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RELATIONSHIPS BETWEEN RESISTANCE TO CROSS-LINKING AGENTS AND GLUTATHIONE METABOLISM, ALDEHYDE DEHYDROGENASE ISOZYMES AND ADENOVIRUS REPLICATION IN HUMAN TUMOUR CELL LINES

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Abstract—In a panel of 10 human tumour cell lines with no prior exposure to drugs *in vitro*, resistance to cisplatin correlated with resistance to the nitrogen mustard derivatives Asta Z-7557 (mafosfamide, an activated form of cyclophosphamide), melphalan and chlorambucil. Simultaneous treatment with DL-buthionine-*S,R*-sulfoximine did not enhance the toxicity of cisplatin or Asta Z-7557, and no correlation was found between drug resistance and cellular levels of metallothioneins (as judged by sensitivity to cadmium chloride), glutathione (GSH), GSH reductase, GSH transferase, or γ-glutamyltranspeptidase. The two cell lines most resistant to Asta Z-7557 expressed aldehyde dehydrogenase cytosolic isozyme 1, found also in normal ovary, but not isozyme 3. Treatment of resistant cells with cisplatin or Asta Z-7557 inhibited cellular DNA synthesis and replication of adenovirus 5 to a lesser extent than in sensitive cells. The virus could be directly inactivated by both drugs prior to infection, subsequent replication being inhibited to the same extent in sensitive and resistant cells. In contrast to Asta Z-7557 and other DNA damaging agents, cisplatin was much more toxic to adenovirus (D_{37} 0.022–0.048 μM) than to cells (D_{37} 0.25–2.5 μM). The adenovirus 5 mutant Ad5ts125 having a G → A substitution was even more sensitive to cisplatin (D_{37} 7–8 nM) than wild type virus and another mutant. Cisplatin was detoxified less by sonicated resistant cells than sensitive cells, as judged by inactivation of Ad5ts125 added to the reaction mixture. It can be inferred that (i) the major differences in cellular resistance to cisplatin and Asta Z-7557 in the present material did not involve enhanced DNA repair or protection by metallothioneins or GSH, but were associated with the ability to continue cellular and viral DNA synthesis during treatment, (ii) resistance was not associated with less template damage, and (iii) the adenovirus genome may be a suitable probe for predicting tumour resistance to cisplatin and for elucidating the DNA sequence dependence of cisplatin toxicity.

Cellular resistance of tumours to present drugs remains an important problem in the chemotherapy of neoplasms such as ovarian cancer where up to 50% of patients respond to DNA cross-linking agents but eventually become resistant to a range of drugs [1, 2].

Resistance to cross-linking agents develops slowly compared with other drugs [3–5] and in mammalian cells may involve a variety of mechanisms including inhibited transport [6, 7], DNA repair [7–11], presentation of alternative targets such as glutathione or metallothioneins [12–17], detoxification by aldehyde dehydrogenase (ALDH¶) [18, 19] and combinations of the above [7, 20]. Human tumour cell lines cross-resistant to cross-linking agents and topoisomerase II inhibitors have been described [12, 13, 21, 22] but the basis for such an association remains undefined. Attempts to enhance the toxicity of cross-linking agents by depletion of intracellular glutathione (GSH) with buthionine sulphoximine (BSO) have given variable results [12, 13]. There is therefore a need to identify those tumours most likely to respond to cross-linking agents, and to understand the genotoxic and epigenetic effects of the drugs in human tumour cells. Adenovirus may serve as a functional probe for some of these purposes.

Human normal and tumour cells provide many of the requirements for replication of the double-stranded DNA genome of adenovirus. Within certain limitations, the effect of an agent on the replication of viral DNA may thus be studied as a model for the replication of cell DNA. Such a probe can be used in two different ways, host cell reactivation (HCR) or viral capacity (VC), to study separately the genotoxic and epigenetic effects of an agent. In the former case, replication of drug-treated virus is compared in sensitive and resistant lines to determine (i) whether the agent is genotoxic at all, viral

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¶ Abbreviations: ALDH, aldehyde dehydrogenase; BSO, DL-buthionine-*S,R*-sulphoximine; HCR, host cell reactivation; VC, viral capacity; ID, infectious dose; PBS, phosphate-buffered saline, pH 7.2; cisplatin, *cis*-diamminedichloroplatinum(II); Asta Z-7557, 4-sulphoethylthiocyclophosphamide; GSH, glutathione; wt, wild type; ts, temperature sensitive.

inactivation being assumed to arise from DNA damage, and (ii) whether resistance arises from enhanced ability of cellular enzymes to repair the viral DNA (HCR assay) [23]. Epigenetic damage on the other hand can be detected by inhibited replication of control virus in drug-treated cells (VC assay), for example as found with hydroxyurea and deoxyadenosine [24].

