Molecular Pharmacology Fast Forward. Published on March 9, 2005 as doi:10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

MOLPHARM/2004/010579

Suppression of hREV1 Expression Reduces the Rate at which Human Ovarian Carcinoma Cells Acquire Resistance to Cisplatin

Tsuyoshi Okuda[1], Xinjian Lin[1], Julie Trang and Stephen B. Howell

Department of Medicine and the Rebecca and John Moores Cancer Center, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0058

MOLPHARM/2004/010579

Running title: Modulation of cisplatin pharmacodynamics by hREV1.

Correspondence should be addressed to Stephen B. Howell, Department of Medicine 0058, University of California, San Diego, La Jolla, CA 92093-0058. Tel: 858- 822-1110; Fax: 858-822-1111; E-mail: showell@ucsd.edu

The number of text pages: 35

The number of tables: 0

The number of figures: 8

The number of references: 62

The number of words in the *Abstract*: 257

The number of words in the *Introduction*: 857

The number of words in the *Discussion*: 1518

The abbreviations used are: EBFP, enhanced blue fluorescent protein; EGFP, enhanced green fluorescent protein; HGPRT, hypoxanthine guanine phosphoribosyl transferase; shRNA, short hairpin interfering RNA.

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

MOLPHARM/2004/010579

ABSTRACT

Replicative bypass of many DNA adducts is dependent on the interaction of hREV1 with DNA polymerase ζ and potentially with members of the Y family of DNA polymerases. To examine the role of hREV1 in the development of cisplatin (DDP) resistance, a subline (2008-shREV1-3.3) of the ovarian carcinoma cell line 2008 was isolated in which stable expression of a short hairpin RNA suppressed *hREV1* expression to 20%, and reduced hREV1 protein level to 43% of that found in the parental cells. The 2008-shREV1-3.3 cells were 1.5-fold more sensitive to the cytotoxic effect of DDP, but 2.6- and 2.7- fold less sensitive, respectively, to the mutagenic effect of DDP as evidenced by reduced ability to induce clones highly resistant to 6-thioguanine or DDP itself in the surviving population. Reduction of hREV1 did not alter the initial rate of DDP adduct removal from DNA but did impair both spontaneous and DDP-induced extra-chromosomal homologous recombination as measured by the recombination-sensitive reporter vector pBHRF. DDP induced an increase in hREV1 protein level. DDP resistance at the population level evolved 2.8- fold more slowly in the 2008-shREV1-3.3 cells than the parental cells during repeated cycles of drug exposure. The results indicate that hREV1 functions to enhance both cell survival and the generation of drug-resistant variants in the surviving population. DDP up-regulates hREV1 suggesting that it may enhance its own mutagenicity. Most importantly, hREV1 controls the rate of emergence of resistance to DDP at the population level. Thus, hREV1 is an important contributor to DDP-induced genomic instability and the subsequent emergence of resistance.

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Cisplatin (DDP) is used to treat many types of solid tumors; however, its clinical efficacy is limited by the rapid development of resistance. Most tumors that are initially sensitive to this drug become resistant over the course of 4-6 cycles of treatment. The evolution of resistance appears to involve both selection for pre-existing resistant variants and mutational generation of new highly drug-resistant clones in the surviving population (Fink et al., 1998; Lin and Howell, 1999; Schabel et al., 1980). DDP produces both intra- and interstrand crosslinks in DNA and these are believed to be important to both the cytotoxicity and the mutagenicity of the drug. DDP is a mutagen in both bacterial (Yarema et al., 1995; Yarema et al., 1994) and mammalian cell systems (Cariello et al., 1992; Johnson et al., 1980; Lin and Howell, 1999; Lin et al., 2001; Turnbull et al., 1979; Wiencke et al., 1979). While DDP triggers recombinational events, most of the mutations generated by DDP appear to result from bypass replication across DDP adducts by the eukaryotic DNA polymerases β, μ and ζ (Havener et al., 2003; Masutani et al., 2000; Vaisman and Chaney, 2000; Vaisman, 1999; Vaisman et al., 2000; Yuan et al., 2000). When non-error-prone mechanisms for repairing DNA damage such as base excision repair, nucleotide excision repair and homologous recombination, are disabled or overwhelmed, bacteria and yeast cells make increased use of specialized low-fidelity error-prone DNA polymerases to bypass DNA lesions that block normal replicative polymerases (Johnson et al., 1999). Such increased reliance on error-prone translesional bypass appears to contribute to the mutagenicity of DDP adducts (Hoffmann et al., 1997).

In *S. cerevisiae* the majority of DNA damage-induced mutagenesis arises as a result of a translesional replication pathway mediated by Rev1 and DNA polymerase ζ (reviewed in (Lawrence, 2002)). This pathway is required for mutagenesis induced by UV radiation,

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

acetylaminofluorene and apurinic sites (Baynton et al., 1998; Morrison, 1989; Nelson et al., 2000), and for the bypass of adducts at the N^2 position of guanine situated in the minor groove (Washington et al., 2004). Yeast Rev1 has a unusual dCMP transferase activity that inserts a C opposite not only normal template guanine but also a variety of adducted forms of guanine (Guo et al., 2004; Haracska et al., 2002; Lin et al., 1999; Nelson et al., 1996; Zhang et al., 2002), and pol ζ has the unique ability to extend nucleotide incorporation from this poorly matched base (Guo et al., 2004; Haracska et al., 2002; Washington et al., 2004). While yeast Rev1 is required for translesional synthesis by pol ζ, its dCMP transferase activity does not appear to be required for the bypass of all types of lesions (Baynton et al., 1999; Lawrence, 2002; Nelson et al., 2000). The damage-induced mutagenesis pathway in which yeast Rev1 operates is evolutionarily conserved in human cells, and the genes of this pathway identified in yeast as controlling platinum-containing drug sensitivity have mammalian homologues that include HHR6A, HHR6B, hRAD18, hREV1, hREV3 and hREV7. In addition to interacting with the REV7 subunit of pol ζ, mouse REV1 binds to pol κ, η, and ι, and all the interactions occur at the same site on REV 1 (Guo et al., 2003). This suggests that in mammalian cells, REV1 may have a role in supporting translesional synthesis carried out by multiple DNA polymerases.

Several investigators have previously reported that loss of hREV1 function markedly reduces UV-induced HGPRT mutations in human cells engineered to contain reduced levels of *hREV1* mRNA through expression of an *hREV1* antisense RNA (Gibbs et al., 2000) or a ribozyme that cleaves endogenous *hREV1* mRNA (Clark et al., 2003). It has also been demonstrated that inactivation of the *REV1* gene in chicken DT40 cells renders them hypersensitive to a wide variety of DNA damaging agents including DDP (Simpson and Sale, 2003).

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

If REV1 is centrally involved in the translesional bypass of DDP adducts, then there is the possibility that disabling hREV1 function in human cancer cells may both enhance sensitivity to the cytotoxic effect of DDP and also reduce its mutagenesis. In this study, we used constitutive expression of an interfering RNA (Paddison et al., 2002) to suppress the level of *hREV1* mRNA in the human ovarian cancer cell line 2008. The sensitivity of these cells to the cytotoxic and mutagenic effects of DDP was compared with that of the parental cells transfected with an empty vector. We report here that reduction in *hREV1* mRNA rendered cells more somewhat sensitive to the cytotoxic effect of DDP but substantially decreased its mutagenicity. Most importantly, it significantly reduced the rate at which the whole population of cells acquired resistance to DDP during repeated cycles of drug exposure analogous to the clinical use of this drug. These results support the hypothesis that when a cell is faced with the challenge of replicating its DNA while the genome is burdened with a large number of DDP adducts, it is highly dependent on hREV1 error-prone bypass replication for survival, that the resulting mutations generate highly drug-resistant clones in the surviving population, and that these clones play a central role in the emergence of DDP resistance at the population level.

