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Nucleotide excision repair in the human ovarian carcinoma cell line (2008) and its cisplatin-resistant variant (C13*)

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Abstract Repair of cisplatin-damaged DNA was investigated in a human ovarian carcinoma cell line (2008) and its cisplatin-resistant variant (C13*) using a host-cell reactivation (HCR) assay. The HCR of cisplatin-damaged adenovirus (Ad) was not significantly different in C13* cells compared to 2008 cells. The cisplatin concentrations required to reduce the amount of viral DNA replicated to 50% were 0.12 ± 0.02 μM and 0.10 ± 0.01 μM after 48 h of repair in 2008 and C13* cells respectively. Similarly, the cisplatin concentration required to reduce the expression of a reporter gene inserted in the viral DNA was not significantly altered in C13* cells compared to the parental line (IC_{50} values were 0.28 ± 0.04 μM in 2008 cells and 0.17 ± 0.06 μM in C13* cells after 48 h of repair). Pretreatment of the cells with cisplatin, immediately prior to Ad infection, did not significantly alter the HCR of cisplatin-damaged Ad in either cell type. In addition, a cisplatin-sensitive variant derived from the C13* cells, namely the RH4 cells, did not differ significantly from either the 2008 or C13* cells in their ability to reactivate cisplatin-damaged Ad. Furthermore, a component of the nucleotide excision repair (NER) pathway, DNA polymerase α, was investigated using the competitive inhibitor aphidicolin. The combination of cisplatin and aphidicolin resulted in similar synergistic growth inhibition in both the 2008 and C13* cells providing additional support to the HCR results which suggest that enhanced NER is not responsible for the cisplatin resistance in C13* cells.

Key words Host cell reactivation · Cisplatin · DNA repair

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Introduction

Cisplatin-induced cytotoxicity is generally believed to be mediated through its ability to form intrastrand adducts and interstrand crosslinks within the cellular genome which can inhibit the transcription of genes and the replication of DNA (reviewed in references 5, 7, 12, 28 and 34). Thus, investigations into the mechanism(s) underlying cisplatin resistance have concentrated on the repair of these potentially lethal lesions. However, a consensus on the role of DNA repair in cisplatin resistance has not been reached. The variability of tumor type, cisplatin dose and the type of lesion investigated (intrastrand or interstrand), has obscured the importance of DNA repair in cisplatin resistance. The multifactorial nature of cisplatin-induced resistance makes it difficult to delineate a single mechanism that contributes to the resistance. In addition to enhanced repair of cisplatin-damaged DNA, mechanisms including altered drug transport, increased metallothionein or glutathione levels, mitochondrial alterations, and altered DNA adduct formation have been reported to contribute to cisplatin resistance [1, 7, 44]. It has been shown that equivalent extracellular cisplatin concentrations can result in a difference in cisplatin-DNA adduct formation in cisplatin-sensitive and cisplatin-resistant cells (as a result of decreased

accumulation or increased scavenging of cisplatin) [43]. Since the induction of certain genes (i.e. DNA damage-inducible genes) may be dependent on the amount of DNA damage, as is the case with UV irradiation [11], the initial amount of cisplatin-DNA damage may influence the rate of DNA repair. Therefore, inducing similar amounts of DNA damage in cisplatin-sensitive and cisplatin-resistant cells may be more appropriate when comparing DNA repair capacities in different cell types.

Zhen et al. [43] investigated total genomic and gene-specific repair of cisplatin intrastrand adducts and interstrand crosslinks in human ovarian carcinoma cells and suggested that enhanced repair of gene-specific interstrand crosslinks, but not intrastrand adducts, contributes to cisplatin resistance. Their study used atomic absorption spectroscopy (AAS) to assess the repair of cisplatin-DNA damage and as a result of the detection limits of AAS, cells were treated with supralethal cisplatin concentrations (as high as 1000 times the IC_{50}); it remains unclear how drug concentrations in this range affect the relative distribution of lesions and cellular repair capacity [38, 41]. Also, different initial levels of cisplatin-DNA damage were induced in the parental line and the cisplatin-resistant variant and as previously mentioned, the amount of DNA damage may influence the rate of DNA repair [11]. Data further supporting the idea that the amount of DNA damage may influence DNA repair rates have been reported by Johnson et al. [17]. They found that when the initial DNA platination levels are similar, repair of cisplatin damage from total genomic DNA is greater in the cisplatin-resistant variant A2780/CP70 relative to the parental A2780 line, after 8 h of repair time, whereas Zhen et al. [43] found no differences in repair between these cells at that time point when initial adduct formation differed in the two cell types. Parker et al. [25] have also shown in A2780 cells that the initial cisplatin adduct level can influence the rate of adduct removal.

