorubicin (200:1)	6.793 μM	0.880	1.619	0.866	0.603
A2780E6	ZD0473	6.039 μM	0.778			
	Paclitaxel	0.017 μM	0.755			
	ZD0473 + paclitaxel (1000:1)	2.424 μM	0.879	0.542	0.543	0.629
	ZD0473	6.039 μM	0.778			
	Gemcitabine	0.010 μM	0.655			
	ZD0473 + gemcitabine (1000:1)	4.208 μM	0.883	1.142	0.803	0.682
	ZD0473	6.039 μM	0.778			
	Topotecan	0.044 μM	0.874			
	ZD0473 + topotecan (250:1)	2.881 μM	0.816	0.585	0.772	1.046
	ZD0473	6.039 μM	0.778			
	Vinorelbine	0.003 μM	0.444			
	ZD0473 + vinorelbine (200:1)	5.510 μM	0.774	1.266	1.031	1.126
	ZD0473	1.699 μM	0.917			
	Doxorubicin	23.87 μM	0.956			
	ZD0473 + doxorubicin (200:1)	2.438 μM	0.971	1.945	1.470	1.132
CH1	ZD0473	4.002 μM	0.888			
	Paclitaxel	0.004 μM	0.565			
	ZD0473 + paclitaxel (1000:1)	1.062 μM	0.869	0.549	0.468	0.400
	ZD0473	4.002 μM	0.888			
	Gemcitabine	0.024 μM	0.451			
	ZD0473 + gemcitabine (1000:1)	2.145 μM	0.866	0.625	0.642	0.721
	ZD0473	4.002 μM	0.888			
	Topotecan	0.014 μM	0.915			
	ZD0473 + topotecan (250:1)	1.600 μM	0.965	0.555	0.506	0.470
	ZD0473	4.002 μM	0.888			
	Vinorelbine	0.002 μM	0.695			
	ZD0473 + vinorelbine (200:1)	1.890 μM	0.948	0.672	0.676	0.753

(*continued on next page*)

Table 1 (*continued*)

Cell Line	Compound	IC_{50}	*r*	CI_{50}	CI_{75}	CI_{90}
	ZD0473	0.519 μM	0.969			
	Doxorubicin	4.25 μM	0.922			
	ZD0473 + doxorubicin (200:1)	1.359 μM	0.972	4.215	4.056	3.985

Values represent the mean calculated from 3–5 individual experiments. Ratios are fixed ratios of concentrations used for ZD0473 versus the comparison drug.

[a] Individual IC_{50} values are shown for each drug and *r* = the goodness of fit for the pooled data (where *r* = 1 is a perfect fit).

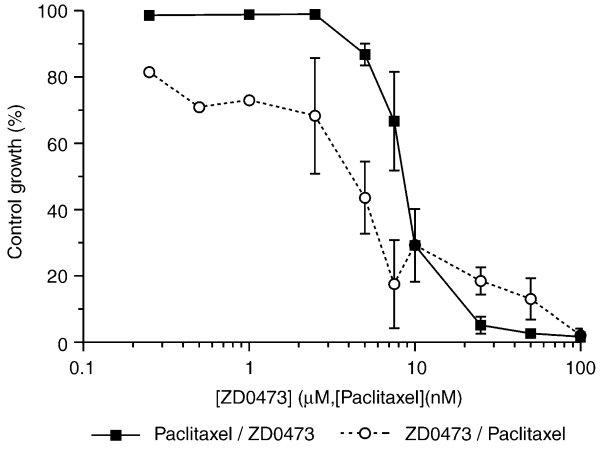

Fig. 2. Dose–response curves for sequential ZD0473 and paclitaxel exposure in A2780CisR cells; paclitaxel/ZD0473 (■), ZD0473/paclitaxel (○). Drug exposure was for 24h to each drug with drug washed off after each exposure period, total incubation time was 96h. A fixed ratio of drugs was used of 1000:1 ZD0473 to paclitaxel; e.g. 1 μM ZD0473 to 1 nM paclitaxel (see abscissa). Curves are pooled data from 3–5 experiments. Error bars = mean ± standard deviation (S.D.).

3.3. Flow cytometric analyses

We hypothesised that the sequence-dependent effects observed with ZD0473 and paclitaxel may be related to the individual effects of the two drugs on the cell cycle. Therefore, we performed a cell-cycle analysis on A2780CisR cells (the line showing the greatest sequence dependency) using 24-h exposure to either paclitaxel (4 nM), ZD0473 (15 μM) or to both agents administered sequentially. These concentrations represent approximate IC_{50} values for 96-h (and 24-h) drug exposure. Results are shown in Fig. 3. A comparison of cell cycle distribution with paclitaxel followed by ZD0473 (antagonistic) versus the reverse synergistic sequence did not reveal any striking differences either upon immediate harvesting or delayed harvesting following 24-h incubation in drug-free growth medium. For example, percentage values (delayed harvest) were G1—14.5 and 15.1, S—57.8 and 54.3, G2—27.7 and 30.6 for paclitaxel/ZD0473 and ZD0473/paclitaxel, respectively. Each individual drug appeared to cause some build-up in G2, especially ZD0473.