This approach has been applied to compare the action of carcinogens in sensitive and resistant mammalian cells [23, 24], and to demonstrate deficient repair of cisplatin-treated SV-40 DNA in xeroderma pigmentosum cells [25]. We have compared the effects of two clinically important cross-linking agents, cisplatin and activated cyclophosphamide, on adenovirus replication and cellular DNA synthesis in a panel of sensitive and naturally-resistant human tumour cell lines. In addition, indices of GSH metabolism, ALDH isozyme activity and metallothionein function were measured in order to detect other mechanisms of resistance.

MATERIALS AND METHODS

The origins of HeLa-S_3 and the human melanoma cell lines MM96L, MM127, MM253cl and MM418 have been described elsewhere [26, 27]. MM474F and MM489F strains are of normal fibroblasts established from human melanoma biopsies. The human ovarian tumor cell line CI-80-13S [28] was provided by Dr R. Bradley, Cancer Institute, Melbourne. The human ovarian tumour lines GG [29] and JAM [30] were obtained from Dr B. Ward, University of Queensland. Asta Z-7557 (Astawerke, F.R.G.) was provided by Dr P. Smith, of this Institute. Melphalan (1 mg) was dissolved in 100 μL DMSO and diluted directly into virus suspensions or, for cell treatments, diluted first into culture medium. Cisplatin was diluted from a 10 mg/mL solution in NaCl (David Bull, Melbourne, Australia). The other drugs were dissolved in culture medium.

Cells were cultured in 5% CO_2/air at 37° in Roswell Park Memorial Institute medium 1640 (Flow Laboratories, Sydney, Australia) supplemented with 1 mM pyruvate, 200 μM nicotinamide, 100 I.U./mL penicillin, 100 μg/mL penicillin, 100 μg/mL streptomycin, 3 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid and 10% fetal calf serum. Assays for *Mycoplasma* by culture on agar were negative. Cell survival was determined by a modified colony assay because most of the cell lines did not form discrete colonies readily counted by eye. This method, which gives similar results to visual counting of colonies for a variety of agents including cross-linking agents [31, 32], involved addition of drug to duplicate cultures (2×10^3cells/16-mm well) seeded 24 hr previously and, after 6–7 days of continuous exposure without change of medium, labelling the cultures with [^{3}H]thymidine for 2 to 4 hr. Cells were detached (0.02% trypsin and 0.1 mM EDTA in PBS), lysed with water and harvested onto glass fibre disks for liquid scintillation counting of thymidine incorporation. The D_{37} (dose required to give 37% survival), D_0 (dose giving a reduction of 0.37 in the linear portion of the survival curve) and D_q (size of the shoulder) were calculated from dose–response curves obtained using five doses.

GSH was determined by reaction of 0.4 N perchloric acid-precipitated cell supernatant (2×10^7 cells/mL) with *o*-phthalaldehyde [33]. For assay of GSH enzymes, cells were lysed in PBS containing 1% Triton X-100 (2×10^7/mL). GGT and GSH transferase were assayed with the substrates L-γ-glutamyl-*p*-nitroanilide and 1-chloro-2,4-dinitrobenzene, respectively [34]. GSH reductase [35] and GSH peroxidase [36] were determined as described previously.

In the adenovirus experiments, duplicate cultures (5×10^3/6-mm well) seeded 24 hr previously in wells treated with 20 μg/mL poly-L-lysine for 1 hr were infected with 10-fold dilutions of adenovirus 5 for 1 hr and then washed once with medium. Viral replication was determined after 2 days by counting the number of virus-infected, immunoperoxidase-labeled cells [24]. One ID was defined as the amount of virus required to produce one infected cell. Unless otherwise stated, virus (1/10 dilution) was treated with the agent at 37° for 24 hr in PBS, dialysed overnight against PBS and then diluted further as described above for replication in cells. Controls (without drug) were incubated in parallel. The pH was found to remain constant (pH 7.2) during drug treatment. In some experiments, virus was treated in PBS diluted to give 4 mM chloride. The Ad5hr7 adenovirus mutant has a restricted host range due to an altered E1B gene [37], and the Ad5ts125 mutant encodes a temperature-sensitive DNA binding protein [38].