We confirmed using both AAS and a polyclonal antibody to cisplatin adducts that equivalent cisplatin concentrations resulted in decreased adduct formation in the cisplatin-resistant variant compared with the parental line. Furthermore, supralethal cisplatin concentrations were required to obtain detectable levels of DNA adducts in both assays. To avoid the complications of supralethal cisplatin concentrations or different initial levels of DNA damage, we used a host cell reactivation (HCR) assay to investigate the repair capacities of the cells in our study. In the HCR assay, adenovirus type 5 (Ad 5) is incubated with cisplatin in order to induce cisplatin-viral DNA adducts. The cells are then infected with the cisplatin-damaged virus and their ability to replicate or transcribe the damaged viral DNA serves as an indicator of the repair capacity of the cell. Since the virus is exposed to cisplatin prior to infection, the cells are not directly exposed to the drug and only nanomolar concentrations of cisplatin are required to produce sufficient amounts of damage to the viral DNA. In addition, each cell type is exposed to an equal amount of cisplatin-damaged DNA.

Ad 5 can be used to investigate the repair of cisplatin damage because Ad 5 DNA is a linear duplex and its repair and replication are dependent on the enzyme pathways that are used in the cellular DNA of the host [14, 27, 32]. HCR of Ad has been used to examine repair capacities of DNA repair-deficient syndromes such as xeroderma pigmentosum (XP) [32, 39]. Fibroblasts from patients with XP are extremely sensitive to UV irradiation as a result of a deficiency in one or more of the steps in the nucleotide excision repair (NER) pathway [35]. NER involves several steps: recognition of the damaged DNA, incision of the DNA near the damage, excision and degradation of the damaged DNA strand, replacement of the damaged DNA strand and ligation of the replaced DNA strand [3, 13]. NER is also believed to be responsible for the repair of cisplatin adducts (XP patients are also hypersensitive to cisplatin) [5, 30]. In addition, the HCR assay system using either a plasmid [6, 25, 36] or a virus [23, 26, 27, 30] has been used to examine the ability of cells to repair damage induced by a number of agents including cisplatin.

Therefore, we used two variations of the HCR assay, viral DNA replication and expression of a reporter gene, to examine the repair capacities of a human ovarian carcinoma cell line, 2008, its cisplatin-resistant variant, C13*, and a rhodamine 123 revertant, RH4. The role of DNA polymerase α, a component of the NER pathway, in cisplatin resistance was also investigated.

Materials and methods

Materials

Cisplatin (*cis*-Diamminedichloroplatinum(II)), aphidicolin, and chloroquine diphosphate were obtained from Sigma Chemical Co., St. Louis, Mo. The 5-dodecanoylaminofluorescein di-ß-D-galactopyranoside (C_{12}FDG) was obtained from Molecular Probes, Eugene, Ore. Pronase and Ad 2 DNA were purchased from Boehringer Mannheim, Laval, Quebec, Canada and Gibco BRL, Burlington, Ontario, Canada respectively. Wild-type Ad 5 and Ad 5 HCMVSp1lacZ were provided by Dr. Frank Graham, McMaster University, Hamilton, Ontario, Canada.

Cell lines and culture conditions

The human ovarian carcinoma cell line, 2008 and its variants, C13* and RH4 were a generous gift from Dr. Paul Andrews, Georgetown University, Rockville, Md. The 2008, C13*, and RH4 cells were grown in RPMI-1640 medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. The AA8 and UV20 cells were provided by Dr. Larry Thompson, Lawrence Livermore National Laboratory, Livermore, Calif., with help from Dr. Gordon Whitmore,

Ontario Cancer Institute, Toronto, Ontario, Canada, and were grown in α-minimal essential medium and supplemented with 10% fetal bovine serum, 2 m*M* glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. All cultures were maintained at 37°C and in an atmosphere of air containing 5% CO_2 in a humidified incubator.

Quantification of cisplatin-DNA adduct formation

Cells in log-phase growth were seeded overnight in 150 × 25-mm dishes. The cells were exposed to various concentrations of cisplatin (prepared in phosphate-buffered saline, pH 7.2) for 1 h for AAS or for 2 h for the competitive ELISA. AAS and ELISA were performed as previously described [2, 24].

Virus treatment

Wild-type Ad 5 and Ad-5-HMCVSp1lacZ were treated with various concentrations of cisplatin for 24 h at 37°C in low chloride phosphate-buffered saline (4 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4; pH 7.2). The virus was diluted tenfold in serum-free medium prior to infection.

