Cancer Chemother Pharmacol (2008) 61:979–987
DOI 10.1007/s00280-007-0554-0

ORIGINAL ARTICLE

Dihydrodiol dehydrogenases regulate the generation of reactive oxygen species and the development of cisplatin resistance in human ovarian carcinoma cells

Jianli Chen · Mahesha Adikari · Rajash Pallai · Hemant K. Parekh · Henry Simpkins

Received: 6 March 2007 / Accepted: 26 June 2007 / Published online: 28 July 2007

Abstract We have previously demonstrated that overexpression of dihydrodiol dehydrogenase isoform 1 (DDH1) or DDH2 leads to the induction of drug resistance to platinum based drugs in human ovarian, lung, cervical and germ cell tumor cell lines. DDH belongs to a family of aldoketo reductases that are involved in the detoxification of several endogenous and exogenous substrates. DDH1 and DDH2 in particular have been shown to be involved in the detoxification (activation?) of polycyclic aromatic hydrocarbons (PAH). Based on the involvement of DDH in the detoxification of electrophilic PAH intermediates, the effect of DDH on the production of reactive oxygen species (ROS) in a cisplatin-sensitive and -resistant human ovarian carcinoma cell line was investigated in the current study. In addition to the overexpression of DDH1 and DDH2, increased expression of DDH3 was demonstrated in the cisplatin-resistant 2008/C13* cells, compared to the parental 2008 cells. However, as assessed by RT-PCR, neither cell line expressed DDH4. The 2008/C13* cells were eightfold resistant to cisplatin, and transfection experiments utilizing cisplatin-sensitive 2008 cells suggest that this could be mediated by overexpression of either DDH1, DDH2, or DDH3. The 2008/C13* cells had lower basal intracellular ROS level as compared to the 2008 cells and ROS production was decreased in the recombinant 2008 cells with forced, constitutive overexpression of either, DDH1, DDH2, or DDH3. Transfection of siRNA against DDH1 or DDH2 in the cisplatin-resistant 2008/C13* cells not only significantly decreased their cisplatin-resistance index (as assayed by MTT and colony formation assay) but also led to an increase in the basal levels of ROS production (although transfection of siRNA against DDH3 resulted in cell death). The 2008/C13* cells were found to be cross-resistant to the cytotoxic effects of hydrogen peroxide and *tert*-butyl hydroperoxide and knockdown of either DDH1 or DDH2 expression (using siRNA) resulted in sensitization of the resistant cells to these agents. These results support the conclusion that the increased levels of DDH in the 2008/C13* cells are directly responsible for the reduced production of ROS and that this may play a role in the development of cisplatin resistance.

Keywords Reactive oxygen species · Dihydrodiol dehydrogenases · Cisplatin resistance · Human ovarian carcinoma cells

Introduction

Cisplatin and carboplatin are among the most widely used drugs for the treatment of solid organ cancers. They are curative for most patients with advanced testicular cancer [23] and they are used in standard protocols for the treatment of ovarian, bladder, cervical, head and neck and lung cancer [16]. The main site of action of this drug (thought to cause its cytotoxicity) is its interaction with DNA. Unfortunately, the efficacy of cisplatin is restricted due to the existence of intrinsic tumor cell resistance or by the acquisition

This research was supported by NIH Grant CA98804 to H.S.

J. Chen · M. Adikari · R. Pallai · H. K. Parekh · H. Simpkins (✉)
Department of Pathology and Laboratory Medicine,
Temple University School of Medicine, Room 206,
OMS, 3400 N. Broad Street, Philadelphia, PA 19140, USA
e-mail: hsimpkins@siuh.edu

H. K. Parekh · H. Simpkins
Fels Institute of Cancer Research and Molecular Biology,
Temple University School of Medicine, 3400 N. Broad Street,
Philadelphia, PA 19140, USA

of tumor cell resistance subsequent to drug treatment [32]. In order to decipher the mechanisms of cisplatin resistance, the human ovarian carcinoma cell line (2008) and its cisplatin-resistant variant (2008/C13*) have been utilized. Several biochemical alterations thought to be associated with cisplatin resistance have been identified in the 2008/C13* cells. Previous work in our and other laboratories has demonstrated that the 2008/C13* cells exhibit a decreased intracellular accumulation of cisplatin [1], increased replicative bypass of cisplatin-DNA adducts [17], reduced expression of membrane-associated β-tubulin [6], and decreased expression of the intermediate filament, cytokeratin 18 [24]. Moreover, the mitochondria in the 2008/C13* appear morphologically aberrant and these cells are hypersensitive to lipophilic cations as compared to the parental cells [2]. Variations in the activation of protein kinase C activity [14] and in the cAMP signal transduction pathway [18] have also been observed in 2008/C13* cells. An increased level of expression of the oncogene *c-fos* in the 2008/C13* cells has recently been reported and partial reversal of the cisplatin resistance phenotype was achieved by treatment of the 2008/C13* cells with an antisense oligonucleotide directed against *c-fos* [20]. Examination of the basal levels of the drug-detoxifying enzyme glutathione S-transferase and the drug transport pump (Multiple drug resistance-associated protein; MRP) revealed no significant difference between the parental 2008 and the cisplatin-resistant 2008/C13* cells ([19] and unpublished observations]. All this data indicates that there are multiple mechanisms interconnected in a very complex way producing cisplatin resistance in the 2008/C13* cell line some of which may have clinical relevance. None of these alterations have been shown to be consistently associated with the development of the cisplatin resistance phenotype in tumor cells from different tissue sources. However, the ubiquitous induction of cisplatin and carboplatin resistance in human ovarian, cervical, germ cell and lung carcinoma tumor cells upon forced, constitutive overexpressing of isoforms 1 and 2 of dihydrodiol dehydrogenase [9, 10] was recently demonstrated.

DDH belongs to a superfamily of monomeric, cytosolic NADP(H)-dependent oxidoreductases that catalyze the metabolic reduction and either activation or inactivation of several xenobiotics [26–28]. In fact, increased expression of a carbonyl reductase has been demonstrated in a doxorubicin resistant tumor cell line [3]. Additionally, increased expression of DDH has been shown in an ethacrynic acid-resistant colon carcinoma cell line [8].