Table 2
Combination Index at Fa 0.5 (CI_{50}), 0.75 (CI_{75}) and 0.90 (CI_{90}) for sequential exposure to ZD0473 and other anticancer agents[a]

Cell Line	Compound	IC_{50} (μM)	R	CI_{50}	CI_{75}	CI_{90}
A2780	ZD0473	0.856	0.700			
	Paclitaxel	0.003	0.813			
	ZD0473 + paclitaxel (1000:1)	1.096	0.801	1.609	1.358	1.258
	Paclitaxel + ZD0473 (1:1000)	8.780	0.882	12.9	7.1	4.3
A2780CisR	ZD0473	10.516	0.675			
	Paclitaxel	0.014	0.892			
	ZD0473 + paclitaxel (1000:1)	4.144	0.804	0.697	0.707	0.790
	Paclitaxel + ZD0473 (1:1000)	8.782	0.882	1.477	0.876	0.571
A2780E6	ZD0473	8.474	0.836			
	Paclitaxel	0.005	0.732			
	ZD0473 + paclitaxel (1000:1)	3.019	0.837	0.915	1.137	1.417
	Paclitaxel + ZD0473 (1:1000)	3.354	0.879	1.016	1.086	1.165
CH1	ZD0473	0.982	0.702			
	Paclitaxel	0.003	0.819			
	ZD0473 + paclitaxel (1000:1)	0.998	0.704	1.304	2.345	4.496
	Paclitaxel + ZD0473 (1:1000)	1.602	0.770	2.092	2.012	2.062

Values represent the mean calculated from 3–5 individual experiments. Ratios are fixed ratios of concentrations used for ZD0473 versus comparison drug.

[a] Drug exposure was for 24 h to each drug with drug aspirated off at the end of each exposure. Control wells were treated similarly. Individual IC_{50} values are shown for each drug and *r* = the goodness of fit for the pooled data (where *r* = 1 is a perfect fit).