Replication of cisplatin-damaged Ad DNA

Cells seeded at a density of 2×10^5 cells per well in 24-well plates were infected with cisplatin-treated virus at a multiplicity of infection of 40 plaque forming units per cell for 90 min (agitated gently every 15 min) at 37°C in a humidified incubator. Following the infection period, medium containing unabsorbed virus was removed and the cells were incubated in fresh growth medium (0.5 ml per well) for 2–4 h. At this time, 0.2 ml of a lysis buffer (4 mg/ml pronase, 40 m*M* Tris pH 8.0, 40 m*M* EDTA pH 8.0, 2.4% sodium dodecyl sulfate) was added to one set of wells for each cell line to determine the extent of viral infection at time zero. Approximately 2 h after the addition of the lysis buffer, the samples were collected and stored at − 20°C until extraction of the DNA was performed. The remaining samples received lysis buffer 48 or 72 h after infection with the damaged virus and handled as previously described.

Total DNA (cellular and viral) was extracted with phenol/chloroform (1:1) and centrifuged at 12 000 *g* for 2 min. The aqueous phase was re-extracted with chloroform/isoamyl alcohol (25:1) and centrifuged at 12 000 *g* for 1 min. The DNA was precipitated with 2 volumes of absolute ethanol and 65 m*M* NaCl (stored overnight at − 20°C) and centrifuged at 12 000 *g* for 30 min at 4°C. The supernatant was removed and the DNA was allowed to dry. The DNA was then resuspended in TE buffer (10 m*M* Tris, 1 m*M* EDTA; pH 8.0) and stored at − 20°C. The DNA was slot-blotted and the amount of viral DNA was detected using a ^{32}P-labelled Ad 2 DNA probe. The viral DNA was quantitated using a the phosphoimager (Molecular Dynamic, Sunnyvale, Calif.).

Transcription of a reporter gene from cisplatin-damaged Ad DNA

The platination of the viral DNA and the infection procedure were identical to the procedures previously described except that the Ad-5-HMCVsp1lacZ virus which has the *lacZ* gene inserted into the E1a region of the viral genome was used and the cells were seeded at a density of 2×10^4 cells per well in 24-well plates. Following the 90-min infection period, the cells were incubated 48 h later with medium containing 300 μ*M* chloroquine diphosphate for 30 min at 37°C protected from light. The chloroquine was then removed and medium containing 25 μ*M* C_{12}FDG was added to each well and the cells were incubated at 37°C protected from light [42]. The protein transcribed from the *lacZ* gene, β-galactosidase, converts C_{12}FDG to a fluorescent substrate. The amount of fluorescence (an indicator of β-galactosidase activity) was determined at various time intervals using excitation and emission wavelengths of 485 nm and 530 nm, respectively, on a Cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ontario, Canada). The amount of fluorescence served as an indicator of the amount of gene transcription.

For the enhanced HCR assay, the procedure used was identical to that described for transcription of a reporter gene from cisplatin-damaged Ad DNA except that the cells were pretreated with cisplatin (IC_{50} or IC_{90} as determined from our survival assays) for 1 h immediately prior to infection with the cisplatin-damaged virus.

Survival assays

Cells in log-phase growth were seeded overnight at a cell density of 2000 and 1000 cells per well for 2008 and C13* cells, respectively, in 96-well plates and treated with cisplatin (prepared fresh for each experiment in phosphate-buffered saline, pH 7.2) alone for 1 h or aphidicolin (prepared in absolute ethanol and stored at − 20°C) alone for 26 h. The treatment protocol used to investigate the interaction of aphidicolin and cisplatin was aphidicolin for 1 h, cisplatin and aphidicolin for 1 h followed by a medium change, exposure to aphidicolin for an additional 24 h and concluding with 3 days in drug-free medium. Cell growth was assessed 4 days after the initiation of drug treatment on a Cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ontario, Canada) using the H33258 DNA fluorochrome method as previously described [31].

The nature of the interaction between cisplatin and aphidicolin was assessed using isobologram analysis at the 50% effect level. A combination index (CI) was calculated for the interaction of cisplatin and aphidicolin in both the 2008 and C13* cells. A CI of 1 indicates that the two drugs interact in an additive fashion while CIs of < 1 and > 1 indicate that the two drugs interact in a synergistic and antagonistic fashion, respectively [33].

Statistical analysis

All values are means ± SEM unless otherwise specified. $P < 0.05$ was considered statistically significant.

Results

Cisplatin-DNA adduct formation

The number of cisplatin-DNA adducts formed following treatment with different cisplatin concentrations was determined by both AAS and a competitive ELISA. A plot of cisplatin-DNA adducts versus dose was used to calculate the slope of adduct formation (data not shown). The 2008 cells had a significantly greater slope than the C13* cells (the 2008 cells had slopes that were 3.6-fold and 2.4-fold greater than the C13* cells as detected by AAS and ELISA, respectively) indicating that at equivalent cisplatin concentrations more cisplatin-DNA adducts were formed in the 2008 cells than in the C13* cells.