Materials and Methods

Drugs. DDP was a gift from Bristol-Myers Squibb (Princeton, NJ). A stock solution of 1 mM cisplatin in 0.9% NaCl was stored in the dark at room temperature. 6-Thioguanine was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in 0.2 N sodium hydroxide to form a 20 mM stock solution and stored at –20 ^{o}C.

Design and Cloning of shRNAs. shRNAs were designed by using software available from the website of the laboratory of Dr. Gregory Hannon (www.cshl.org/public/SCIENCE/

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

hannon.html). Two complementary ≈ 72-nt DNA oligonucleotides shRNA sequences targeted to *REV1* mRNA were annealed and cloned directly into the plasmid vector pSHAG-1 (Paddison et al., 2002). When transfected into 2008 cells this vector constitutively expresses a short interfering RNA targeted to *REV1* mRNA from a RNA polymerase III-specific U6 promoter. The resultant vector has been named pSHAG-REV1. The mRNA targeting sequence is 5'-AATGTCCAACTCCTGGTAGATTGGTCACGAAGCTTGGTGATCAGTCTACTAGGAGTT GGACGTTCAATTTTTT-3'.

Cell Culture, Transfection, and Selection. The human ovarian carcinoma cell line 2008 was grown in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were co-transfected using Fugene 6 (Roche, Indianapolis, IN) with pcDNA3.1(-) (Invitrogen, Carsbad, CA) and either pSHAG-REV1 or pSHAG-1 as an empty vector control according to the manufacturer's recommendations. The transfection mixtures contained pcDNA3.1(-) in a 10-fold molar excess to the pSHAG vectors. Cells expressing the G418-resistance marker from pcDNA3.1(-) were then selected by exposure to 400 μg/ml G418, and individual clones were screened for REV1 mRNA expression level by real-time PCR and for REV1 protein level by Western blotting. One clone, designated 2008-shREV1-3.3, was found to have a substantial stable reduction in REV1 mRNA and protein level, was chosen for all subsequent experiments. 2008-shREV1-3.3 cells were maintained in medium supplemented with 400 μg/ml G418. The cells transfected with the pSHAG-1 empty vector control were designated 2008-EV.

Quantitation of *hREV1* mRNA by RT-PCR. Total RNA was extracted with TRIzol^{R} reagent (Invitrogen). First strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time PCR was performed using the BIO-RAD iCycler iQ detection system in the presence of SYBR-Green I dye (Bio-Rad laboratory,

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

Inc.). For the *REV1* gene expression, the forward (5'-AAGGCTGATGCAATCG-3') and reverse (5'-CCACCTGGACATTGTCAAGAATAA-3') primers were used for amplification with a iCycler protocol consisting of a denaturation program (95 ^{0}C for 3 min), amplification and quantification program repeated 40 times (95 ^{0}C for 10 sec and 55 ^{0}C for 45 sec), and melting curve analysis. A melt-curve analysis immediately followed amplification and was executed using 95 ^{0}C for 1 min then 55 ^{0}C for 1 min, followed by 80 repeats of heating for 10 sec, starting at 55 ^{0}C with 0.5 ^{0}C increments. The data were analyzed by using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The ΔCt values from each cell line were obtained by subtracting the values for 18S Ct from the sample Ct. A 1 unit difference of Ct value represents a 2-fold difference in the level of mRNA.

Western Blot Analysis. The nuclear proteins were extracted as previously described (Schreiber et al., 1989), heated and a sample containing 10 μg was then subjected to electrophoresis on a 4-15 % SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane that was blocked with 5% skimmed milk in buffer (0.35 M NaCl, 10 mM Tris-HCl (pH 8.0)) containing 0.05% Tween 20 for 1 h at room temperature and then incubated overnight at 4°C with a 1:100 dilution of polyclonal antibody against hREV1 and a 1:500 dilution of a goat polyclonal antibody against histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then washed and exposed for 1 h to a 1:500 dilution of a horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and bands were detected using the ECL Western blotting detection system (Amersham, Buckinghamshire, United Kingdom) and analyzed densitometrically by a ChemiImager (Alpha Innotech Corporation, San Leandro, CA).

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

Immunofluorescent Imaging. Cells were fixed by treatment for 15 min with 3.7% formaldehyde in phosphate buffered saline and stained as previously reported (Safaei et al., 2004). Briefly, cells were stained with 1 μg/ml Hoechst 33324 and 0.4 μg/ml Alexa Flour 647 phalloidin (Molecular Probes, Eugene, OR) along with a primary goat polyclonal antibody to REV1 (Santa Cruz Biotechnology, Santa Cruz, CA) and Texas red-conjugated donkey anti-goat secondary antibodies (Jackson Immuno-Research Laboratories Inc., West Grove, PA). Microscopy was performed at UCSD Cancer Center Digital Imaging Shared Resource using a DeltaVision deconvoluting microscope system (Applied Precision, Inc., Issaquah, WA.). Images were captured from 0.2 μM sections by 100x, 60x and 40x lenses and SoftWorx software (Applied Precision, Inc) was used for deconvoluting data. Image quantification was performed with Data Inspector program in SoftWorx or by NearCount software.

Clonogenic and Enrichment Assays. Clonogenic assays were performed as previously reported (Lin et al., 2001) by seeding 250 cells into 35 mm dishes, adding the requisite concentration of DDP for 1 h, washing the drug away and allowing colonies to form for 10 – 14 days. Each experiment was performed a minimum of 3 times using triplicate cultures for each drug concentration. IC_{50} values were determined by log-linear interpolation. For enrichment assays a cell population containing 10% 2008-EV cells constitutively expressing GFP and 90% 2008-shREV1-3.3 cells was prepared by mixing appropriate numbers of the two cell types, and the actual fraction of 2008-EV cells was documented by flow cytometry. Enrichment assays were performed by plating 10,000 cells from the mixed cell population in 100-mm tissue culture dishes for 24 h and then exposing the cells to increasing concentrations of DDP for 1 hr. The untreated cell population served as a control. Flow cytometric analysis was repeated 5 days later

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

to determine the fraction of GFP-expressing 2008-EV cells in the treated and in the control group. Each experiment was performed 3 separate times for each drug concentration.

Measurement of Rate of Generation of Resistant Variants and DDP Mutagenicity. The rate at which highly drug-resistant variants spontaneously appeared in the population was measured using the "maximum likelihood estimation" technique (Glaab and Tindall, 1997) as previously reported (Lin and Howell, 1999). The sensitivity of cells to the mutagenic effects of DDP was measured by determining the frequency of variants highly resistant to either 10 μM 6TG or to 12 μM DDP itself in the surviving population 20 days after a 1 h exposure to increasing concentrations of DDP as previously reported (Lin and Howell, 1999; Lin et al., 2001). Each experiment was performed a minimum of 3 times and the data is presented as mean ± SEM. When testing for 6TG-resistant variants the cells were grown in HAT medium containing 0.4 μM aminopterin, 16 μM thymidine and 100 μM hypoxanthine for a minimum of 14 days prior to testing to eliminate pre-existing HGPRT mutants.

Measurement of Pt in DNA. DNA was isolated using Wizard Genomic DNA Purification Kit (Promaga, Madison, WI) according to the manufacturer's instructions. Aliquots of the DNA were digested in 70% nitric acid at 65 ˚C for 2 h and diluted to 5% nitric acid by adding appropriate volume of double distilled deionized water. The pg platinum/μg DNA in the hydrolysate was quantified by inductively coupled plasma mass spectroscopy as previously described (Katano et al., 2002). When assessing the time course of the loss of Pt from DNA, the cells were treated with 200 μM DDP for 1 h in order to obtain quantifiable levels of Pt over the entire period of the experiment. DNA was isolated at 0, 6, 12, 18, 24 h after drug exposure.