It has been postulated that the mechanism by which DDH induces cisplatin resistance may involve free radicals. In addition to the formation of DNA adducts, cisplatin has been shown to induce oxidative stress in a variety of tumor cells. Also, several different classes of xenobiotics, some of which are known to induce oxidative stress, have been shown to increase the expression of DDH [5]. These observations suggest that DDH induction may be a component of a counter-response to oxidative stress. Thus an investigation as to whether there is an association between DDH and the production of reactive oxygen species (ROS) in cisplatin-sensitive and -resistant human ovarian carcinoma cell lines was undertaken.

Materials and methods

Reagents

Cell culture reagents and gentamycin were obtained from Cellgro (Herndon, Virginia). RNAzol B was purchased from Tel-test Inc. (Friendswood, TX). The enhanced chemiluminescence reagents were from Pierce Biochemicals (Rockford, IL). The antibodies utilized in this study and their suppliers were; rabbit polyclonal HO-2 antibody was from BD Biosciences (San Diego, CA), and the HRP-conjugated anti-rabbit antibody was from Pierce Biochemicals (Rockford, IL). Cisplatin was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Methylindoxylacetate (MIA) (1-H-indol-3-ol, 1 methyl-acetate (ester)) was purchased from Sigma (St Louis, MO). H_2DCFDA was from Molecular Probes (Eugene, OR). All of the components of the RT and the PCR reaction were purchased from Qiagen (Valencia, CA). The primers used in this study were designed using the software program Primer Express, version 1.0 from Applied Biosystems (Foster City, CA). The forward and reverse primer sequence for DDH1, DDH2, DDH3 and DDH4 genes, as well as the Glyceraldehyde-6-phosphate dehydrogenase (GAPDH) gene for routine RT-PCR as well as for real-time RT-PCR analysis is available upon request.

Cell lines

The cisplatin-sensitive and -resistant (2008 and 2008/C13* cells, respectively) human ovarian carcinoma cells were grown in RPMI 1640 medium supplemented with 10% FBS and gentamycin at a final concentration of 10 μg/ml as described previously [9, 24].

Stable overexpression of DDH1, DDH2 or DDH3 in cisplatin-sensitive cells

In order to correlate the function of each isoform of DDH with the cisplatin resistance phenotype, it was necessary to evaluate the effects of forced overexpression of the recombinant protein produced by each of these genes on anticancer drug cytotoxicity. Thus, primer pairs from the mRNA

sequence of each of the candidate genes (using the full-length sequence data from the sequence deposited in the GenBank database; see [13]) were designed to enable us to generate a full-length cDNA. These were then cloned into the eukaryotic expression vector (pCDNA4/HisMax-TOPO TA, Invitrogen, San Diego, CA). Orientation of the full-length cDNA as well as its sequence was determined by restriction enzyme digestion and automated DNA sequencing, respectively. The expression vector with the insert in the right orientation was then transfected into sub-confluent parental human tumor cell lines using the lipofectamine reagent (GIBCO-Life Sciences) as described previously [9, 24]. The transfected cells were propagated in a medium containing 500–1,000 μg/ml geneticin (G418 sulphate) for 3–5 weeks. Individual G418-resistant colonies were picked (20 colonies for each transfection experiment), grown and screened for the expression of the recombinant mRNA utilizing RT-PCR as described below. The clones that expressed a high level of the recombinant message were then subjected to growth inhibition assays in the presence of different concentrations of cisplatin. The cytotoxic effects of these drugs were assessed using a tetrazolium dye as described previously [9, 24].

Stable expression of DDH siRNA in cisplatin-resistant cells

siRNAs oligonucleotides specific for individual DDH isoforms were initially procured from Ambion (Austin, TX). Preliminary experiments demonstrated the efficacy of individual siRNA oligonucleotides in suppressing the expression of DDH isoforms (data not presented). Thereafter, double stranded oligonucleotides were designed such that these could be cloned into an expression vector for stable knockdown of the corresponding DDH isoform. These were cloned into pSilencer vector (Ambion, Austin, TX) with the neomycin resistance marker gene. siRNA-associated plasmid transfection and generation of stable clones with reduced expression of individual DDH isoforms was carried out as described above. The clones with significant knockdown of the endogenous DDH1 or DDH2 levels were then subjected to growth inhibition assays in the presence of different concentrations of cisplatin. The cytotoxic effects of these drugs were assessed using a tetrazolium dye as described previously [9, 24].

In addition, colony formation assays were also performed to assess the cytotoxicity of cisplatin against 2008, 2008/C13*, and 2008/C13* cells transfected with DDH1-siRNA or DDH2-siRNA. 1×10^5 cells of were seeded in 6-well plates and incubated for 24 h and then treated with different concentrations of cisplatin. After a 4 h exposure, the medium was aspirated and the wells were washed in drug-free medium. Cells were trypsinized with 0.25% Trypsin-0.02% EDTA and 100, 200, and 500 cells were seeded in 6-well followed by one week incubation in drug-free complete medium to allow colony formation. At the end of this incubation, medium was aspirated and cells were fixed and stained with 0.5% (*w*/*v*) methylene blue in 50% (*v*/*v*) ethanol for 40 min at room temperature. The plates were gently washed with water and allowed to air-dry. Visible colonies were counted to determine the percent colony formation after each drug treatment. Values were expressed as the mean ± SD from triplicate experiments.

RT-PCR analysis

RNA extraction and reverse transcription-PCR were performed essentially as described previously [9, 10]. Each reverse transcription reaction consisted of 1 μg of RNA, 4 units of Omniscript RT, 1 μM oligo-dT primer, 0.5 mM dNTP, 10 units of RNase inhibitor, and 1× RT buffer. Reverse transcription was performed at 37°C for 1 h followed by incubation at 93°C for 3 min to inactivate the reverse transcriptase enzyme. Thereafter, equal volume of cDNA was amplified by PCR using gene specific primer pairs. Each PCR reaction consisted of 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 2.5 units of *Taq* polymerase, and 0.2 mM gene-specific forward and reverse primers. The PCR conditions were as follows; an initial denaturation at 94°C for 3 min, followed by 94°C for 15 s, 55°C (or 57°C for DDH3) for 30 s, 72°C for 30 s for the number of cycles optimized for each primer. A final elongation step was performed for 10 min at 72°C. RT-PCR amplification of GAPDH transcript was used as the internal control to verify that equal amounts of RNA were used from each cell line.