In attempts to recover the infectivity of adenovirus treated for 24 hr with 0.02–0.05 μM cisplatin in 4 mM NaCl, samples were incubated with 1 mM sodium diethyldithiocarbamate, 100 μM thiouracil or 10 mM KCN at 37° for 4 hr. Cells were then infected with the virus for 1 hr, washed twice and assayed for virus replication as described above. To compare the detoxification of cisplatin by different cell lines, 3×10^7cells in 0.2 mL PBS were sonicated at 2° (2 min at 50 W) and then incubated with 100 μM cisplatin in the same volume at 37° for 30 min. Ad5ts125 (in 20 μL PBS; 2×10^6 ID) was added and incubation was continued for 120 min. The reaction was terminated by dilution with culture medium (minimum of 100-fold), followed by titration of viral infectivity in HeLa cells at 32° using immunoperoxidase detection as described above.

Cellular DNA synthesis was measured by prelabeling cultures (5×10^4cells/16-mm well) with [2-C^{14}]thymidine (0.005 μCi/mL; 20 Ci/mol) for 24 hr, followed by drug treatment and then pulse labeling for 45 min with [*methyl*-^{3}H]thymidine (5 μCi/mL; 40 Ci/mmol). The cells were detached with trypsin, lysed, harvested onto glass fibre discs with H_2O and solubilized in Soluene 350 (Packard Instruments, Zurich, Switzerland) prior to liquid scintillation counting. The $^3H/^{14}C$ ratio was expressed as a percentage of untreated controls harvested at the same time.

Statistical tests for significant differences between cell lines were carried out with the Student's *t*-test, applied to the slope and SE of the slope of the

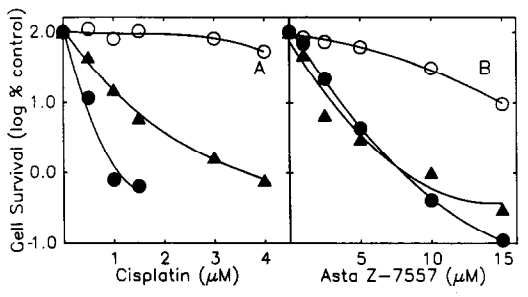

Fig. 1. Dose–response of cell survival after continuous treatment with cisplatin (A) or Asta Z-7557 (B). (●) HeLa; (▲) MM127; (○) CI-80-13S. Points are means of duplicates.

response curve or to the mean and SE of the D_{37} from separate experiments.

RESULTS

Cellular resistance to cross-linking agents in human tumour cell lines

A panel of tumour cell lines and two fibroblast strains with no prior exposure to drugs *in vitro* was assayed for resistance to the two most commonly used DNA cross-linking agents, cisplatin and cyclophosphamide. Since the latter drug requires metabolic activation, Asta Z-7557, which hydrolyses spontaneously under aqueous conditions releasing 4-hydroxycyclophosphamide [39], was used.

The dose–response curves for cell survival were generally semi-logarithmic in the 1–100% survival range for sensitive cell lines (Fig. 1). Some of the resistant lines exhibited a shoulder (D_q) at low doses on the survival curve. The relative resistances of cell lines were therefore compared by the dose giving 37% survival (D_{37}), a parameter which is influenced both by D_q and D_0. In this study, D_{37} was similar to D_0 in all cell survival curves. The results in Fig. 1 and Table 1 showed a 10-fold variation in the D_{37} for cisplatin in the cell lines studied, and a 5-fold range for Asta Z-7557. The validity of the thymidine incorporation method was confirmed by colony assay using the CI-80-13S and HeLa lines treated with cisplatin. No correlation between resistance and cell parameters such as histological type, doubling time, size or DNA content were found for either drug (results not shown). The highly melanized MM418 cell line was in the resistant group.

Cellular resistance to $CdCl_2$ was determined in some of the cell lines, as a functional test for resistance associated with elevated levels of metallothioneins [14–17]. No cross resistance was found however, between $CdCl_2$ and the cross-linking agents (Table 1).

Resistance to melphalan and chlorambucil was determined in a smaller number of cell lines (Table 2 and Fig. 2). Again, no correlation of drug resistance with known cell parameters was found. A high degree of cross-resistance was found between cisplatin and Asta Z-7557 ($r = 0.88$), chlorambucil ($r = 0.96$), or melphalan ($r = 0.99$). No correlation was evident in these cell lines with resistance to a DNA methylating agent [26] or topoisomerase II inhibitors (unpublished results).

In a previous study of human melanoma cells [11], the dose–response of cell survival to melphalan did not follow the expected concentration ×time ($C \times t$) relationship in some cell lines. A similar study was therefore made of cisplatin and Asta Z-7557 with CI-80-13S and HeLa, in which the stability of the drug in culture medium, measured by loss of toxicity, was determined simultaneously with the exposure time required for the drug to exhibit toxicity in sensitive and resistant cells, at doses of equitoxicity. The results (not shown) indicated that cisplatin and Asta Z-7557 exhibited a linear $C \times t$ relationship to toxicity in both cell lines.