Measurement of the Frequency of Extra-Chromosomal Homologous Recombination. Homologous recombination was assayed by determining the extent of

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

recombination between 2 GFP sequences in plasmid DNA as previously described (Slebos and Taylor, 2001). The pBHRF vector contains an intact "blue" variant of GFP (EBFP) that includes a ≈300 nucleotide sequence with perfect homology to a second truncated nonfunctional copy of GFP. In the absence of homologous recombination within the vector only EBFP is expressed, however, homologous recombination between the EBFP and truncated GFP sequences creates a functional GFP, and if this occurs the cell expresses GFP as well as EBFP that is expressed from other plasmids in the cell that have not undergone recombination. Cells were seeded into 6-well plates overnight and then exposed to 0 or 10 μM DDP for 1 h. The untreated or surviving cells were then transfected with pBHRF 24 h later with siPORT^{TM} *XP-1* transfection agent (Ambion Inc., Austin, TX) in the presence of serum according to the manufacturer's instruction. Four h after transfection, BoosterExpress reagent (Gene Therapy Systems, Inc., San Diego, CA) was added and the cells were then analyzed by two color flow cytometry 48 h after transfection. The recombination frequency was calculated as RF = [(EBFP$^+$ and GFP$^+$) + (GFP$^+$)]/[(EBFP$^+$ and GFP$^+$) + (GFP$^+$) + (EBFP$^+$)] where (EBFP) and (GFP) represent the number of blue and green fluorescent cells, respectively, in the sample.

Relative Rate of Development Resistance to DDP. The rate at which the cell population became resistant to DDP during repeated cycles of 1 h exposure to the drug was determined by measuring the IC_{50} for DDP by constructing a concentration-survival curve that included a minimum of 5 DDP concentrations using a clonogenic assay following each round of selection. The degree of resistance was determined by dividing the IC_{50} for the multiple DDP-treated population to the IC_{50} for the untreated cells for each cell line individually. The DDP concentration used for selection was the IC_{90} for the population under study. For each round of selection, 10^6 cells were exposed to DDP for 1 h. When the cells had recovered to 90%

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

confluence, an aliquot was used to determine cell number and the slope of the DDP concentration-survival curve in a clonogenic assay, and another aliquot was again exposed to DDP. Total cell number and plating efficiency was determined at each step; this information, along with the exact number cells sub-cultured, was used to calculate population doubling according to the equation described above. The rate of acquisition of resistance to DDP was then calculated by plotting the slope of the DDP concentration-survival curve as a function of population doubling; population doubling = (Ln[total number of cells]- Ln[number of cells plated x plating efficiency])/Ln2. The slope of this plot yields the rate of relative resistance development.

Statistics. All data were analyzed by use of a two-sided paired Student's *t* test with the assumption of unequal variance.

Results

Characterization of 2008-shREV1-3.3 Cells with Stable shRNA-Mediated Knockdown of hREV1. The ovarian carcinoma cell line 2008 was co-transfected with either a pSHAG-1 empty vector (2008-EV cells) or a vector expressing a *hREV1*-specific shRNA (2008-shREV1-3.3 cells) in combination with pcDNA3.1(-) which provided a selectable marker. Individual clones were expanded and the resulting populations screened for stable suppression of *REV1* mRNA level by RT-PCR. The endogenous *hREV1* mRNA level in the 2008-shREV1-3.3 clone was found to be reduced to 20 ± 2 % ($p<0.001$) of that in 2008 cells transfected with the empty pSHAG-1 vector, and this population was used for subsequent studies. Fig.1A shows that the 80% reduction in level of the *hREV1* mRNA was accompanied by a reduction in the 138 kDa form of REV1 protein in the 2008-shREV1-3.3 cells to 43% of that in the empty vector control cells as

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

determined by Western blot analysis after normalization for histone H1 levels. The effect of stable expression of hREV1 shRNA was also assessed by confocal digital deconvoluting microscopic examination of cells immunofluorescently stained with a polyclonal anti-REV1 antibody. As shown in Fig.1B, staining for REV1 was abundant in the 2008-EV cells but markedly reduced in the 2008-shREV1-3.3 cells.

Effect of REV1 Reduction on the Spontaneous Rate of Generation of Resistant Variants. The spontaneous rate of generation of variants resistant to 6TG was determined by repeatedly measuring the frequency of resistant variants in expanding populations of the 2008-EV and 2008-shREV1-3.3 cells. The results, presented in Fig. 2, show that the hREV1 shRNA-expressing 2008-shREV1-3.3 cells exhibited a 2.9-fold decrease in spontaneous rate of generation of variants resistant to 6TG as compared to the empty vector-transfected 2008-EV cells ($p<0.01$). The population doubling times were nearly identical for the two cell types being 22.8 ± 1.0 (SD) h for the 2008-EV cells and 22.9 ± 0.5 (SD) h for 2008-shREV1-3.3 cells. There was also no significant difference in cell cycle phase distribution as determined by flow cytometric analysis of propidium iodide-stained cells (2008-EV cells: G_1 49%, S 14%, G_2/M 37%; 2008-shREV1-3.3 cells: G_1 46%, S 9%, G_2/M 45%). Thus, the difference in spontaneous rate of development of 6TG-resistant variant can not be attributed to differences in growth rate. The reduced rate observed in the 2008-shREV1-3.3 cells is consistent with the loss of Rev1 function on spontaneous mutagenesis in a yeast system (Kalinowski et al., 1995) and validates this experimental system for the study of the effect of hREV1 on DDP pharmacodynamics.

Effect of REV1 Reduction on Sensitivity to the Cytotoxic Effect of DDP. Clonogenic assays were used to determine the effect of reduced hREV1 on sensitivity to the cytotoxic effect of DDP. Fig. 3A shows the survival of 2008-EV and 2008-shREV1-3.3 cells as a function of

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

drug concentration following a 1-hr exposure to DDP. The sensitivity of the empty-vector transfected cells did not differ from that of the parental non-transfected 2008 cells (data not shown); however, the 2008-shREV1-3.3 cells were 1.5-fold more sensitive to the cytotoxic effect of DDP than the empty vector-transfected 2008-EV cells (IC_{50} 5.4 ± 0.69 (SEM) μM *vs* 8.0 ± 0.62 (SEM) μM; n=3; $p<0.05$). Thus, impairment of hREV1 function by down-regulation of its mRNA enhanced sensitivity to the cytotoxic effect of DDP. The ability of DDP to select for hREV1-proficient cells in a tumor cell population under conditions in which the hREV1-proficient cells were only 1.5-fold less sensitive was tested by preparing a population containing 10% GFP-expressing 2008-EV cells and 90% hREV1 shRNA-expressing 2008-shREV1-3.3 cells. This mixed population was exposed to increasing concentrations of DDP for 1 h, and the fraction of hREV1-proficient GFP-expressing cells was determined by flow cytometric analysis after 5 days of subsequent growth in drug-free medium. Fig. 3B shows that the fraction of GFP-expressing hREV1-replete 2008-EV cells increased in a DDP concentration-dependent manner. Five days after a single 1-hr exposure to the highest concentration of DDP tested (20 μM) the treated populations contained up to 66.6% GFP-expressing 2008-EV cells ($p< 0.001$). The greater sensitivity of 2008-shREV1-3.3 cells as measured by clonogenic assay and the marked selection against these cells in the enrichment assay indicate that hREV1 is a determinant of the ability of the cell to tolerate damage induced by DDP.