For real-time RT-PCR analysis, SYBR Green dye was utilized in each of the PCR reactions using an Eppendorf Real-Plex PCR system

Nonspecific esterase assay and ROS detection

Nonspecific esterases have been previously reported to be associated with resistance to chemotherapeutic agents, heavy metals, and insecticides [22, 33]. Thus, the activity of nonspecific esterases in the cisplatin-sensitive and -resistant human ovarian carcinoma cells was assessed. *N*-methylindoxyacetate (MIA) was used as a substrate to determine nonspecific esterase activity [13]. Fresh stock solutions of 1 mg/ml MIA (5.3 mM) were made weekly in methanol and kept at −20°C in the dark. The enzyme activity was measured after 24 h incubation utilizing 4×10^4 cells seeded in 96-well plates. At the end of this time, the growth medium was aspirated and 100 μl of warm (37°C water bath-preincubated) HBSS buffer (pH 7.4) and MIA was added to each well and incubated further at 37°C for 30 min. The cells were then washed with warm HBSS twice and the intracellular nonspecific esterases activity

(that hydrolyzes MIA and result in an increase in fluorescence) was assessed using a multi-well fluorescence microplate reader (TECAN GENios) with excitation wavelength set at 405 nm and emission wavelength set at 505 nm.

For estimation of intracellular ROS levels, a cell membrane permeable and oxidant-sensitive fluorescent dye 5-(6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) was employed [31]. H_2DCFDA, by itself, is non-fluorescent, but once inside the cells is hydrolyzed to H_2DCF by nonspecific esterases. The H_2DCF is an ROS-sensitive intermediate whose degradation by intracellular peroxides results in generation of fluorescent DCF [21]. Briefly, 4×10^4 cells were seeded in 96-well plates for 24-h incubation. Thereafter, the growth medium was aspirated and 100 µl of warm (37°C water bath-preincubated) HBSS buffer (pH 7.4) and H_2DCFDA was added to each well (final concentration at 25 µM) and incubated further at 37°C for 30 min. The cells were then washed with warm HBSS twice and the generation of ROS (measured as fluorescence intensity) was measured on a multi-well fluorescence microplate reader (TECAN GENios, Durham, NC) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The data are expressed as fluorescent intensity per 4×10^4 cells

Western blotting analysis

The 2008, 2008/C13* and 8n23 (DDH1 siRNA transfected 2008/C13* cells) cells were seeded at a density of 1×10^6/ml and incubated under normal growth conditions for 24 h. Thereafter, the cells were left untreated or treated with cisplatin for the indicated time periods. At the end of each time period, the cells were washed with chilled PBS (3×). The whole cell lysate was prepared from each of the cell line by scraping cells into a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (*v*/*v*) Triton-X-100, 0.5% (*v*/*v*) Nonidet P40, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride and 1× protease inhibitor cocktail and incubating on ice for 15 min. Then, the lysate was centrifuged at 13,000*g* for 20 min and the supernatant was transferred to a fresh tube and stored at −80°C until use. Proteins were separated on a SDS-PAG and transferred to a PVDF membrane. Western blotting analysis was performed using rabbit polyclonal antibody against HO-2 (BD Biosciences, San Diego, CA) and enhanced chemiluminescence reagents from Pierce Biochemicals (Rockford, IL).

Statistical analysis

The linear regression analysis and paired *t*-test were performed using the SigmaStat Statistical Analysis System, Version 1.01. $P < 0.05$ was considered significant.

Results

Expression of DDH isoforms in 2008 and 2008/C13* cells

RT-PCR analysis was employed to determine the expression levels of the DDH isoforms in cisplatin-sensitive (2008) and cisplatin-resistant (2008/C13*) human ovarian carcinoma cells. The results shown in Fig. 1 indicate that the expression of DDH1, DDH2, and DDH3 was significantly higher in the 2008/C13* cells compared to the 2008 cells. However, expression of DDH4 was not detected in the cisplatin-sensitive or the cisplatin-resistant cells (data not shown). The latter observation was not completely unexpected considering that DDH4 is known to be a liver-specific enzyme [25]. Real time RT-PCR analysis was then performed to quantify the differential expression of the various DDH isoforms in the cisplatin-sensitive and -resistant cells. The 2008/C13* cells were found to express 130-, 69- and 32-fold higher levels of DDH1, DDH2 and DDH3 mRNA compared to the 2008 cells.

Recombinant 2008 cell clones overexpressing the DDH isoforms and recombinant 2008/C13* cell clones with DDH1 or DDH2 knockdown

In order to determine the association of DDH overexpression with the cisplatin resistance phenotype and the production of intracellular ROS, recombinant cell clones of 2008 cells (Table 1) were generated that display forced, constitutive

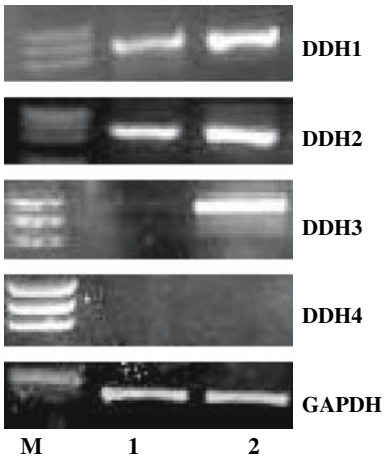

Fig. 1 The differential expression of DDH1, DDH2 and DDH3 in the 2008 and 2008/C13* cells assessed by RT-PCR: RNA was prepared from the 2008 (lane *2*) and 2008/C13* (lane *3*) cells using the RNAzol B reagent (TelTest Inc., Friendswood, TX). cDNA was synthesized using the Qiagen omniscript RT using 1 µg of RNA as template. Thereafter, the PCR reaction using gene-specific primers and 1.5 mM $MgCl_2$ was performed as described in "Materials and methods" section. Each of the RT-PCR reactions was performed with three different batches of RNA to assess differential gene expression. A representative ethidium bromide stained agarose gel is shown. Lane *1* contains 1 kb marker DNA ladder