In attempts to modulate the toxicity of cross-linking agents with BSO it was found that at the low cell densities required for the clonogenic survival

Table 1. Sensitivity of human cells to cisplatin, Asta Z-7557 and $CdCl_2$

Cell	Cisplatin (μM)				Asta Z-7557 (μM)				$CdCl_2$ (μM)	
	N	D_{37}	D_0	D_q	N	D_{37}	D_0	D_q	N	D_{37}
Ovarian tumour										
CI-80-13S	9	2.5 ± 0.6	2.2 ± 0.6	0.31 ± 0.43	5	5.6 ± 1.7	5.0 ± 1.8	0.50 ± 0.63	1	3.1
GG	3	0.82 ± 0.36	0.82 ± 0.36	0	2	6.9 ± 0.8	5.9 ± 0.7	1.41 ± 1.41	1	27
JAM	3	0.52 ± 0.15	0.52 ± 0.15	0	3	2.2 ± 0.3	2.2 ± 0.3	0	1	35
Melanoma										
MM96L	5	0.65 ± 0.22	0.54 ± 0.21	0.10 ± 0.16	3	2.1 ± 0.7	1.8 ± 0.8	0.24 ± 0.33	1	13
MM127	5	0.28 ± 0.15	0.28 ± 0.15	0	3	1.1 ± 0.09	1.1 ± 0.09	0	NT	
MM170	2	0.91 ± 0.18	0.78 ± 0.30	0.11 ± 0.11	1	2.9	2.9	0	NT	
MM253cl	2	0.32 ± 0.14	0.32 ± 0.14	0	1	1.52	0.71	0.61	1	13
MM418	3	1.10 ± 0.24	0.98 ± 0.19	0.08 ± 0.11	3	3.5 ± 1	3.5 ± 1	0	1	9.3
Others										
HeLa	10	0.24 ± 0.07	0.24 ± 0.07	0	5	2.0 ± 0.7	1.7 ± 0.7	0.21 ± 0.41	1	62
MM474F	2	0.67 ± 0.05	0.51 ± 0.11	0.16 ± 0.16	NT					NT
MM489F	1	0.33	0.33	0	NT					NT

N, number of experiments.
Values are means ± SD.
NT, not tested.

Table 2. Sensitivity of human cells to melphalan and chlorambucil

Cell line	Melphalan (μM)				Chlorambucil (μM)			
	N	D_{37}	D_0	D_q	N	D_{37}	D_0	D_q
CI-80-13S	2	4.7 ± 0.2	4.2 ± 0.3	0.37 ± 0.37	3	8.4 ± 2	7.8 ± 1.2	0.49 ± 0.69
MM96L	2	2.0 ± 0.3	2.0 ± 0.3	0	2	5.0 ± 3.6	3.5 ± 2.1	1.4 ± 1.4
MM127	2	1.1 ± 0.6	1.1 ± 0.6	0	2	2.2 ± 0.4	2.2 ± 0.4	0
MM418	1	2.6	2.6	0	NT			
HeLa	2	1.0 ± 0.2	0.64 ± 0.04	0.36 ± 0.28	1	2.3	2.3	0

N, number of experiments.
Values or means ± SD.
NT, not tested.

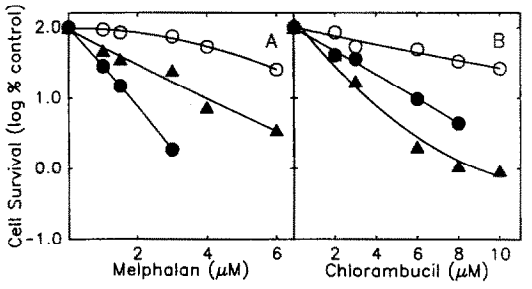

Fig. 2. Dose–response of cell survival for melphalan (A) and chlorambucil (B). (●) HeLa; (▲) MM127; (○) CI-80-13S. Points are means of duplicates.

assay BSO alone exhibited significant toxicity. At the non-toxic level of 2 μM, BSO had little effect upon the dose-responses for survival of several cell lines after simultaneous treatment with cisplatin or Asta Z-7557 (Table 3). Four cellular properties involved in GSH metabolism were determined in untreated sensitive and resistant cell lines (Table 3). Neither the GSH level itself nor the activities of enzymes likely to regulate GSH (GGT, GSH reductase) or to conjugate electrophilic agents (GSH transferase) correlated with cellular resistance to the cross-linking agents. No GSH transferase could be detected in HeLa cells.