Effect of REV1 Reduction on the Ability of DDP to Generate Resistant Variants. DDP is a mutagen in human cells, and generates mutations that result in high-level resistance to many classes of drugs (Lin and Howell, 1999; Lin et al., 1999; Lin et al., 2001). To determine the role of hREV1 in DDP-induced mutagenesis, the 2008-EV and 2008-shREV1-3.3 cells were exposed to 10 μM DDP for 1 h and then 20 days later the fraction of clonogenic cells

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

demonstrating high-level resistance to 6TG or to DDP itself was determined. As shown in Fig. 4A, the frequency of 6TG-resistant variants induced by exposure to 10 μM DDP was significantly lower (2.6-fold) in the 2008-shREV1-3.3 cells than in the 2008-EV cells ($p<0.01$). Fig. 4B shows that following exposure to DDP, the hREV1-proficient cells yielded 2.7-fold more colonies that were highly resistant to DDP itself than the hREV1-shRNA expressing cells ($p<0.01$). Thus, DDP was able to generate variants in the surviving population that were highly resistant to 6TG or to DDP itself, and reduction in hREV1 level decreased this mutagenic effect. This indicates that hREV1 plays an important role in generating mutations that lead to drug resistance when DDP adducts are present in DNA.

Effect of REV1 Reduction on the Removal of Platinum from DNA. The changes in sensitivity to the cytotoxic and mutagenic effects of DDP could be explained by differences in initial adduct levels or their persistence if loss of hREV1 interferes with DNA adduct removal. The rate of disappearance of platinum from the DNA accurately mirrors the rate of removal of the most common DDP adducts (Djit et al., 1988; Johnson et al., 1994). The initial pg Pt/μg DNA and the rate of disappearance of platinum from total cellular DNA was measured in both cell lines following a 1-hr exposure to 200 μM DDP. The initial adduct levels were nearly identical being 6.64 ± 1.21 (SEM) and 6.79 ± 1.03 (SEM) pg Pt/μg DNA, respectively, for the 2008-EV and 2008-shREV1-3.3 cells. Fig. 5 shows that there was no difference in the kinetics of platinum disappearance from DNA in the two cell lines for the initial period of 12 h. More platinum remained in the 2008-shREV1-3.3 cells at 18 h and 24 h, 52.3% and 48.5%, respectively, than in the 2008-EV cells, 41.2% and 32.4%, respectively, but this difference did not reach the level of statistical significance ($p>0.05$). Since nucleotide excision repair is responsible for removal of the majority of DDP adducts during the initial phase of repair, this

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

suggests that the degree of impairment of hREV1 function in the 2008-shREV1-3.3 cells did not interfere significantly with this pathway of DNA repair.

Effect of REV1 Reduction on Spontaneous and DDP-induced Homologous Recombination. DDP induces sister chromatid exchange, and homologous recombination may play a role in the ability of DDP to generate highly drug resistant variants. The pBHRF recombination-sensitive reporter vector described by Slebos and Taylor (Slebos and Taylor, 2001) was used to assess the effect of reduced hREV1 function on basal and DDP-induced rates of homologous recombination. This plasmid constitutively expresses an intact, emission-shifted, "blue" variant of GFP (EBFP), and also contains a carboxy-terminally truncated form of GFP in which there exists a 300-bp homologous sequence for recombination with identical nucleotide stretch within the intact EBFP sequence. In the absence of homologous recombination within or between the GFP sequences in the vector only EBFP is expressed in transfected cells; however, an homologous recombination event can create a functional GFP, in which case the cell expresses both EBFP and GFP since most cells acquire multiple copies of the vector during transfection only some of which recombine. The hREV1-proficient 2008-EV and hREV1-shRNA expressing 2008-shREV1-3.3 cells were exposed to 10 μM DDP for 1 h or left untreated, and the pBHRF was transfected into the cells 24 h later. After a further 48 h, the fraction of BFP-positive cells that also expressed GFP was determined by flow cytometry. As shown in Fig. 6, the spontaneous homologous recombination frequency was 1.4-fold lower in the 2008-shREV1-3.3 cells than the empty vector-transfected 2008-EV cells ($p<0.05$). Exposure to 10 μM DDP for 1 h increased the recombination frequency in both cell types; however, the frequency was still significantly lower (1.6-fold, $p<0.05$) in the 2008-shREV1-3.3 than in the 2008-EV cells. Thus,

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

hREV1 function appears to be important to both spontaneous and DDP-induced extra-chromosomal homologous recombination.

Effect of DDP on hREV1 Protein Levels. Given its ability to promote both survival and mutagenicity following a DDP exposure, it was of interest to determine whether DDP induces an increase in hREV1 level during the injury response following drug exposure. Fig. 7A shows that DDP induced a concentration-dependent increase in hREV1 protein level in the 2008-EV cells when measured at 24 h after a 1-hr drug exposure. Interestingly, although smaller in magnitude, DDP also induced an increase in hREV1 protein level in the hREV1 shRNA-expressing cells, suggesting that the ability of the shRNA to mediate hREV1 degradation may be overwhelmed by an increase in endogenous *hREV1* mRNA production or that DDP impairs the production or action of the interfering RNA. Fig. 7B shows the change in hREV1 protein level as a function of time following exposure to 10 μM DDP for 1 h. The hREV1 protein level continued to increase for up to 24 h in both cell lines by 3.3- and 1.9- fold induction in the 2008-EV and 2008-shREV1-3.3 cells, respectively. However, the increase was generally lower in the shRNA-expressing 2008-shREV1-3.3 cells than in the 2008-EV cells. By 48 h the hREV1 protein levels had declined in the both cell lines. These results indicate that DDP induced an increase in hREV1 protein level that peaked at a time similar to the time of maximum cell cycle arrest produced by DDP (Lin et al., 2001).

Effect of REV1 Reduction on the Rate of Development of DDP Resistance. The development of drug resistance during repeated cycles of DDP exposure may be due to enrichment for resistant clones already present in the population, or it may be due to DDP-induced generation of new resistant clones, or both effects. As shown above, loss of hREV1 function reduced the ability of DDP to generate drug-resistant variants in the surviving

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

population. If this ability of DDP is central to the emergence of acquired DDP resistance in the whole population, then reduction of hREV1 activity would be expected to reduce the rate at which resistance emerges. We measured the rate of development of resistance in the whole population of 2008-EV and 2008-shREV1-3.3 cells starting with a total 500,000 cells. The cells were exposed to an IC_{90} concentration of DDP for 1 h, and the exposure was repeated again as soon as log phase growth resumed. After each round of drug treatment, the sensitivity of the whole population to DDP was measured by determining survival over 2 logs of cell kill as a function of DDP concentration in a clonogenic assay. The degree of resistance after repeated cycles of DDP exposure was expressed as the ratio of the IC_{50} values for the treated cells relative to the untreated cells for each line separately. Fig. 8 shows that after the first drug treatment the degree of resistance relative to non-DDP-treated cells increased linearly with population doubling for the 2008-EV cells. In the case of the 2008-shREV1-3.3 cells there was an abrupt development of 2.1-fold resistance relative to non-DDP-treated 2008-shREV1-3.3 cells after just a few population doublings in the face of DDP treatment, but thereafter resistance was acquired 2.8-fold more slowly than for the 2008-EV cells ($p < 0.05$). Thus, hREV1 plays a central role in the acquisition of DDP resistance at the population level. Since loss of hREV1 does not appear to alter the extent of adduct formation or the time course of platinum removal from DNA, these results are consistent with the concept that mutagenic translesional synthesis across DDP adducts is responsible for generating drug-resistant variants that become enriched in the population by subsequent rounds of DDP exposure.