Table 1 List of recombinant clones generated and utilized in this study

Cell-line transfected	cDNA/siRNA transfected	Clone designation	DDH expression levels
2008	DDH1 cDNA	AK6	Increased DDH1
	DDH2 cDNA	D19	Increased DDH2
	DDH3 cDNA	A11	Increased DDH3
2008/C13*	DDH1 siRNA	8n23	Decreased DDH1
	DDH2 siRNA	7n1	Decreased DDH2

overexpression of DDH1, DDH2 or DDH3 mRNA (as assessed by RT-PCR, Fig. 2a). Thus, the cell clones AK6 (2008/DDH1), D19 (2008/DDH2) and A11 (2008/DDH3) with increased expression of the indicated DDH isoform were obtained.

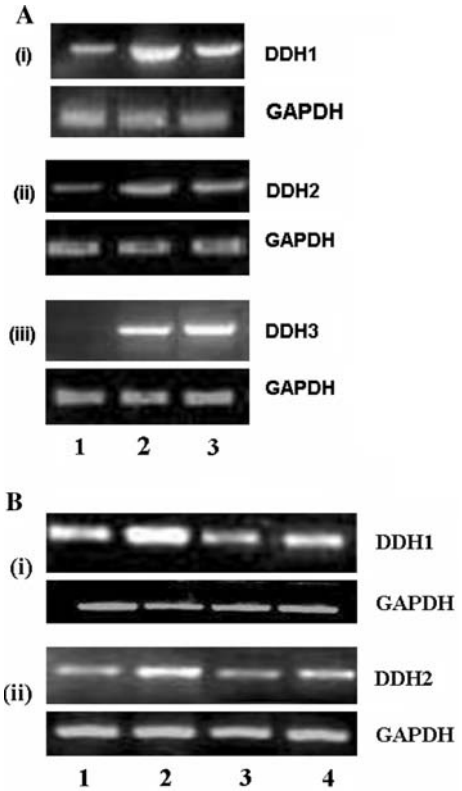

Fig. 2 **a** RT-PCR analysis of the expression of DDH1 (i), DDH2 (ii) and DDH3 (iii) mRNA in the recombinant 2008 cells transfected with the respective full length cDNA. RT-PCR was performed as described in the legends to Fig. 1. Lane *1* = 2008 cells; lane *2* = 2008/C13* cells; and lane *3* = 2008 cells transfected with the DDH cDNA indicated to the right of the figure. **b** RT-PCR analysis of the expression of DDH1 (i), DDH2 (ii) mRNA and DDH (iii) protein in the 2008/C13* cells transfected with the respective siRNA. RT-PCR was performed as described in the legends to Fig. 1. Western blot analysis was performed as described previously [24] utilizing a monoclonal DDH antibody that recognizes DDH1 and DDH2 [9]. Lane *1* = 2008 cells; lane *2* = 2008/C13* cells; lane *3* = 8n23 (2008/C13* cells transfected with siRNA directed against DDH1) cells; lane *4* = 7n1 (2008/C13* cells transfected with the siRNA directed against DDH2)

Similarly, recombinant 2008/C13* cell clones that were transfected with plasmids containing the double stranded siRNA directed against DDH1, or DDH2 were prepared. Preliminary investigations (data not shown) indicated that siRNA oligonucleotides directed against DDH1 were able to reduce the mRNA levels between 48 and 72 h post-transfection in the 2008/C13* cells. Thereafter, the levels of DDH1 recovered and increased to levels observed in the untreated cell lines within an additional 72 h. In order to ensure stable knockdown of the gene under investigation, transfection with siRNA cloned into plasmid vectors was utilized—this resulted in a clone termed 8n23; which exhibit stable knockdown of the DDH1 gene (20% expression compared to untransfected 2008/C13*cells, Fig. 2b, Top panel, lane 3). Similarly, a clone termed 7n1 was isolated that exhibited stable knockdown of the DDH2 gene (30% expression compared to untransfected 2008/C13*cells, Fig. 2b, middle panel, lane 4). In addition, utilizing a monoclonal antibody against DDH1/2 [9], we also demonstrate a greater than 50% reduction in the expression of the DDH protein in both the 8n23 (DDH1-siRNA transfected cells) and 7n1 (DDH2-siRNA transfected cells) compared to the 2008/C13* cells (Fig. 2b, bottom panel, compare lanes 3 and 4 with lane 2). Despite repeated attempts, generation of a 2008/C13* cell clone with stable knockdown of DDH3 gene has not been possible since transfection of 2008 cells with siRNA against DDH3 resulted in cell death.

Cisplatin sensitivity of recombinant clones

The cisplatin sensitivity of the various recombinant clones was assessed and compared to the parental cisplatin-sensitive and -resistant cells utilizing the MTT assay [9, 24]. As shown in Table 2, the 2008/C13* cells were found to be sevenfold resistant to the cytotoxic effects of cisplatin compared to the 2008 cells. Forced constitutive expression of DDH1 (AK6 cells) or DDH2 (D19 cells) in the 2008 cells led to induction of cisplatin resistance. Thus, the AK6 cells were sixfold resistant to cisplatin compared to the parental 2008 cells and the D19 cells were 4.3-fold resistant to cisplatin compared to the 2008 cells. The cisplatin sensitivity of the vector only transfected 2008 cells was found to be similar to that of the 2008 cells (data not shown). Interestingly, overexpression of DDH3 in the 2008 cells (A11 cells) also led to induction of 2.3-fold cisplatin resistance.