Replication of cisplatin-damaged Ad DNA

The Ad was exposed to cisplatin in a solution of low chloride phosphate-buffered saline in an attempt to mimic intracellular chloride concentrations [27, 45]. The use of the low chloride phosphate-buffered saline also permitted us to decrease the amount of cisplatin necessary to induce the cisplatin-viral DNA damage since it is the hydrated form of cisplatin that binds to DNA and hydration of cisplatin is favoured in the presence of low concentrations of chloride ions [27, 45]. It was also determined that temperature and duration of drug exposure could influence the amount of cisplatin-induced damage in the viral genome. Therefore, incubation of the virus at 37°C for 24 h in a low chloride environment with nanomolar cisplatin concentrations provided sufficient damage to the viral DNA.

The extent of viral infection was determined in each cell type by harvesting the cells 4 h after the end of the viral infection period. The virus used for determining the extent of infectivity was treated with the highest dose of cisplatin (0.4 μM) to ensure that the cisplatin treatment did not prevent infection by the virus. The amount of viral DNA was similar in all three cell types indicating that the cisplatin treatment did not inhibit infection by the virus and the extent of viral infection was similar in 2008, C13* and RH4 cells.

To determine the ability of the HCR assay to detect differences in the replication of cisplatin-damaged Ad DNA, AA8 and UV20 cells were used as controls. UV20 cells are UV repair-deficient mutants of the Chinese hamster ovary cell line AA8. The UV20 cells are also hypersensitive to cisplatin because they lack the incision step of the excision repair pathway which is necessary for removal of cisplatin-DNA adducts [8]. Figure 1 shows a representative experiment indicating that at varying degrees of cisplatin-induced viral DNA damage, UV20 cells were less efficient than AA8 cells at replicating the cisplatin-damaged Ad DNA. Replication of the Ad DNA was assessed 72 h after the infection of the cells with damaged virus. Thus, the HCR assay can detect differences in the capacity of cells to replicate cisplatin-damaged Ad DNA. Figure 2 illustrates the ability of 2008 and C13* cells to replicate cisplatin-damaged Ad DNA. Replication was assessed at 48 and 72 h after viral infection and there was no significant difference in the capacities of the two cell types to replicate the damaged Ad DNA.

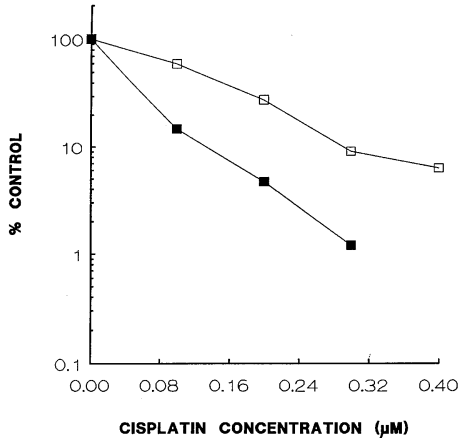

Fig. 1 Replication of cisplatin-damaged Ad DNA in AA8 (□) and UV20 (■) cells after 72 hours of repair. Cells were infected with virus treated with different concentrations of cisplatin and the ability of the cells to replicate the damaged viral DNA was assessed. The amount of viral DNA replication of cisplatin-treated virus is expressed relative to the amount of viral replication of undamaged virus. This is a representative experiment