ALDH isozymes in resistant cell lines

Cell lysates and human tissue standards (skin, liver, stomach and ovary) were electrophoresed on isoelectric focusing gels and stained for NAD-dependent ALDH activity using *n*-hexanal as the substrate. The results (Fig. 3) showed high activity of the ALDH 1 isozyme, typical of normal ovary and the liver cytosolic form [40, 41], in the Asta Z-7557 resistant CI-80-13S and GG lines but not in the JAM line which had significant activity of ALDH 3 or in the other lines which had little activity of any type. No activity was found in controls (no NAD).

Ability of cells to replicate drug-treated adenovirus (HCR)

Virus was treated with drug and either dialysed or diluted at least 100-fold before infection, ensuring that no cytotoxic level of the agent was carried over to the cell cultures. Cisplatin reacts with DNA via the hydrated complex, the concentration of which is inversely dependent on the chloride ion concentration [10]. Replication of adenovirus treated with cisplatin

Table 3. GSH metabolism in human tumour cells, relative to the CI-80-13S line

Cell line	GSH content	GSH transferase	GSH reductase	GGT	Effect of BSO (2 μM) on drug toxicity*	
					Cisplatin	Asta Z-7557
CI-80-13S†	1.0	1.0	1.0	1.0	76	88
HeLa	0.47	<0.01	0.87	110	91	73
MM96L	0.76	0.91	1.8	13	NT	NT
MM127	NT	0.59	0.39	0.51	NT	NT
MM253cl	1.1	0.23	0.92	24	NT	NT
MM418	0.92	0.37	0.98	22	NT	NT

* Per cent of D_{37} for the combination expressed as a percentage of the D_{37} for the cross-linking agent used alone. Both drugs were added at the same time and left on for 6–7 days.

† Absolute values for CI-80-13S were: GSH, 6.1×10^{-15}mol/cell; GSH transferase, 144nmol/min/mg protein; GSH reductase, 25 nmol NADPH oxidized/min/mg protein; GGT, 0.29 nmol/min/mg protein.

NT, not tested.

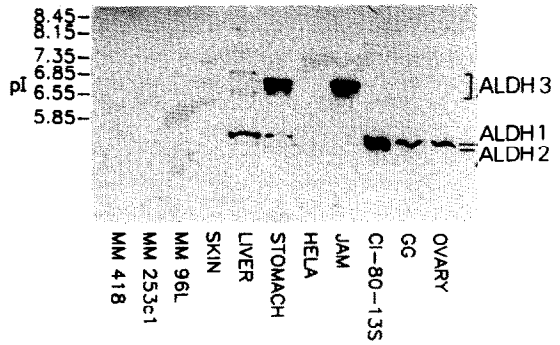

Fig. 3. ALDH isozymes in human tumor cell lines. Cell lysates (approximately 10 μg protein per lane) were subjected to isoelectric focusing on agarose gels and stained for ALDH activity with 1 mM *n*-hexanal as the substrate, as described previously [40, 41]. Bars indicate pI standards.

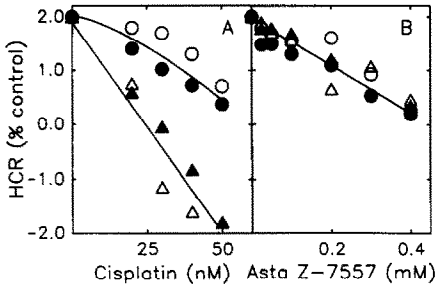

Fig. 4. Replication of adenovirus strains Ad5wt (○, ●) and Ad5ts125 (△, ▲) treated with cisplatin (A) or Asta Z-7557 (B). HeLa (○, △); CI-80-13S (●, ▲).

in 4 mM chloride, corresponding to the intracellular chloride concentration [10], was consistently inhibited to the same extent in sensitive and resistant cells, and at cisplatin levels much lower than those required to kill cells (Fig. 4A and Table 4). Since the concentration and mobility of anions in the nucleus may lower the reactivity of cisplatin, virus was also treated with cisplatin in high chloride. Virus treated in 150 mM NaCl for 1 hr (D_{37} 20–30 μM for four cell lines) was inactivated less than when treated for 24 hr (D_{37} 0.75–1.0 μM) but again no significant difference was observed between sensitive and resistant cell lines. The reactivity of the nitrogen mustard compounds with DNA should also be higher in low chloride but with a half-life of 1–2 hr in 150 mM chloride, the 24-hr virus treatment time precludes any major difference. This was confirmed for the inactivation of adenovirus by Asta Z-7557 at both chloride concentrations (results not shown). Unlike cisplatin, the D_{37} for virus inactivation by Asta Z-7557 was much higher than for cell toxicity. No major difference in the HCR of cisplatin or Asta Z-7557-treated virus was found amongst sensitive and resistant cell lines (Fig. 4 and Table 4). Similar results were obtained with virus treated for 1 hr (results not shown).