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The biochemical pathways that mediate resistance to DDP are poorly defined, as are the mechanisms by which these pathways become altered in the resistant cells. The DDP resistant phenotype is stable over many cell generations indicating that it is the result of genetic changes. The results of the studies reported here indicate that hREV1 plays a role in producing these genetic changes, and that disabling its function slows the emergence of resistance to this important chemotherapeutic agent.

The DNA adducts produced by DDP are important to its ability to kill the cell. Cytotoxicity is proportional to the extent of adduct formation, and cells with defects in the major DNA repair mechanism that removes these adducts, nucleotide excision repair, are hypersensitive to DDP (Damia et al., 1998; Furuta, 2002). Recently, REV1-deficient chicken DT40 lymphocytes were shown to be hypersensitive to the cytotoxic effect of a variety of mutagens including DDP. These cells have normal resting and damage-induced sister chromatid exchange, but have an increased frequency of chromosome and chromatid breaks after exposure to UV irradiation (Simpson and Sale, 2003). The results of the current studies indicate that impairment of hREV1 function in human ovarian carcinoma cells caused a moderate increase in sensitivity to the cytotoxic effect of DDP without altering the rate at which total platinum adducts were removed from DNA during the early phase of DNA repair. This suggests that, as for other types of adducts that block the progression of the replicative polymerases (Bradley et al., 1993; Chaney and Vaisman, 1999), hREV1 is involved in a pathway that normally carries out enough translesional synthesis to allow some cells to complete DNA synthesis and survive.

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

The mechanisms that generate mutations in eukaryotes have been studied most intensively in the budding yeast *S. cerevisiae*. In this organism, translesional bypass replication mediated by Rev1 and pol ζ accounts for a large fraction of the mutations induced by DNA-damaging agents (Lawrence and Hinkle, 1996). Pol ζ, consisting of the Rev3 and Rev7 subunits, is required for translesion replication. Rev1 participates in bypass replication but also has an independent dCMP transferase activity (Lin et al., 1999; Masuda and Kamiya, 2002; Nelson et al., 1996; Zhang et al., 2002). Deletion of the genes coding for either Rev1, Rev3, or Rev7 results in a similar phenotype that includes failure to generate as many mutations after exposure to a variety of mutagenic agents. It has been reported that both pol ζ and REV1 are important for error-prone translesion synthesis across bulky guanine adducts in yeast cells (Guo et al., 2004; Washington et al., 2004).

The pol ζ mutagenesis pathway also exists in human cells (Gibbs et al., 2000; Gibbs et al., 1998; Lin et al., 1999; Lin et al., 1999). We have previously reported that reduction of the expression of *hREV3* mRNA in human fibroblasts produces a phenotype with many of the same characteristics as that observed in the 2008-shREV1-3.3 cells, including hypersensitivity to the cytotoxic effect of DDP and a marked reduction in sensitivity to its mutagenic effects (Wu et al., 2004). The similarity of the phenotypes produced by reduction of *REV1* and *REV3* mRNA, at least with respect to cytotoxic sensitivity and mutagenesis, suggests that these two proteins act in the same process, possibly as elements of a single complex. However, since reduction of either of these proteins may destabilize the other, it is not possible to determine whether it is pol ζ activity or a pol ζ-independent activity of REV1 that is key to error-prone bypass replication across DDP adducts. Likewise, since REV1 has been shown to interact with other DNA polymerases they two may play a role (Guo et al., 2003).

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

The observation that reduction in the level of hREV1 decreased the ability of DDP to generate highly drug-resistant clones in the surviving population provides strong evidence that the translesional synthesis pathway in which hREV1 functions is error-prone when it bypasses DDP adducts in mammalian cells. It appears that this pathway normally fosters the development of resistance to DDP both by permitting the survival of cells that contain mutagenic adducts in their DNA, and by generating new mutations in genes that mediate the resistant phenotype.

The role hREV1 plays in mammalian DNA repair mechanisms has not been clearly defined. The observation that, despite being 1.5-fold hypersensitive to the cytotoxic effect of DDP, the time course of removal of the majority of platinum from DNA was the same in the hREV1-proficient and deficient cells suggests that nucleotide excision repair is not highly dependent on hREV1. However, reduction in hREV1 level did have an effect on both the endogeneous and DDP-induced frequency of homologous recombination as detected by the pBHRF vector. This suggests a role for hREV1, pol ζ or one of the other proteins with which hREV1 interacts in homologous recombination, consistent with another recent report (Sonoda et al., 2003). Homologous recombination is important to the survival of cells following DDP exposure (Aloyz et al., 2002; Stracker et al., 2002; Zhong et al., 1999), and it may be essential for repair of interstrand crosslinks (Keller et al., 2001; McHugh et al., 2001; Thompson and Schild, 2001). The finding that DDP exposure enhances pBHRF recombination suggests that DDP up-regulates this putatively non-mutagenic repair mechanism, an effect expected to improve the ability of the cell to survive the DNA damage produced by this agent.

The expression of a large number of genes is known to be altered following DDP exposure (Johnsson et al., 2001), and the current study indicates that *hREV1* belongs to the family of genes whose expression is up-regulated by DDP-induced injury. hREV1 protein levels

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

increased in proportion to the extent of injury, and the level peaked at 24 h following a 1 h exposure to 10 μM DDP. It is not known whether the increase in hREV1 protein directly reflects increased enzyme activity but the data are consistent with the concept that DDP increases hREV1 activity and thus further enhances its own mutagenicity. A completely independent line of evidence suggesting the importance of hREV1 in platinum drug resistance has emerged from cDNA microarray-based studies of genes differentially expressed in isogenic oxaliplatin-sensitive and resistant cells (Manorek et al., 2004). Among the 12,300 genes assayed, hREV1 was one of a very small number that were statistically significantly up-regulated in the resistant member of 3 or more of the 5 pairs of cells examined. Of even greater interest was the discovery that the two genes that lie immediately adjacent to hREV1 in the human chromosome are also significantly up-regulated in the oxaliplatin-resistant cells (Samimi et al., 2004).

The studies reported here were performed on a clonal population of 2008 cells in which REV1 expression was suppressed. Caution is needed in generalizing the results of these studies since the RNAi may be produced off-target effects, and integration of the vector may have produced an effect on other genes as well. Nevertheless, the phenotypic characteristics of the 2008-shREV1-3.3 are consistent with those of other cell systems in which the expression of REV1 has been disabled (Simpson and Sale, 2003), and can not be attributed to differences in population growth rate or cell cycle phase distribution.

Perhaps the single most important observation to emerge from these studies is that reduction of hREV1 impedes the development of resistance to DDP at the population level. Preventing the emergence of resistance during DDP treatment is a key clinical goal. Previous studies have established that exposure to DDP results in the generation of clones in the surviving population that are highly resistant to DDP (Lin and Howell, 1999), and this was confirmed in

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

the current study. However, whether these clones play a central role in the change in sensitivity of the entire population of tumor cells remained unknown. The results reported here indicate that acquisition of resistance by the entire population is not just due to enrichment for drug-resistant clones that existed in small numbers prior to drug exposure. They also indicate that the genes involved in high level DDP resistance are susceptible to attack by DDP with the formation of adducts that can be mutagenically bypassed by hREV1 acting in combination pol ζ or other polymerases. It is noteworthy that the magnitude of the effect of REV1 depletion on the rate of resistance development was quite large. Thus, hREV1 is of interest as a target for the development of drugs capable of stabilizing the genome following DNA damage and reducing the rate of development of resistance in patients treated with DDP.