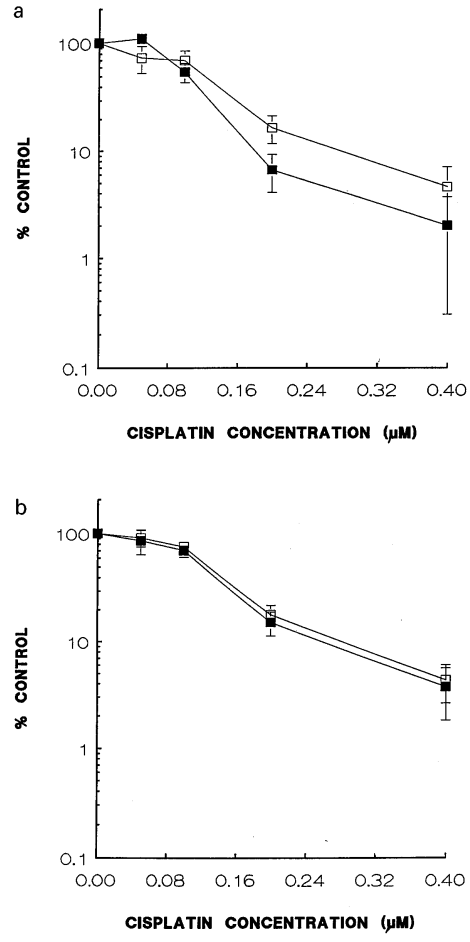

Fig. 2a,b Replication of cisplatin-damaged Ad DNA in 2008 (□) and C13* (■) cells after 48 h (**a**) and 72 h (**b**) of repair. Cells were infected with virus treated with different concentrations of cisplatin and the ability of the cells to replicate the damaged viral DNA was assessed. The amount of viral DNA replication of cisplatin-treated virus is expressed relative to the amount of viral replication of undamaged virus. Values are expressed as percentages of control ± SEM ($n = 3$)

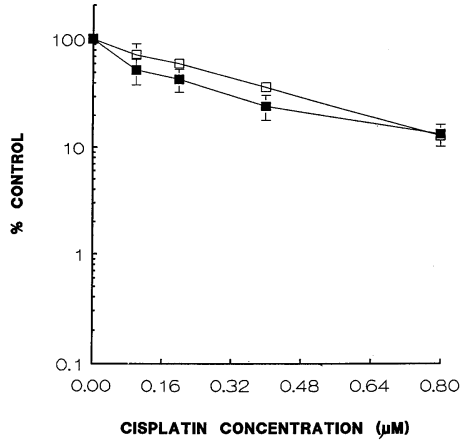

Fig. 3 Transcription of a reporter gene from cisplatin-damaged Ad DNA in 2008 (□) and C13* (■) cells. Cells were infected with virus treated with different concentrations of cisplatin and the ability of the cells to transcribed the *lacZ* gene was assessed after 48 h of repair. The amount of functional protein (ß-galactosidase) produced from cisplatin-treated virus was expressed relative to the amount of functional protein produced from undamaged virus. Values are expressed as percentages of control ± SEM ($n = 3$)

Transcription of a reporter gene from cisplatin-damaged Ad DNA

Some investigators have indicated that the repair of cisplatin damage is more efficient in actively transcribed gene regions than in the overall genome [3, 4, 19, 21, 22, 43]. In addition, some repair-deficient mutants (e.g. cells from patients with Cockayne's syndrome) repair damage from the total genome at levels similar to cells from normal subjects but are deficient in preferential repair of actively transcribed genes [3, 4, 23, 40]. Therefore, we examined the ability of 2008 and C13* cells to express a reporter gene from cisplatin-damaged Ad DNA after 48 h of repair. There was no significant difference between 2008 cells and its cisplatin-resistant variant, C13*, in their ability to express the *lacZ* gene in cisplatin-damaged Ad as shown in Fig. 3.

Enhanced HCR

Since the cells are not exposed to cisplatin in the standard HCR assay, we investigated whether cisplatin pretreatment affected the cells' ability to express the *lacZ* gene from cisplatin-damaged Ad. Each of the cell types was exposed to equitoxic cisplatin concentrations (IC_{50} and IC_{90}) and an equal cisplatin concentration (the IC_{50} dose for C13* cells and the IC_{90} dose for the 2008 cells were both 21 μ*M* of cisplatin) for 1 h immediately prior to virus infection. Figure 4 shows that pretreatment with cisplatin did not alter the ability of either 2008 or C13* cells to express a reporter gene from cisplatin-damaged Ad DNA relative to cells which received no drug pretreatment after 48 h of repair. However, pretreatment of the cells with an equal concentration of cisplatin (21 μ*M*) increased transcription of the *lacZ* gene from undamaged virus 5.24 ± 1.1-fold in the 2008 cells but only 1.64 ± 0.2-fold in the C13* cells (Table 1).