The adenovirus mutant Ad5hr7 showed an HCR response similar to that of Ad5wt virus. The temperature-sensitive Ad5ts125 mutant, however, was considerably more sensitive to inactivation by cisplatin than Ad5wt (Fig. 4A and Table 4). This was not due to the lower temperature required for replication of Ad5ts125 because Ad5wt was inactivated to the same extent at 32° as at 37°. Asta Z-7557 had similar toxicity to both virus strains (Table 4). Cisplatin-treated virus was incubated with sodium diethyldithiocarbamate, thiouracil or sodium cyanide to reverse platinum–thiol and platinum–DNA complexes [42]. These agents, used at the maximum doses permitting cell viability, increased the titre of virus treated with 0.02 μM cisplatin by approximately 50% but had little effect on virus treated with 0.03 or 0.05 μM cisplatin.

The sensitivity of Ad5ts125 to inactivation by cisplatin prompted use of the former to bioassay the detoxification of cisplatin by sonicated cells. The chloride concentration of PBS was used because of residual PBS in the cell pellets prior to sonication, and the incubation times were brief in order to minimize proteolysis after sonication. Incubation of Ad5ts125 for 2 hr with sonicated cells (1.5×10^8/mL) had no effect on infectivity. In the presence of 100 μM cisplatin, the infectivity of virus treated in PBS alone was reduced to 0.03% of untreated virus. Considerable protection was afforded by treatment of virus in the presence of HeLa (0.89%) and CI-80-13S lysates (0.3%). In repeated experiments, detoxification of cisplatin by HeLa cells was more effective than CI-80-13S by a factor of 3.3 ± 0.3.

Inhibition of cellular DNA synthesis and adenovirus replication in drug-treated cells

Inhibition of cellular DNA synthesis required >3 hr treatment with cisplatin and >6 hr treatment with Asta Z-7557, the maximum difference between sensitive (HeLa) and resistant (CI-80-13S) cells being obtained with 24-hr treatment (Fig. 5). The dose–response curve determined at 24 hr showed that DNA synthesis, being inhibited to 37% by five times the cellular D_{37} levels, was relatively insensitive to these agents compared with cell survival (Fig. 6A and B). Cisplatin was more effective than Asta Z-7557 in inhibiting DNA synthesis, compared at equitoxic doses.

The ability of untreated adenovirus 5 to replicate in drug-treated cells (VC) was also tested in sensitive and resistant cells as an independent index of DNA synthesis. The results using Ad5wt (Fig. 6C and D) showed inhibition of VC by cisplatin and Asta Z-7557, up to doses causing overt cell death and detachment. The latter effect precluded obtaining a complete dose–response curve for Asta Z-7557 in HeLa cells (Fig. 6D). VC was inhibited more in sensitive cells than in resistant cells, particularly with cisplatin. This was not caused by direct reaction of drug with virus in the cells or culture medium because the hypersensitive Ad5ts125 mutant gave the same dose-response as Ad5wt (results not shown).

DISCUSSION

In this study, the high degree of natural resistance

Table 4. HCR of adenovirus strains treated with cross-linking agents

Cell line	D_{37} (μM) for Ad5wt replicated at 37°			D_{37} (μM) for viruses replicated at 32°				
				Cisplatin			Asta Z-7557	
	Cisplatin*	Asta Z-7557	Melphalan†	Ad5wt	Ad5hr7	Ad5ts125	Ad5wt	Ad5ts125
CI-80-13S	0.024	80	1000	0.027 ± 0.003‡	0.032	0.0075 ± 0.0004	100	110
GG	0.026	—	—	0.014	—	0.007	—	—
JAM	0.022	—	—	—	—	0.008	—	—
HeLa	0.040	65	1200	0.024 ± 0.006	0.05	0.0073 ± 0.0005	85	100
MM96L	—	—	350	—	—	—	—	—
MM127	0.048	—	500	—	—	—	—	—
MM170	0.038	—	—	—	—	—	—	—

* Cisplatin treatments carried out in 4 mM chloride.
† Virus treated for 1 hr; all other treatments were for 24 hr.
‡ Mean ± SD of 2–4 separate experiments.