Knockdown of DDH1 and DDH2 expression in the 2008/C13* cells was achieved utilizing gene-specific siRNA. This led to a significant decrease in the resistance index of the 2008/C13* recombinant clones (Table 2). The 8n23 cells (2008/C13* cells with a stable knockdown of DDH1 expression) were found to be only 1.9-fold cisplatin resistant compared to the 2008 cells. This demonstrates a

Table 2 Cisplatin sensitivity of DDH cDNA or DDH siRNA transfected clones: The IC_{50} values were determined by the MTT assay as described in the "Materials and methods" section

Cells	IC_{50} (μM) ± SD	Fold resistance (compared to 2008 cells)	*P* values
2008	1.00 ± 0.11	1	
AK6 (2008/DDH1)	6.42 ± 4.64	6.4	<0.01[a]
D19 (2008/DDH2)	4.46 ± 0.49	4.5	<0.01[a]
A11 (2008/DDH3)	2.33 ± 0.27	2.3	<0.01[a]
2008/C13*	7.29 ± 0.83	7.3	<0.01[a]
8n23 (2008/C13*/ DDH1 siRNA)	1.89 ± 0.35	1.9	<0.01[a,b]
7n1 (2008/C13*/ DDH2 siRNA)	5.41 ± 0.36	5.4	<0.01[a]; <0.05[b]

The values presented are mean ± SD and are from at least three independent experiments, each performed in triplicate. The degree of resistance was calculated as the ratio of anticancer drug IC_{50} value of individual transfected clonal cell lines to the anticancer drug IC_{50} value of the parental cells

[a] Compared to the 2008 cells

[b] Compared to the 2008/C13* cells

near complete reversal of the cisplatin resistant index of the 2008/C13* cells. Similarly, the 7n1 cells (2008/C13* cells with a stable knockdown of DDH2 expression) displayed a small but consistent (25%) decrease in the cisplatin resistance index of the 2008/C13* cells.

In addition to the MTT assay, colony formation assays were also performed to assess the cisplatin sensitivity of these cells. The 2008/C13* cells (IC_{50} = 4.46 ± 0.41 μM) were found to be eightfold resistant to cisplatin compared to the 2008 cells (IC_{50} = 0.56 ± 0.16 μM) and knockdown of endogenous DDH1 expression in the 2008/C13* cells (8n23 cell clone) led to a near complete reversal of the cisplatin resistance (cisplatin IC_{50} = 0.85 ± 0.14 μM) in the colony formation assay. Similarly, the 7n1 cell clone (DDH2-siRNA transfected 2008/C13* cells) was also sensitized to the cytotoxic activity of cisplatin (IC_{50} = 1.84 ± 0.39 μM) compared to the 2008/C13* cells.

Nonspecific esterase activity in 2008 and 2008/C13* cells

Within the concentration range of 0.6625–5.3 mM of the substrate, MIA, the nonspecific esterases activity in the 2008 and 2008/C13* cells was found to be similar (data not shown). This observation ruled out the role of a nonspecific esterase in producing spurious ROS generation results.

Production of ROS and its association with the level of DDH expression

We employed H_2DCFDA as probe to evaluate intracellular ROS levels in the cisplatin-sensitive and -resistant cells as well as the recombinant clones generated by transfection with either full-length DDH cDNA or the DDH siRNA. Initial results demonstrated that that the cisplatin-sensitive 2008 cells had significantly higher ROS levels compared to the cisplatin-resistant 2008/C13* cells ($P < 0.01$, Table 3).

Forced, constitutive overexpression of DDH1 in 2008 cells (AK6 cells) led to a significant decrease ($P < 0.01$) in the intracellular ROS levels compared to the 2008 cells (Table 3). Similarly, increased expression of DDH2 (D19 cells) and DDH3 (A11 cells) in the 2008 cells led to a 30–40% decline in the intracellular levels of ROS in these recombinant cell clones. These results clearly indicate that in addition to its involvement in the development of cisplatin resistance in 2008 cells, DDH overexpression is also associated with reducing the production of ROS in the 2008 cells. Interestingly, the ectopic overexpression of DDH in the 2008 cells reduced the intracellular ROS levels in the recombinant clones to the levels observed in the 2008/C13* cells. The fact that the 2008/C13* cells display increased expression of DDH1, DDH2 and DDH3 (Fig. 1), suggests that the lower levels of ROS in these cells compared to the 2008 cells may be directly associated with the overexpression of the DDH isoforms in the 2008/C13* cells.

To further validate the regulation of intracellular ROS levels by DDH we assessed the ROS levels in the recombinant 2008/C13* clones expressing the DDH1 siRNA or DDH2 siRNA. Transfection of DDH1 siRNA in 2008/C13* cells (8n23 cells) reduced the DDH1 mRNA levels by 78% (as assessed by real-time RT-PCR analysis; data not shown) and a 37% increase in the generation of intracellular ROS was observed in the 8n23 cells (Table 3).

Table 3 The intracellular ROS levels in the cisplatin-senstive and -resistant human ovarian carcinoma cells. After incubating the cells overnight the intracellular ROS levels was measured utilizing the fluorescent probe H_2DCFDA as described in "Materials and methods" section

Cells	ROS levels (relative fluorescence unit)	Change (compared to untransfected cells) (%)	*P* values
2008	39528 ± 2517	100	
2008/C13*	18936 ± 1577	48	<0.01[a]
AK6 (2008/DDH1)	16141 ± 220	41	<0.01[a]
D19 (2008/DDH2)	27420 ± 605	69	<0.01[a]
A11 (2008/DDH3)	24539 ± 1897	62	<0.01[a]
8n23 (2008/C13*/ DDH1 siRNA)	25978 ± 5526	137	<0.05[b]
7n1 (2008/C13*/ DDH2 siRNA)	30841 ± 3021	163	<0.01[b]

Values presented are means ± SD of at least three independent experiments with each performed in duplicates

[a] Compared to the 2008 cells

[b] Compared to the 2008/C13* cells

Similarly, a marked decrease (65%) in the DDH2 mRNA in the 7n1 cells (DDH2 siRNA transfected 2008/C13* cells) was associated with a 63% increase in the intracellular levels of ROS in these cells (see Table 3).

Hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (*t*-BHP) sensitivity in 2008 and C13

To further validate the hypothesis that DDHs can act as antioxidants to attenuate intracellular ROS the sensitivity of 2008 and 2008/C13* cells to H_2O_2 and *t*-BHP was investigated. Previous studies have clearly demonstrated the association of DDH overexpression with the development of cisplatin resistance in the 2008/C13* cells compared to the 2008 cells and in this study DDH has been shown to control the production of ROS. Considering that ROS production has been shown to induce cytotoxic effects on a variety of cellular systems, it was hypothesized that the overexpression of DDHs would diminish the cytotoxic effects of H_2O_2 and *t*-BHP in the 2008/C13* cells compared to the 2008 cells. Our results show that the 2008/C13* cells are indeed 1.9 to 2.2-fold resistant to cytotoxic effects of H_2O_2 and *t*-BHP compared to the 2008 cells (Table 4). Moreover, knockdown of either DDH1 or DDH2 in 2008/C13* utilizing siRNA, resulted in a significant sensitization of the cells to the cytotoxic effects of *t*-BHP (Table 4). Whilst the cytotoxic effects of H_2O_2 against 2008/C13* cells with stable knockdown of either DDH1 or DDH2 were decreased (albeit to a lesser extent than to *t*-BHP), this were found not to be statistically significant.

Expression of Hemeoxygenase-2 (HO-2) in the cisplatin-sensitive and -resistant cells

Activation of Hemeoxygenase family of enzymes (HO-1 and HO-2) has been shown to be an ubiquitous cellular response to oxidative stress. In fact, exposure of cells to a variety of oxidative stress has been shown to induce the expression of hemeoxygenases [15]. A biochemical consequence of the observations (decreased levels of ROS in cells overexpressing DDH) made in the current study could be differential regulation of the expression of HO-1 and/or HO-2 in the 2008 and 2008/C13* cells. Indeed, as shown in Fig. 3, the cisplatin-resistant 2008/C13* cells display a significant decrease in the expression of the HO-2 compared to the 2008 cells. Interestingly, expression of HO-1 was not observed in either the 2008 or the 2008/C13* cells (data not shown). RNA interference-mediated knockdown of either DDH1 expression in the 2008/C13 cells (8n23 cells) or DDH2 expression in 2008/C13* cells (7n1 cells) led to an induction of HO-2 expression. These observations provides further confirmation of the role of DDH in reducing ROS levels in the cisplatin-resistant 2008/C13* cells and the role of DDH in the development of the cisplatin-resistance phenotype.

Discussion

In the present study a new function of DDH, a member of the aldo-keto reductase family of enzymes, in the detoxification of intracellular ROS in cisplatin-resistant human ovarian carcinoma cells has been highlighted. ROS production in the cisplatin-resistant 2008/C13* cells was significantly lower compared to the 2008 cells and the cisplatin-resistant cells were shown to have increased expression of DDH1, DDH2 and DDH3 and ectopic expression of either one of the DDH isoforms in the cisplatin-sensitive 2008 cells led to a decrease in the intracellular levels of ROS as well as induction of cisplatin-resistance in the recombinant cells. Reciprocally, siRNA-mediated knockdown of DDH1 or DDH2 in the cisplatin-resistant 2008/C13* cells increased the basal production of ROS and sensitized the recombinant cells to the cytotoxic effects of cisplatin. It may be pointed

Table 4 Cytotoxic effects of H_2O_2 and *t*-BHP in the 2008 cells, 2008/C13* and the 2008/C13* cells transfected with siRNA directed against DDH1 (8n23 cells) and DDH2 (7n1 cells)

Cell line	H_2O_2		*t*-BHP	
	IC_{50} (μM) ± SD	Fold-resistance	IC_{50} (μM) ± SD	Fold-resistance
2008	29.9 ± 4.6	1.0	36.7 ± 3.3	1.0
2008/C13*	58.0 ± 10.4[a]	1.9	81.3 ± 17.3[a]	2.2
8n23 (DDH1 siRNA)	38.6 ± 6.5[b]	1.3	38.5 ± 5.4[c]	1.1
7n1 (DDH2 siRNA)	44.8 ± 5.2[b]	1.5	42.2 ± 1.4[c]	1.1

The IC_{50} values were determined by the MTT assay as described in the "Materials and methods" section. The values presented are as mean ± SD and are from at least three independent experiments, each performed in triplicate. The fold- resistance was calculated as the ratio of IC_{50} value against 2008/C13* cells (or siRNA transfected 2008/C13* cells) to the IC_{50} value against the parental 2008 cells

[a] $P < 0.05$ compared to the 2008 cells

[b] Not significant compared to the 2008/C13* cells

[c] $P < 0.05$ compared to the 2008/C13* cells

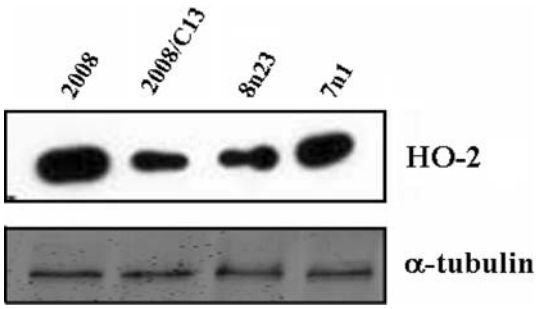

Fig. 3 Expression of HO-2 in the cisplatin-sensitive and -resistant cells. The 2008 (lanes *1–3*), 2008/C13* (lanes *4–6*) and the 8n23 (lanes *7–9*) cell lines were seeded at a density of 1×10^6 cells/plate for 24 h. Thereafter, the cells were left untreated (lanes *1*, *4*, and *7*) or treated with 25 mM cisplatin for 1 h (lanes *2*, *5*, and *8*) or 4 h (lanes *3*, *6*, and *9*). At the end of the time period, the cells were washed with chilled PBS and the whole cell lysate was prepared from each of the cell line by scraping into a buffer containing 20 mM *Tris*-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (*v*/*v*) Triton-X-100, 0.5% (*v*/*v*) Nonidet P40, 2.5 mM Na pyrophosphate, 1 mM NaOV, 50 mM NaF and 1 × protease inhibitor cocktail and incubated on ice for 15 min. The lysate was then centrifuged at 13,000*g* for 20 min and the supernatant was transferred to a fresh tube and stored at −80°C until use. Proteins (25 μg/lane) were separated on a SDS-PAG and transferred to a PVDF membrane. Western blotting analysis was performed using rabbit polyclonal antibody against HO-1 and enhanced chemiluminescence reagents. Expression of α-tubulin was evaluated in the same lysates to ensure equal protein concentrations in each sample