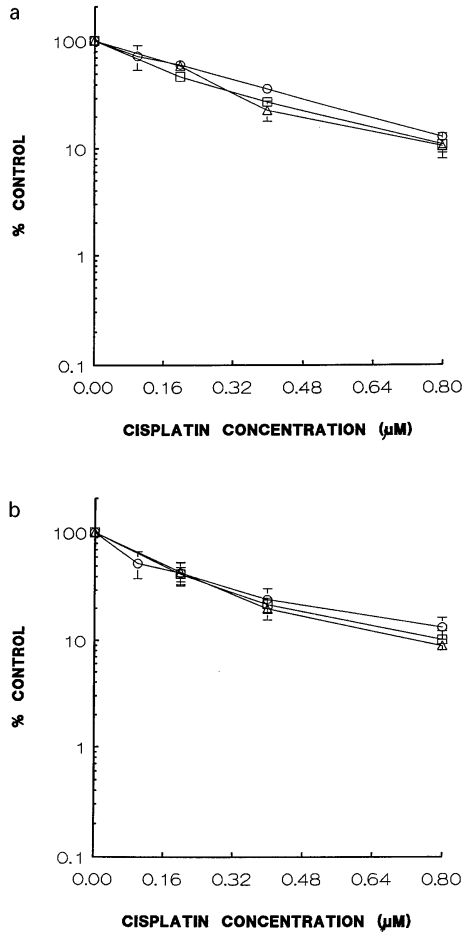

Fig. 4a, b Transcription of a reporter gene from cisplatin-treated Ad DNA after 48 h of repair in 2008 (**a**) and C13* (**b**) cells exposed to no cisplatin pretreatment (○), or pretreatment with the IC_{50} (□) or IC_{90} (△) concentration for 1 h immediately prior to virus infection. Values are expressed as percentages of control ± SEM ($n = 3$)

HCR of cisplatin-damaged Ad by RH4 cells

In addition to the 2008 and C13* cells, the HCR response of RH4 cells was investigated. RH4 cells were derived from C13* cells through selection for resistance to rhodamine 123 [44]. Not only did the selection procedure reduce the mitochondrial membrane potential, it also reverted the cisplatin sensitivity of the RH4 cells to a level comparable to the 2008 cells [44]. Table 2 shows that replication of cisplatin-damaged Ad DNA and the expression of a reporter gene from cisplatin-damaged Ad in RH4 cells was similar to both 2008 and C13* cells with or without cisplatin pretreatment.

Table 1 Induction of *lacZ* gene expression from undamaged virus in cells treated with cisplatin (-fold increase). The IC_{50} concentrations of cispaltin were 5 μ*M* for 2008 and RH4 cells and 21 μ*M* for C13* cells and the IC_{90} concentrations of cisplatin were 21 μM for 2008 and RH4 cells and 100 μM for C13* cells

Cisplatin dose	2008	C13	RH4
IC_{50}	2.11 ± 0.2	1.64 ± 0.2	1.51 ± 0.1
IC_{90}	5.24 ± 1.1	1.98 ± .03	1.86 ± 0.1

Interaction of aphidicolin and cisplatin

C13* cells were found to be resistant to cisplatin yet equally sensitive to aphidicolin relative to 2008 cells (Fig. 5). Isobologram analysis was used to determine the nature of the interaction between cisplatin and aphidicolin when used in combination and revealed a similar degree of synergy in both cell types; the CIs were 0.79 ± 0.06 and 0.77 ± 0.07 ($n = 4$) in 2008 and C13* cells, respectively, as determined at the 50% effect level.

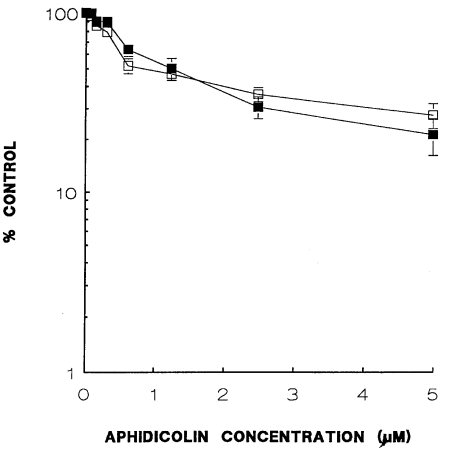

Fig. 5 Effect of aphidicolin on 2008 (□) and C13* (■) survival. Cytotoxicity was assessed by a DNA fluorometric assay 72 h after a 26-h aphidicolin exposure. Values are expressed as percentages of control ± SEM ($n = 3$)

Discussion

One of the many mechanisms that has been suggested as contributing to cisplatin resistance is the enhanced repair of cisplatin-induced DNA damage [5, 7, 12, 28, 34]. Owing to the limitations of the detection techniques used (i.e. AAS), supralethal doses of cisplatin are frequently used for repair studies. Since it remains unclear how a cell responds to damage beyond its capacity to repair [38, 41], we utilized the HCR assay to assess cellular repair capacity. In the HCR assay, cisplatin-DNA adducts are induced in a viral genome and the damaged virus is infected into the host cell. The ability of the host cell to replicate the cisplatin-damaged viral DNA or to express a reporter gene from cisplatin-damaged viral DNA is used as an indirect indicator of the cell's ability to repair cisplatin damage from its own DNA. This assay system permits repair to be assessed using low cisplatin concentrations and thus allows the comparison of our results with those reported by others using high cisplatin concentrations [27, 43].