The finding that inhibition of hREV1 expression in the human cancer cells markedly reduces the mutagenicity of DDP indicates an important role for hREV1 in DNA damage induced mutagenesis and identifies hREV1 as being of particular interest with respect to the mechanism underlying emergence of the multi-drug resistant phenotype that so frequently accompanies the development of DDP resistance. The hREV1 protein is an attractive target for therapeutic intervention to simultaneously enhance DDP sensitivity and reduce the risk of development of drug resistance. Studies are now needed of the extent to which expression of hREV1 varies amongst different types of tumors, and whether its expression is linked to clinical response.

Acknowledgements

We gratefully acknowledge Dr. R.J.C. Slebos for kindly providing the pBHRF vector.

References

Aloyz R, Xu ZY, Bello V, Bergeron J, Han FY, Yan Y, Malapetsa A, Alaoui-Jamali MA, Duncan AM, and Panasci L (2002) Regulation of cisplatin resistance and homologous recombinational repair by the TFIIH subunit XPD. *Cancer Res* **62:**5457-62.

Baynton K, Bresson-Roy A, and Fuchs RP (1999) Distinct roles for Rev1p and Rev7p during translesion synthesis in Saccharomyces cerevisiae. *Mol Microbiol* **34:**124-133.

Baynton KJ, Bresson-Roy A, and Fuchs RP (1998) Analysis of damage tolerance pathways in Saccharomyces cerevisiae: a requirement for Rev3 DNA polymerase in translesion synthesis. *Mol Cell Biol* **18:**960-966.

Bradley LJ, Yarema KJ, Lippard SJ, and Essigmann JM (1993) Mutagenicity and genotoxicity of the major DNA adduct of the antitumor drug cis-diamminedichloroplatinum (II). *Biochemistry (Mosc)* **32:**982-988.

Cariello NF, Swenberg JA, and Skopek TR (1992) *In vitro* mutational specificity of cisplatin in the human hypoxanthine guanine phosphoribosyltransferase gene. *Cancer Res* **52:**2866-73.

Chaney SG and Vaisman A (1999) Specificity of platinum-DNA adduct repair. *J Inorg Biochem* **77:**71-81.

Clark DR, Zacharias W, Panaitescu L, and McGregor WG (2003) Ribozyme-mediated REV1 inhibition reduces the frequency of UV-induced mutations in the human HPRT gene. *Nucleic Acids Res* **31:**4981-8**.**

Damia G, Guidi G, and D'Incalci M (1998) Expression of genes involved in nucleotide excision repair and sensitivity to cisplatin and melphalan in human cancer cell lines. *Eur J Cancer* **34:**1783-1788.

Djit FJ, Fichtinger-Schepman AMJ, Berends F, and Reedijk J (1988) Formation and repair of cisplatin-induced addcts to DNA in cultured normal and repair-deficient human fibroblasts. *Cancer Res* **48:**6058-6062.

Fink D, Nebel S, Norris PS, Baergen RN, Wilczynski SP, Costa MJ, Haas M, Cannistra SA, and Howell SB (1998) Enrichment of DNA mismatch repair-deficient cells during treatment with cisplatin. *Int J Cancer* **77:**746.

Furuta T, Ueda, T.,Aune, G. Sarasin, A., Kraemer, K., Pommier, Y. (2002) Transcription coupled-nucleotide excision repair as a determinant of cisplatin sensitivity of human cells. *Cancer Res* **62:**4899-4902.

Gibbs PE, Wang XD, Li Z, McManus TP, McGregor WG, Lawrence CW, and Maher VM (2000) The function of the human homolog of Saccharomyces cerevisiae REV1 is required for mutagenesis induced by UV light. *Proc Natl Acad Sci U S A* **97:**4186-91.

Gibbs PEM, McGregor WB, Maher VM, Nisson P, and Lawrence CW (1998) A human homolog of the Saccharomyces cervisiae REV3 gene, which encodes the catalytic subunit of DNA polymerase. *PNAS* **95:**6876-6880.

Glaab WE and Tindall KR (1997) Mutation rate at the *hprt* locus in human cancer cell lines with specific mismatch repair-gene defects. *Carcinogenesis* **18:**1-8.

Guo C, Fischhaber PL, Luk-Paszyc MJ, Masuda Y, Zhou J, Kamiya K, Kisker C, and Friedberg EC (2003) Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis. *Embo J* **22:**6621-30.

Guo D, Xie Z, Shen H, Zhao B, and Wang Z (2004) Translesion synthesis of acetylaminofluorence-dG addcuts by DNA polymerase zeta is stimulating by yeast Rev1 protein. *Nucleic Acids Res* **32:**1122-1130.

Haracska L, Prakash S, and Prakash L (2002) Yeast Rev1 protein is a G template-specific DNA polymerase. *J Biol Chem* **277:**15546-51.

Havener JM, McElhinny SA, Bassett E, Gauger M, Ramsden DA, and Chaney SG (2003) Translesion synthesis past platinum DNA adducts by human DNA polymerase mu. *Biochemistry (Mosc)* **42:**1777-88.

Hoffmann JS, Pillaire MJ, Lesca C, Burnouf D, Fuchs RP, Defais M, and Villani G (1997) Fork-like DNA templates support bypass replication of lesions that block DNA synthesis on single-stranded templates. *Proc Natl Acad Sci U S A* **93:**13766-13769.

Johnson NP, Hoeschele JD, Rahn RO, O'Neill JP, and Hsie AW (1980) Mutagenicity, cytotoxicity, and DNA binding of platinum (II)- chloroammines in Chinese hamster ovary cells. *Cancer Res* **40:**1463-68.

Johnson RE, Washington MT, Prakash S, and Prakash L (1999) Bridging the gap: A family of novel DNA polymerases that replicate faulty DNA. *Proc Natl Acad Sci U S A* **96:**12224-12226.

Johnson SW, Perez RP, Godwin AK, Yeung AT, Handel LM, Ozols RF, and Hamilton TC (1994) Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. *BiochemPharmacol* **47:**689-697.

Johnsson A, Byrne P, de Bruin R, Weiner D, Wong J, and Los G (2001) Identification of gene clusters differentially expressed during the cellular injury responses (CIR) to cisplatin. ***Br J Cancer*** **85:**1206-10.

Kalinowski DP, Larimer FW, and Plewa MJ (1995) Analysis of spontaneous frameshift mutations in REV1 and rev1-1 strains of Saccharomyces cerevisiae. *Mutat Res* **331:**149-159.

Katano K, Kondo A, Safaei R, Holzer A, Samimi G, Mishima M, Kuo Y-M, Rochdi M, and Howell S (2002) Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. *Cancer Res* **62:**6559-65.

Keller K, Overbeck-Carrick T, and Beck D (2001) Survival and induction of SOS in Escherichia coli treated with cisplatin, UV-irradiation, or mitomycin C are dependent on the function of the RecBC and RecFOR pathways of homologous recombination. *Mutat Res* **486:**21-29.

Lawrence C and Hinkle D (1996) DNA polymerase zeta and the control of DNA damage induced mutagenesis in eukaryotes. *Cancer Surv* **28:**21-31.

Lawrence CW (2002) Cellular roles of DNA polymerase zeta and Rev1 protein. *DNA Repair* **1:**425-435.

Lin W, Wu X, and Wang Z (1999) A full-length cDNA of hREV3 is predicted to encode DNA polymerase zeta for damage-induced mutagenesis in humans. *Mutat Res* **433:**89-98.

Lin W, Xin H, Zhang Y, Wu X, Yuan F, and Wang Z (1999) The human REV1 gene codes for a DNA template-dependent dCMP transferase. *Nucleic Acids Res* **27:**4468-4475.