Table 5. Comparison of the inhibition of adenovirus replication and cell survival by treatment with genotoxic agents

Agent	Reference	Anticellular activity/ antiviral activity
UV	[23]	20*
Methylating agents	[24]	100–200
Melphalan	This work	22–100
Asta Z-7557	This work	NT20–85
Cisplatin	This work	0.01–0.14 (4 mM Cl^-)†
		0.2–1 (150 mM Cl^-)†

* Ratio of D_{37} values for virus inactivation (HCR assay) and cellular toxicity.
† Concentration of chloride during virus treatment.

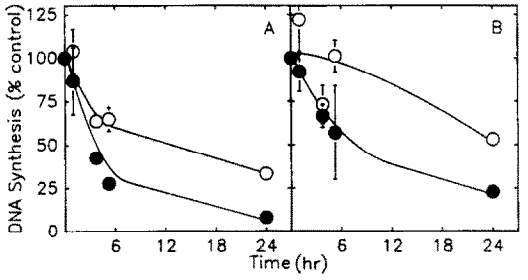

Fig. 5. Temporal response of cellular DNA synthesis after treatment with 5 μM cisplatin (A) or 15 μM Asta Z-7557 (B). (○) CI-80-13S; (●) HeLa. Points are means of duplicates.

in certain human tumour cell lines to cross-linking agents of different structures could not be accounted for by several known mechanisms. Treatment-time dependent resistance and DNA repair inferred from a previous study of melphalan [11] was not found with cisplatin and Asta Z-7557. Elevated metallothionein levels would appear to be unlikely because of lack of resistance to cadmium chloride; and detoxification of cisplatin by cell constituents in

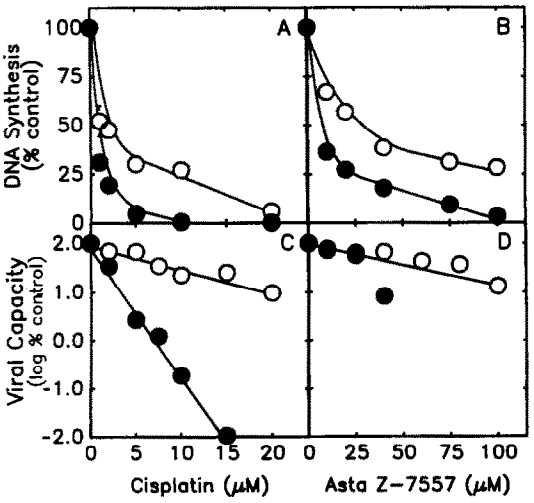

Fig. 6. Inhibition of DNA synthesis (A, B) and adenovirus replication (C, D) in human tumour cells treated with cross-linking agents for 24 and 48 hr respectively. (●) HeLa; (○) CI-80-13S. Points are means of duplicates.

general appeared to be more effective in sensitive than in resistant cells. The minimal effect of BSO on drug toxicity and the absence of any correlation between resistance and four cellular components of the GSH pathway including the level of GSH itself indicate that GSH is not an important mediator of drug resistance. HeLa cells for example, were not exceptionally sensitive despite having undetectable levels of GSH transferase, an enzyme likely to directly conjugate GSH with electrophilic agents such as the nitrogen mustard group. It should be noted, however, that the maximum level of BSO tolerated by cells at the cell density used in the survival assay may have been insufficient to effectively deplete the cells of GSH.

It is unlikely that resistant cells have enhanced repair of early or late DNA adducts because HCR of virus treated with drug for 1 or 24 hr was similar in sensitive and resistant cells. Although not addressed in this study, diminished transport of drugs into cells is considered to be an unlikely general mechanism of resistance because of the structural diversity of cisplatin compared with the aliphatic and aromatic nitrogen mustards. This leaves other mechanisms of resistance to be considered.

The high ALDH1 activities of the CI-80-13S and GG cell lines suggest that detoxification of aldophosphamide by ALDH found in resistant murine cells [18, 19] may also be associated with cyclophosphamide resistance in human tumour cells. In contrast the ALDH-3 isozyme, active in JAM cells, was not associated with resistance. Since normal ovary expresses ALDH1, it is possible that cyclophosphamide sensitivity arises during tumourigenesis. Characterization of the isozymes for substrate specificity and intracellular localization should now be possible, as well as assay of ALDH isozymes in patients' tumours. The reason for the cross-resistance of ALDH1 active cells to other cross-linking agents is not clear. An ovarian tumour line with induced resistance to cisplatin had elevated lactate dehydrogenase activity [43], another enzyme which contains SH groups. Sulfhydryl enzymes present in sufficient quantity may conceivably sequester electrophilic drugs, as suggested for metallothioneins [14–17]. Alternatively, co-ordinate activation of different resistance mechanisms for each agent may have occurred in these cells.