The Chinese hamster ovary cell line AA8 and its UV-repair deficient mutant UV20 served as controls in the HCR protocol. UV20 cells are deficient in the incision step of excision repair and are hypersensitive to cisplatin in survival assays [8, 15]. This study demonstrated that UV20 cells were not as efficient at replicating the cisplatin-damaged viral DNA as AA8 cells, and thus the HCR assay can detect differences in the capacity of cells to replicate cisplatin-damaged Ad DNA. However, it is interesting to note that even though UV20 cells were 50-fold more sensitive to cisplatin than AA8 cells, they were only approximately 2.5-fold less efficient at replicating the cisplatin-damaged viral DNA. The lack of correlation between cisplatin sensitivity and viral replication capacity may reflect the inability of the HCR assay to detect differences in the repair of interstrand crosslinks. It has been shown that fibroblasts from patients with Fanconi's anemia (FA), which are thought to be defective in the repair of DNA interstrand crosslinks, replicate

Table 2. Cisplatin concentrations required to inhibit viral DNA replication or expression of a reporter gene by 50% in 2008, C13* and RH4 cells. Statistical analysis was performed using the Tukey test and $P < 0.05$ was considered significant. The IC_{50} cisplatin concentrations used were 5 μM for 2008 and RH4 cells and 21 μM for C13* cells and the IC_{90} cisplatin concentrations used were 21 μ*M* for 2008 and RH4 cells and 100 μ*M* for C13* cells

	2008	C13	RH4
Replication of cisplatin-damaged Ad DNA			
48 h	0.117 ± 0.20 μ*M*	0.101 ± 0.011 μ*M*	0.118 ± 0.004 μ*M*
72 h	0.160 ± 0.033 μ*M*	0.121 ± 0.009 μ*M*	0.134 ± 0.050 μ*M*
Expression of a reporter gene			
No pretreatment	0.276 ± 0.040 μ*M*	0.174 ± 0.059 μ*M*	0.189 ± 0.053 μ*M*
IC_{50} pretreatment	0.184 ± 0.009 μ*M*	0.164 ± 0.029 μ*M*	0.190 ± 0.038 μ*M*
IC_{90} pretreatment	0.235 ± 0.018 μ*M*	0.173 ± 0.042 μ*M*	0.182 ± 0.031 μ*M*

cisplatin-damaged viral DNA to the same extent as control fibroblasts even though FA cells are hypersensitive to cisplatin [9, 29, 30]. Therefore, reduced replication of cisplatin-damaged Ad DNA may only reflect the ability of cells to repair intrastrand adducts but not interstrand crosslinks from the total viral genome.

C13* cells were not more efficient in their ability to replicate cisplatin-damaged Ad DNA than 2008 cells. Similarly, preliminary results on the repair of UV-induced viral DNA damage indicated that C13* cells did not have an enhanced ability to replicate UV-damaged viral DNA compared with 2008 cells (data not shown). These results suggest that the ability of the NER system to repair intrastrand adducts from the total viral genome is not enhanced in the cisplatin-resistant variant relative to the parental cell line. Our results are consistent with those previously reported for 2008 cells and their cisplatin-resistant variant. Zhen et al. [43] and Jekunen et al. [16] have found that there are no significant differences in repair of total genomic intrastrand adducts between the parental line and its cisplatin-resistant variant as assessed by AAS and an isotopic analogue of cisplatin ([^{3}H]dichloro (ethylenediamine)platinum(II)), respectively.

Since it has recently been demonstrated that cells repair DNA damage, including cisplatin-DNA adducts, from actively transcribed gene regions more efficiently than from non-transcribed regions of DNA [3, 4, 19], we examined the cells' ability to repair cisplatin intrastrand adducts from a reporter gene within the viral genome. Using Ad 5 with the *lacZ* gene inserted into the E1a region of the viral genome, it was found that the cisplatin-resistant variant was not more efficient than the parental line at transcribing a reporter gene from cisplatin-damaged Ad DNA, suggesting that enhanced gene-specific repair of intrastrand adducts does not contribute to the cisplatin resistance in C13* cells. These results are consistent with those obtained by Zhen et al. [43].

To further examine repair capacities in 2008 and C13* cells an enhanced HCR assay was performed. Previous reports have indicated that pretreating cells with physical or chemical agents can enhance the replication of UV-damaged viral DNA (reviewed in reference 32). In this enhanced HCR assay the cells were exposed to cisplatin for 1 h immediately prior to their infection with cisplatin-damaged virus (a temporal relationship between cisplatin pretreatment and viral infection was not investigated in this study). This experiment was performed to determine whether C13* cells required cisplatin-induced cellular damage to stimulate their repair process. Neither of the cell types demonstrated any significant alteration in their ability to repair cisplatin damage following cisplatin pretreatment over the range of concentrations employed and thus there do not appear to be any significant differences in the NER process for cisplatin damage in 2008 and C13* cells.