Lin X and Howell SB (1999) Effect of loss of DNA mismatch repair on development of topotecan-, gemcitabine, and paclitaxel-resistant variants after exposure to cisplatin. *Mol Pharmacol* **56:**390-395.

Lin X, Kim HK, and Howell SB (1999) The role of DNA mismatch repair in cisplatin mutagenicity. *J Inorg Biochem* **77:**89-93.

Lin X, Ramamurthi K, Mishima M, Kondo A, Christen RD, and Howell SB (2001) p53 modulates the effect of loss of DNA mismatch repair on the sensitivity of human colon cancer cells to the cytotoxic and mutagenic effects of cisplatin. *Cancer Res* **61:**1508-16.

Manorek G, Samimi G, Breaux J, Cheng T, Berry C, Los G, and Howell S (2004) Microarray-based expression analysis of 5 pairs of isogenic oxaliplatin-sensitive and resistant human tumor cell. *Proc Am Assoc Cancer Res* **45:**383.

Masuda Y and Kamiya K (2002) Biochemical properties of the human REV1 protein. *FEBS Lett* **520**:88-92.

Masutani C, Kusumoto R, Iwai S, and Hanaoka F (2000) Mechanisms of accurate translesion synthesis by human DNA polymerase eta. *Embo J* **19:**3100-9.

McHugh P, Spanswick V, and Hartley J (2001) Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet Oncol* **2:**483-490.

Morrison AC, Christensen, RB, Alley, J, Beck, AK, Bernstine, EG, Lemontt, JF, Lawrence, CW (1989) REV3, a Saccharomyces cerevisiae gene whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase. *J Bacteriol* **171:**5659-5667.

Nelson JR, Gibbs PE, Nowicka AM, Hinkle DC, and Lawrence CW (2000) Evidence for a second function for Saccharomyces cerevisiae Rev1p. *Mol Microbiol* **37:**549-554.

Nelson JR, Lawrence CW, and Hinkle DC (1996) Deoxycytidyl transferase activity of yeast REV1 protein. *Nature* **382:**729-731.

Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, and Conklin DS (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16:**948-958.

Safaei R, Katano K, Larson BJ, Samimi G, Holzer AK, Naerdemann W, Tomioka M, Goodman M, Howell SB (2005) Intracellular localization and trafficking of fluorescein-labeled cisplatin in human ovarian carcinoma cells. *Clin Cancer Res* **11:**756-767.

Samimi G, Manorek G, Castel R, Breaux JK, Cheng TC, Berry CC, Los G, Howell SB (2005) cDNA microarray-based identification of genes and pathways associated with oxaliplatin resistance. *Cancer Chemother Pharmacol* **55:**1-11.

Schabel FM, Jr., Skipper HE, and Trader MW(1980) Concepts for controlling drug-resistant tumor cells, in *Breast Cancer: Experimental and Clinical Aspects* (Mouridsen HT and Palshof T, eds) pp199-212, Pergamon Press, Oxford.

Schreiber E, Matthias P, Muller MM, and Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17:**6419.

Simpson LJ and Sale JE (2003) Rev1 is essential for DNA damage tolerance and non-templated immunoglobulin gene mutation in a vertebrate cell line. *Embo J* **22:**1654-64.

Slebos RJ and Taylor JA (2001) A novel host cell reactivation assay to assess homologous recombination capacity in human cancer cell lines. *Biochem Biophys Res Commun* **281:**212-9.

Sonoda E, Okada T, Zhao GY, Tateishi S, Araki K, Yamaizumi M, Yagi T, Verkaik NS, van Gent DC, Takata M, and Takeda S (2003) Multiple roles of Rev3, the catalytic subunit of polzeta in maintaining genome stability in vertebrates. *Embo J* **22:**3188-97.

Stracker TH, Carson CT, and Weitzman MD (2002) Adenovirus oncoproteins inactivate the Mre11 Rad50 NBS1 DNA repair complex. *Nature* **418:**348-52.

Thompson L and Schild D (2001) Homologous recombinational repair of DNA ensures mammalian chromosome stability. *Mutat Res* **477:**131-153.

Turnbull NC, Popescu JA, DiPaolo JA, and Myhr BC (1979) *cis*-platinum (II) diamine dichloride causes mutation, transformation, and sister-chromatid exchanges in cultured mammalian cells. *Mutat Res* **66:**267-75.

Vaisman A and Chaney SG (2000) The efficiency and fidelity of translesion synthesis past cisplatin and oxaliplatin GpG adducts by human DNA polymerase beta. *J Biol Chem* **275:**13017-25.

Vaisman A, Lim,S.E., Patrick, S.M., Copeland,W.C., Hinkle, D.C., Turchi,J.J. Chaney, S.G. (1999) Effect of DNA polymerases and high mobility group protein 1 on the carrier ligand specificity for translesion synthesis past platinum-DNA adducts. *Biochemistry (Mosc)* **38:**11026-11039.

Vaisman A, Masutani C, Hanaoka F, and Chaney SG (2000) Efficient translesion replication past oxaliplatin and cisplatin GpG adducts by human DNA polymerase eta. *Biochemistry (Mosc)* **39:**4575-80.

Washington MT, Mindo IG, Johnson RE, Haracska L, Harris TM, Lloyd RS, Prakash S, and Prakash L (2004) Efficient and error-free replication past a minor groove N^2-guanine adduct by the sequential action of yeast Rev1 and DNA polymerase ζ. *Mol Cell Biol* **24:**6900-6906.

Wiencke JK, Cervenka J, and Paulus H (1979) Mutagenic activity of anticancer agent cis-dichlorodiammine platinum-II. *Mutat Res* **68:**69-77.

Wu F, Lin X, Okuda T, Maher V, and Howell SB (2004) DNA Polymerase zeta regulates cisplatin cytotoxicity, mutagenicity and the rate of development of cisplatin resistance. *Cancer Res* **64:**8029-8035.

Yarema KJ, Lippard SJ, and Essigmann JM (1995) Mutagenic and genotoxic effects of DNA adducts formed by the anticancer drug cis-diamminedichloroplatinum(II). *Nucleic Acids Res* **23:**4066-4072.

Yarema KJ, Wilson JM, Lippard SJ, and Essigmann JM (1994) Effects of DNA adduct structure and distribution on the mutagenicity and genotoxicity of two platinum anticancer drugs. *J Mol Biol* **236:**1034-48.

Yuan F, Zhang Y, Rajpal DK, Wu X, Guo D, Wang M, Taylor JS, and Wang Z (2000) Specificity of DNA lesion bypass by the yeast DNA polymerase eta. *J Biol Chem* **275:**8233-9.

Zhang Y, Wu X, Rechkoblit O, Geacintov NE, Taylor JS, and Wang Z (2002) Response of human REV1 to different DNA damage: preferential dCMP insertion opposite the lesion. *Nucleic Acids Res* **30:**1630-8.

Zhong Q, Chen C-F, Li S, Chen Y, Wang C-C, Xiao J, Chen P-L, Sharp ZD, and Lee W-H (1999) Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. *Science* **285:**747-750.

Footnotes

This work was supported in part by grant CA78648 from the National Institutes of Health and conducted in part by the Clayton Foundation for Research – California Division. Drs. Lin and Howell are Clayton Foundation investigators.

[1]These two authors contributed equally to this work.

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Fig. 1. shRNA-mediated suppression of hREV1 in the 2008-shREV1-3.3 cells. (A) Western blot analysis of hREV1 expression. Fold reduction was calculated as the ratio of hREV1 level in the parental 2008 and 2008-shREV1-3.3 cells relative to that in the 2008-EV cells after normalization to the level of histone H1. (B) shRNA-induced suppression of hREV1 at the single-cell level by immunofluorescence. hREV1 was stained with hREV1-specific antibody (red), β-actin with Alexa Fluor 647 phalloidin (green), and nuclei with Hoechst 33342 (blue).