out that protein expression levels of specific DDH isoforms cannot be ascertained because the DDH antibody available is not specific and reacts with all DDH isoforms. Regulation of ROS by DDH and its association with the cisplatin resistance phenotype of the 2008/C13* cells was further corroborated by the observation of cross-resistance of the 2008/C13* cells to the cytotoxic effects of H_2O_2 and *t*-BHP when compared to the parental 2008 cells and a consequence of the reduced ROS production in the 2008/C13* cells was the observed decrease in HO-2 expression in the cisplatin-resistant cells as compared to the 2008 cells. This was confirmed by the increased expression of HO-2 observed in 8n23 and 7n1 cells (siRNA-mediated DDH1 knockdown in 2008/C13* cells).

Cellular redox homeostasis is essential for the normal functioning of cellular processes as well as to prevent damage to cellular components including lipids, proteins and nucleic acids. Aberrant production of ROS can lead to cell signaling processes necessary for cellular proliferation as well as those processes that lead to irreversible damage and death. In order to prevent the latter, oxidative stress can induce an adaptive response that activates an antioxidant defense mechanism that facilitates detoxification of ROS and normalizes the redox homeostasis within the cell. Considering that the 2008/C13* cells were generated by step-wise exposure of the parental 2008 cells to increasing concentrations of cisplatin, the upregulation of DDH in the resistant 2008/C13* cells may be an antioxidant defense mechanism responsible for attenuating the intracellular levels of ROS.

In this regard it should be noted that in addition to its direct association with DNA, several recent reports have shown that generation of reactive oxygen species (ROS) upon exposure of tumor cell lines to cisplatin appear to be involved in drug-induced apoptosis. Thus, in human melanoma cells expressing low levels of c-myc, cisplatin treatment increases the generation of ROS, which results in increased apoptosis [4]. Indeed, treatment with cisplatin has been shown to increase the release of intracellular hydrogen peroxide (H_2O_2) as well as superoxide anions in murine macrophage cells [29]. Furthermore, increased resistance to cisplatin has also been observed in fibroblast cells that are resistant to oxidative stress which was associated with increased catalase activity [30]. In addition, a recent report showed that increased expression of peroxiredoxin II, an enzyme known to eliminate intracellular H_2O_2, led to cisplatin cross-resistance in gastric carcinoma cells [7]. Whilst, exposure of the 2008 and 2008/C13* cells to acute cisplatin treatment (1–4 h) did not alter the intracellular ROS levels, we speculate that during the process of development of the cisplatin-resistant subline, the oxidative stress (as a result of cisplatin exposure) led to the increased expression of DDH (adaptive response); a model of cisplatin treatment which mimics the clinical situation, where treatment of short duration (approximately 1–3 h) is repeated up to six or more times over a month or more

The biochemical consequences of increased generation of ROS are many and practically all of these are geared towards rendering the cells unable to divide and proliferate. Thus, (to name a few) increased ROS production leads to DNA strand breakage, activation of caspases and activation of c-jun *N*-terminal kinases (JNK's) that in turn activate c-jun [12]. In some cases this indicates that ROS commit cells to the process of apoptosis via diverse signaling mechanisms that either work independently or that may have significant cross-talking ability. The fact that overexpression of DDH reduced the basal intracellular levels of ROS and led to development of cisplatin resistance in the 2008/C13* cells, suggests that the apoptotic machinery downstream of ROS has been compromised in the 2008/C13* cells. Indeed, we have demonstrated that the 2008/C13* cells are deficient in the proteolytic activation of MEKK1 by caspase-3 [11]. This was found to be associated with a lack of activation of JNK and p38MAPK and an increased expression of Bcl-x_L in the cisplatin-resistant 2008/C13* cells. Future studies are aimed at elucidating the precise apoptotic pathway that is(are) disrupted due to upregulation of DDH leading to reduced ROS production in the 2008/C13* cells utilizing the various recombinant cell clones generated in this study.