We found that pretreatment with an equal cisplatin concentration increased the transcription of the *lacZ* gene in undamaged virus to a greater extent in 2008 cells than in C13* cells. This is consistent with the observation that DNA-damaging agents, such as UV radiation also increase the expression of a number of genes, termed damage-inducible genes (reviewed in reference 10). It has also been demonstrated that enhanced gene expression occurs at lower doses of a DNA damaging agent (i.e. UV radiation) in UV repair-deficient mutants compared with repair-proficient controls [10, 11]. Thus, it appears that induction of gene expression may be related to the amount of DNA damage (or the amount of unrepaired DNA damage). Our study demonstrated that the concentration of cisplatin required to enhance expression of a reporter gene was lower in 2008 cells than in C13* cells. This observation may indicate that 2008 cells respond to DNA damage in a manner similar to a repair-deficient cell type compared with C13* cells. However, a more likely explanation for the enhanced expression of the *lacZ* gene in 2008 cells compared with the C13* cells at equivalent cisplatin concentrations is that C13* cells require higher cisplatin concentrations than 2008 cells to induce similar amounts of cisplatin-DNA adducts. ([43]; this study). The low levels of *lacZ* gene induction in the RH4 cells (similar to C13* cells) may indicate that adduct formation in the RH4 cells occurs at levels similar to those observed in C13* cells. Since adduct formation has not been investigated in this cell type, further studies are required.

The repair capacity of RH4 cells was also determined. RH4 cells were derived from C13* cells through selection for resistance to rhodamine 123 [44]. This selection process not only decreased the mitochondrial membrane potential but also increased the cells' sensitivity to cisplatin to a level comparable to the parental 2008 cells. In each of the HCR experiments, the ability of RH4 cells to reactivate cisplatin-damaged Ad DNA was not significantly different from either 2008 or C13* cells. Therefore, there do not appear to be any significant differences in the ability to repair cisplatin-induced viral DNA damage between cisplatin-sensitive and cisplatin-resistant cells in our model system.

Further evidence to support a lack of a difference in NER capacities between 2008 and C13* cells was obtained using aphidicolin, an inhibitor of DNA polymerase α and δ [37]. DNA polymerase α has been proposed as one of the components involved in the repair of cisplatin-DNA adducts [34]. We found that 2008 and C13* cells were equally sensitive to aphidicolin. These results are not consistent with the survival data published by Katz et al. [20] who found that 2008 cells were 3.3-fold more sensitive to aphidicolin than their cisplatin-resistant variant (2008/DDP). It is possible that the different cisplatin selection protocols used to generate the cisplatin-resistant variants 2008/DDP and C13* influenced the

resistant mechanisms utilized by these variants. We also investigated the nature of the interaction between aphidicolin and cisplatin. Using isobologram analysis and assessing the interaction at the 50% effect level, it was found that the interaction was synergistic in both 2008 and C13* cells. Therefore, it appears that polymerase α and/or δ are important components of the cisplatin repair pathway in both 2008 and C13* cells, suggesting that these components of the NER pathway do not contribute to the cisplatin resistance in C13* cells.

Our aphidicolin and cisplatin results differ from those previously reported in which median effect analysis demonstrated synergy between aphidicolin and cisplatin in the cisplatin-resistant variant but only additivity in the parental line [20]. Although the drug treatment protocol and the parental cell line were the same, the formulation of aphidicolin, the cisplatin-resistant variant and the method used to determine the CI were different in our study compared to the study by Katz et al. [20]. It is unclear which of these factors contributed to the different results from the two studies.

Since our assay system appears unable to detect differences in the repair of cisplatin interstrand crosslinks [9, 29, 30], we are unable to comment on the suggestion by Zhen et al. [43] that enhanced repair of gene-specific interstrand crosslinks contributes to the cisplatin resistance in C13* cells. Johnson et al. [18] have also demonstrated a correlation between enhanced repair of cisplatin interstrand crosslinks and cisplatin resistance in a number of cisplatin-resistant variants of the parental A2780 cell line.

In conclusion, enhanced repair of total genomic or gene-specific cisplatin intrastrand adducts does not appear to contribute to cisplatin resistance in C13* cells. This study also suggests that RH4 cells repair intrastrand adducts from cisplatin-damaged Ad DNA with an efficiency similar to both 2008 and C13* cells. Furthermore, the similar synergistic growth inhibition mediated by the combination of cisplatin and aphidicolin in both the parental line and the cisplatin-resistant variant is consistent with the suggestion that enhanced NER does not contribute to the cisplatin resistance in C13* cells.

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