Resistance of treated cells to inhibition of cellular DNA synthesis and even more so of VC was another distinguishing feature of cisplatin-resistant cells found in this study. In the presence of serum, inhibition of DNA synthesis correlated with the antitumour activity of cisplatin [44]. The mechanism of resistance does not directly involve less DNA damage because the untreated viral genome was replicated poorly by sensitive cells in the VC assay. To address the possibility that cellular DNA damage indirectly inhibits DNA synthesis by affecting the transcription and translation of cellular proteins as proposed for Chinese hamster ovary cells [45], a detailed temporal response of VC to brief cisplatin treatments will be required. Such a study should uniquely detect drug-sensitive events in the 8–12 hr period after infection and before viral DNA synthesis commences and such information may help explain why the difference in VC between sensitive and resistant cells was greater than for cellular DNA synthesis. The fact that DNA synthesis was inhibited more in sensitive than in resistant cells as early as 4–6 hr after commencing treatment suggests a direct relationship with sensitivity. At present, it appears that the cisplatin resistance of CI-80-13S cells arises either from ALDH1 protection as suggested above, or from some other epigenetic property involved directly or indirectly in the maintenance of DNA synthesis. This may involve a topological phenomenon, perhaps associated with the resistance of these cells to topoisomerase II inhibitors [46], or ability to continue histone acetylation as found for rodent tumour cells resistant to nitrogen mustard [47].

Enhanced VC should be found in any resistant phenotype which directly or indirectly prevents inhibition of DNA synthesis by cisplatin. At the practical level of predicting individual tumour resistance to cisplatin, the VC assay may therefore provide a sensitive and relatively rapid method applicable to short term cultures of non-proliferating tumour cells, independent of the particular mechanism or combination of mechanisms of resistance. It is also possible that the VC assay will detect cyclophosphamide resistance, if the problem of cell detachment at high doses can be overcome.

Inactivation of a virus usually requires a much higher dose of agent than that needed to kill a cell, presumably because of the smaller target size of the former genome. It is of considerable interest therefore that cisplatin was much more effective than other genotoxic agents for inactivating adenovirus, relative to cell toxicity, even assuming a high chloride level in the nucleus (Table 5). Cisplatin has previously been shown to inhibit the infectivity of phage [48] and SV40 DNA [25], in the latter case at concentrations close to those required to inactivate mammalian cells. DNA–protein cross-links could not have been responsible because purified SV40 DNA was treated with the drug before transfection of cells. In a cell-free system, the cisplatin-induced lesions which blocked the replication of SV-40 DNA by *Escherichia coli* DNA polymerase were found to involve a regulatory sequence (GGGCGG) containing adjacent guanines in the same strand [49]. Apart from confirming the ability of intrastrand cross-links to inhibit DNA replication, those results could offer an explanation for the extreme sensitivity to cisplatin of adenovirus, the genome of which also contains guanine-rich regulatory sequences [50]. In exploring this approach using adenovirus mutants, however, we found that the Ad5ts125 mutant was considerably more sensitive to cisplatin compared with another mutant and wild type virus.

The Ad5ts125 mutant has a base substitution (GC → AT, resulting in proline → serine) in the sequence coding for a DNA-binding protein essential for initiation of viral DNA synthesis [51]. The protein is not present in virions and therefore cannot directly influence the toxicity of cisplatin, for example by becoming cross-linked to viral DNA. Since no difference was found with Asta Z-7557, a member of the nitrogen mustard group where toxicity is associated with purine interstrand cross-links [4, 5, 8],

it is assumed that the base substitution in Ad5ts125 somehow influences the number or consequence of DNA intrastrand cross-links induced by cisplatin. Whether this is due to formation of an exceptionally toxic adduct involving the new adenine, a base rarely substituted by cisplatin [9, 10], or to some indirect effect such as a conformational change in the DNA, remains unknown. A low proportion of d(ApG) adducts were found in treated single stranded DNA [52] and blocked DNA synthesis [53].

Compounds which partly reverse the formation of cisplatin adducts [42] had a limited effect on inactivated adenovirus. This approach applied to simultaneous HCR and VC assays could in principle be used to identify the separate functional effects of cisplatin in human cells but will require modification of the assay protocol in order to optimize the reversal conditions while maintaining virus and cell viability.

Overall, it is apparent that adenovirus mutants of defined sequence might provide a sensitive model for identifying rare but critical genomic targets of cisplatin.

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