References

1. Andrew PA, Velury S, Mann SC, Howell SB (1988) *cis*-diamminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells. Cancer Res 48:68–73
2. Andrews PA, Albright KD (1992) Mitochondrial defects in *cis*-diamminedichloroplatinum (II)—resistant human ovarian carcinoma cells. Cancer Res 52:1895–1901
3. Ax W, Soldan M, Koch L, Maser E (2000) Development of duanorubicin resistance in tumor cells by induction of carbonyl reductase. Biochem Pharmacol 59:293–300
4. Biroccio A, Benassi B, Amodei S, Gabellini C, Del Bufalo D, Zupi G (2001) c-Myc down-regulation increases susceptibility to cisplatin through reactive oxygen species-mediated apoptosis in M14 human melanoma cells. Mol Pharmacol 60:174–182
5. Burczynski ME, Lin HK, Penning TM (1999) Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. Cancer Res 59:607–614
6. Christen RJ, Jekunen AP, Jones JA, Thiebaut F, Shalinsky DR, Howell SB (1993) In vitro modulation of cisplatin accumulation in human ovarian carcinoma cells by pharmacological alteration of microtubules. J Clin Invest 92:431–440
7. Chung YM, Yoo YD, Park JK, Kim YT, Kim HJ (2001) Increased expression of peroxiredoxin II confers resistance to cisplatin. Anticancer Res 21:1129–1133
8. Ciaccio PJ, Stuart JE, Tew KD (1993) Overproduction of a 37.5-kDa cytosolic protein structurally similar to prostaglandin F synthase in ethacrynic acid-resistant human colon cells. Mol Pharmacol 43:845–853
9. Deng HB, Parekh HK, Chow KC, Simpkins H (2002) Increased expression of dihydrodiol dehydrogenase induces resistance to cisplatin in human ovarian carcinoma cells. J Biol Chem 277:15035–15043
10. Deng HB, Adikari M, Parekh HK, Simpkins H (2004) Ubiquitous induction of resistance to platinum drugs in human ovarian, cervical, germ-cell and lung carcinoma tumor cells overexpressing isoforms 1 and 2 of dihydrodiol dehydrogenase. Cancer Chemother Pharmacol 54:301–307
11. Gebauer G, Mirakhur B, Nguyen Q, Shore SK, Simpkins H, Dhanasekaran N (2000) Cisplatin-resistance involves the defective processing of MEKK1 in human ovarian adenocarcinoma 2008/C13 cells. Int J Oncol 16:321–325
12. Gius D, Spitz DR (2006) Redox signaling in cancer biology. Antioxid Redox Signal 8:1249–1252
13. Hamers T, Molin KR, Koeman JH, Murk AJ (2000) A small-volume bioassay for quantification of the esterase inhibiting potency of mixtures of organophosphate and carbamate insecticides in rainwater:development and optimization. Toxicol Sci 58:60–67
14. Isonishi S, Jekunen AP, Hom DH, Eastman A, Edelstein PS, Thiebaut FB, Christen RD, Howell SB (1992) Modulation of cisplatin sensitivity and growth rate of an ovarian carcinoma cell line by bombesin and tumor necrosis factor α. J Clin Invest 90:1436–1442
15. Jeong WS, Jun M, Kong AN (2006) Nrf2: a potential molecular target for cancer chemoprevention by natural compounds. Antioxid Redox Signal 8:99–106
16. Loehrer PJ, Einhorn LH (1984) Drugs five years later. Cisplatin Ann Intern Med 100:704–713
17. Mamenta EL, Poma EE, Kaufmann WK, Delmastro DA, Grady HL, Chaney SG (1994) Enhanced replicative bypass of platinum-DNA adducts in cisplatin-resistant human ovarian carcinoma cell lines. Cancer Res 54:3500–3505
18. Mann SC, Andrews PA, Howell SB (1991) Modulation of *cis*-diamminedichloroplatinum (II) accumulation and sensitivity by forkolin and 3 isobutyl-1 methylxanthine in sensitive and resistant human ovarian carcinoma cells. Int J Cancer 48:866–872
19. Mirakhur B, Parekh HK, Simpkins H (1996) Expression of the cisplatin resistance phenotype in a human ovarian carcinoma cell line segregates with chromosome 11 and 16. Cancer Res 56:2256–2262
20. Moorehead RA, Singh G (2000) Influence of the proto-oncogene c-fos on cisplatin sensitivity. Biochem Pharmacol 59:337–345
21. Mattiasson G (2004) Analysis of mitochondrial generation and release of reactive oxygen species. Cytometry A 62:89–96
22. Ogasawara H, Nishio K, Kanzawa F, Lee YS, Funayama Y, Ohira T, Kuraishi Y, Isogai Y, Saijo N (1995) Intracellular carboxyl esterase activity is a determinant of cellular sensitivity to the antineoplastic agent KW-2189 in cell lines resistant to cisplatin and CPT-11. Jpn J Cancer Res 86:124–129
23. Ozols RF, Williams SD (1989) Testicular cancer. Curr Prob Cancer 13:287–335
24. Parekh HK, Simpkins H (1995) The differential expression of cytokeratin 18 in cisplatin-sensitive and -resistant human ovarian adenocarcinoma cells and its association with drug sensitivity. Cancer Res 55:5203–5206
25. Penning TM, Burczynski ME, Jez JM, Hung CF, Lin HK, Ma H, Moore M, Palackal N, Ratnam K (2000) Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. Biochem J 351:67–77
26. Smithgall TE, Harvey RG, Penning TM (1986) Regio- and stereospecificity of homogeneous 3 alpha-hydroxysteroid-dihydrodiol dehydrogenase for *trans*-dihydrodiol metabolites of polycyclic aromatic hydrocarbons. J Biol Chem 261:6184–6191
27. Smithgall TE, Harvey RG, Penning TM (1988) Oxidation of the *trans*-3,4-dihydrodiol metabolites of the potent carcinogen 7,12-dimethylbenz(a)anthracene and other benz(a)anthracene derivatives by 3 alpha-hydroxysteroid-dihydrodiol dehydrogenase: effects of methyl substitution on velocity and stereochemical course of *trans*-dihydrodiol oxidation. Cancer Res 48:1227–1232
28. Smithgall TE, Harvey RG, Penning TM (1988) Spectroscopic identification of ortho-quinones as the products of polycyclic aromatic *trans*-dihydrodiol oxidation catalyzed by dihydrodiol dehydrogenase. A potential route of proximate carcinogen metabolism. J Biol Chem 263:1814–1820
29. Sodhi A, Gupta P (1986) Increased release of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) by murine macrophages in vitro after *cis*-platin treatment. Int J Immunopharmacol 8:709–714
30. Spitz DR, Phillips JW, Adams DT, Sherman CM, Deen DF, Li GC (1993) Cellular resistance to oxidative stress is accompanied by resistance to cisplatin: the significance of increased catalase activity and total glutathione in hydrogen peroxide-resistant fibroblasts. J Cell Physiol 156:72–79
31. Sung YJ, Cheng CL, Chen CS, Huang HB, Huang FL, Wu PC, Shiao MS, Tsay HJ (2003) Distinct mechanisms account for beta-amyloid toxicity in PC12 and differentiated PC12 neuronal cells. J Biomed Sci 10:379–388
32. Timmer-Bosscha H, Mulder NH, de Vries EGE (1992) Modulation of *cis*-diamminedichloroplatinum(II) resistance: a review. Br J Cancer 66:227–238
33. Zvereva E, Serebrov V, Glupov V, Dubovskiy I (2003) Activity and heavy metal resistance of non-specific esterases in leaf beetle Chrysomela lapponica from polluted and unpolluted habitats. Comp Biochem Physiol C Toxicol Pharmacol 135:383–391