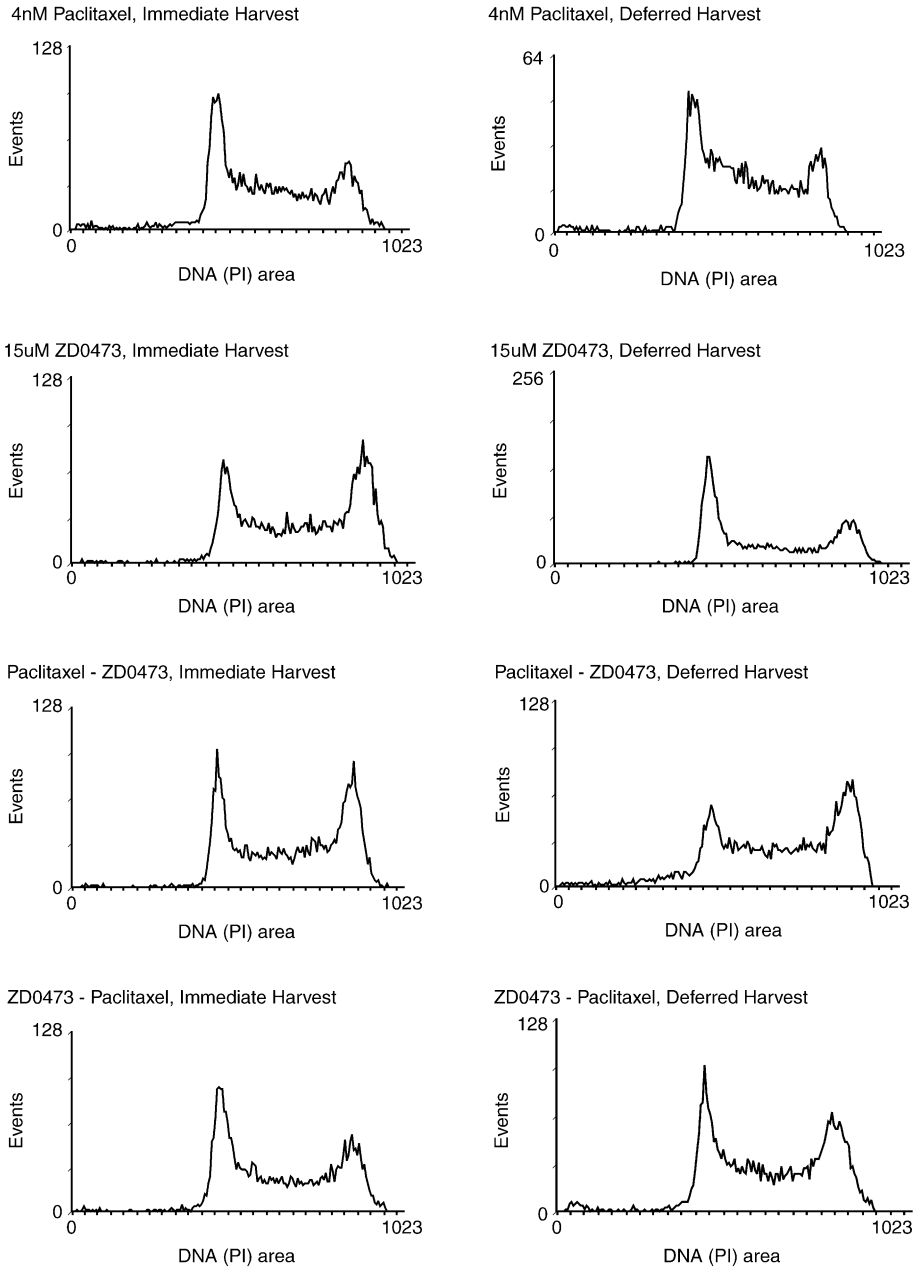

Fig. 3. Cell cycle effects of 24 h exposure to paclitaxel (4 nM) and/or ZD0473 (15 μM) in A2780CisR cells either harvested immediately following agent removal or following 24-h in drug-free growth medium (deferred harvest).

4. Discussion

ZD0473 is an interesting new generation platinum agent selected on the basis of its circumvention of acquired cisplatin resistance in preclinical studies. The drug is currently undergoing extensive phase II clinical evaluation. We have used four human ovarian carcinoma cell lines representative of the clinical situation to investigate the potential of combining ZD0473 with other cytotoxic cancer drugs. Two of the cell lines were 'parent' lines, the A2780 line being derived from a previously untreated patient [22]. Another line, A2780CisR, is representative of acquired cisplatin resistance (through multiple mechanisms including reduced drug transport, increased glutathione and enhanced DNA repair of platinum–DNA adducts) [2] and another where loss of p53 function has been achieved [19]. The most notable finding from the drugs studied was that of consistent synergy, independent of p53 status and acquired cisplatin-resistance status, between ZD0473 and the taxane paclitaxel. This was illustrated by CI_{50} values ranging from 0.31 in A2780CisR cells to 0.61 in the parent line, indicative of synergy. Similarly, synergy has been reported with concomitant exposure of ovarian cell lines to cisplatin or carboplatin with paclitaxel or docetaxel [16,23].

Interestingly, the sequence-dependency results with ZD0473 and paclitaxel are in contrast to those previously published for cisplatin and paclitaxel [13–15]. Our study shows that, in three of the four cell lines, the combination of ZD0473 administered before paclitaxel produced superior growth inhibition in comparison to ZD0473 given after paclitaxel. The exception was the A2780E6 subline where p53 function has been inactivated by stable transfection with the *E6* gene. Published studies with cisplatin and paclitaxel have generally shown greater synergy when cisplatin was administered after, rather than, before paclitaxel [13–15]. It is unclear why there is an apparent difference in schedule dependency with paclitaxel plus cisplatin versus paclitaxel plus ZD0473 (although there has been no direct comparison of both combinations using the same cell lines). However, there is already plenty of evidence indicating that ZD0473 does possess differing cellular pharmacological and DNA binding properties to those of cisplatin [2,3,24] . We proposed that the sequence-dependent findings with ZD0473 and paclitaxel might have been reflected in the corresponding drug-induced changes in the cell cycle. However, this did not appear to be the case as was also true for cisplatin and paclitaxel [13]. Thus far, mechanistic studies reported for the cisplatin/paclitaxel combination have not revealed the mechanism for the synergy although this does not appear to relate to changes in glutathione or platinum transport [13,14].

Greater than additive growth inhibitory effects were also obtained by combining ZD0473 with either the topisomerase I inhibitor, topotecan (in three of four cell lines), gemcitabine (in two of four cell lines) or the vinca analogue vinorelbine (in only the CH1 line). The result with topotecan is interesting in that this was the only drug where, following simultaneous exposure, a different effect was apparent between the isogenic pair of lines differing only in their p53 status. Whereas antagonism was apparent between ZD0473 and topotecan with the parent wild-type p53 A2780 cell line, synergy (CI_{50} of 0.585) occurred in the A2780E6 cells. The finding of synergy between ZD0473 and gemcitabine in two cell lines (A2780CisR and CH1) is in accordance with published reports of synergy between gemcitabine and cisplatin [18]. The synergism appears to be related to an increase in platinum–DNA adduct formation and increased gemcitabine incorporation into DNA and RNA [25].

In summary, these data suggest that ZD0473 may be usefully combined with other cytotoxic drugs such as paclitaxel, gemcitabine or topotecan. However, these *in vitro* findings of additivity/synergy should be viewed in context as they do not address the critical issue of therapeutic gain *in vivo* and thus require confirmation in xenograft or clinical studies. As the dose-limiting toxicities of ZD0473 are rather more 'carboplatin-like' (thrombocytopenia being dose-limiting) than cisplatin-like, it would be of interest to test clinically whether a platelet-sparing effect, as seen with carboplatin/paclitaxel combination studies [26], is also apparent with ZD0473. In accordance with these results, a phase I study of ZD0473 with paclitaxel is already underway [27]. Thereafter, it may be appropriate to conduct additional trials addressing the issue of sequencing as has been reported for cisplatin/paclitaxel [28].

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