Fig. 2. Effect of reducing hREV1 level on the spontaneous rate of generation of 6TG-resistant variants. ♦, 2008-EV cells; ■, *hREV1* shRNA-expressing 2008-shREV1-3.3 cells. Each curve shows the change in the frequency of resistant variants with increasing numbers of population doublings. The rate of generation of resistant variants is given by the slope of the linear regression line. Each data point is the mean of 2 experiments. Vertical bars, SEM.

Fig. 3. Effect of hREV1 reduction on sensitivity to the cytotoxic effect of DDP as determined by clonogenic and enrichment assays. Panel A, DDP concentration-survival curves for the *hREV1* shRNA-expressing 2008-shREV1-3.3 cells (■) and empty vector transfected 2008-EV cells (♦). Panel B, DDP concentration-enrichment histogram. Each bar depicts the fraction of GFP-expressing 2008-EV cells remaining in the population 5 days after a 1-hr exposure to the indicated concentration of DDP. Each point or bar represents the mean of 3 experiments performed with triplicate cultures. Vertical bars, SEM.

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4. Effect of hREV3 reduction on the ability of DDP to generate drug resistant variants in the surviving population. Panel A, number of 6TG-resistant colonies per 10^6 clonogenic cells scored on day 20 after a 1-hr exposure to 10 μM DDP. Panel B, number of DDP-resistant colonies. Each data point represents the mean of 3 experiments. Vertical bars, SEM.

Fig. 5. Effect of hREV3 reduction on the disappearance of platinum from DNA. The 2008-EV and 2008-shREV1-3.3 cells were treated with 200 μM DDP for 1 h. DNA was isolated at the indicated times after treatment and the platinum content quantified. ♦, 2008-EV cells; ■, *hREV1* shRNA-expressing 2008-shREV1-3.3 cells. Each data point represents the mean of 3 experiments. Vertical bars, SEM.

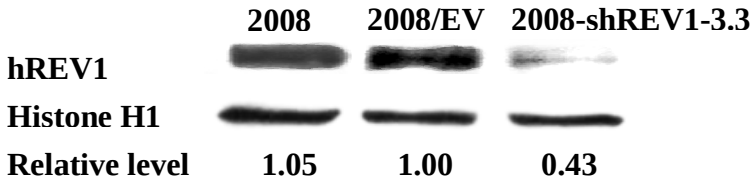
Fig. 6. Effect of hREV1 reduction on the spontaneous and DDP-induced homologous recombination frequency. Closed bars, 2008-EV cells; open bars, 2008-shREV1-3.3 cells . Each data point represents the mean of 3 experiments each performed with duplicate cultures. Vertical bars, SEM.

Fig. 7. The effect of DDP on hREV1 protein level. Panel A, induction of hREV1 protein as a function of DDP concentration at 24 hrs following a 1-hr exposure to DDP. Panel B, time course of change in hREV1 protein levels following exposure to 10 μM DDP for 1 h.

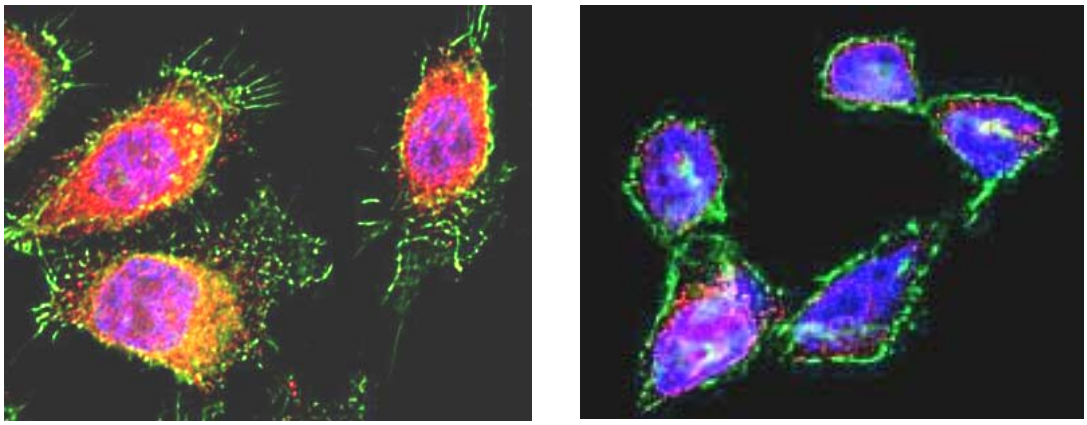
Fig. 8. Effect of hREV1 reduction on the rate of development of DDP resistance. ♦, 2008-EV cells; ■ , *hREV1* shRNA-expressing 2008-shREV1-3.3 cells. Each data point represents the mean of 3 experiments. Vertical bars, SEM.

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

A



B



2008-EV **2008-shREV1-3.3**

Figure 1

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

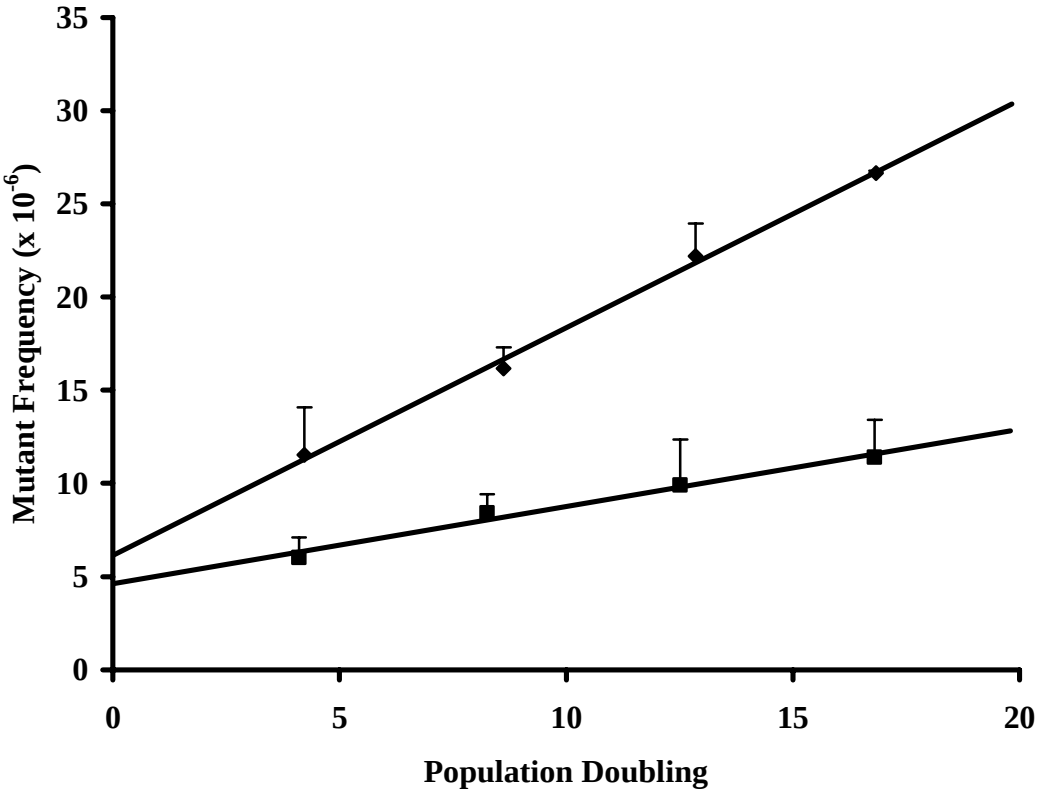


Figure 2

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

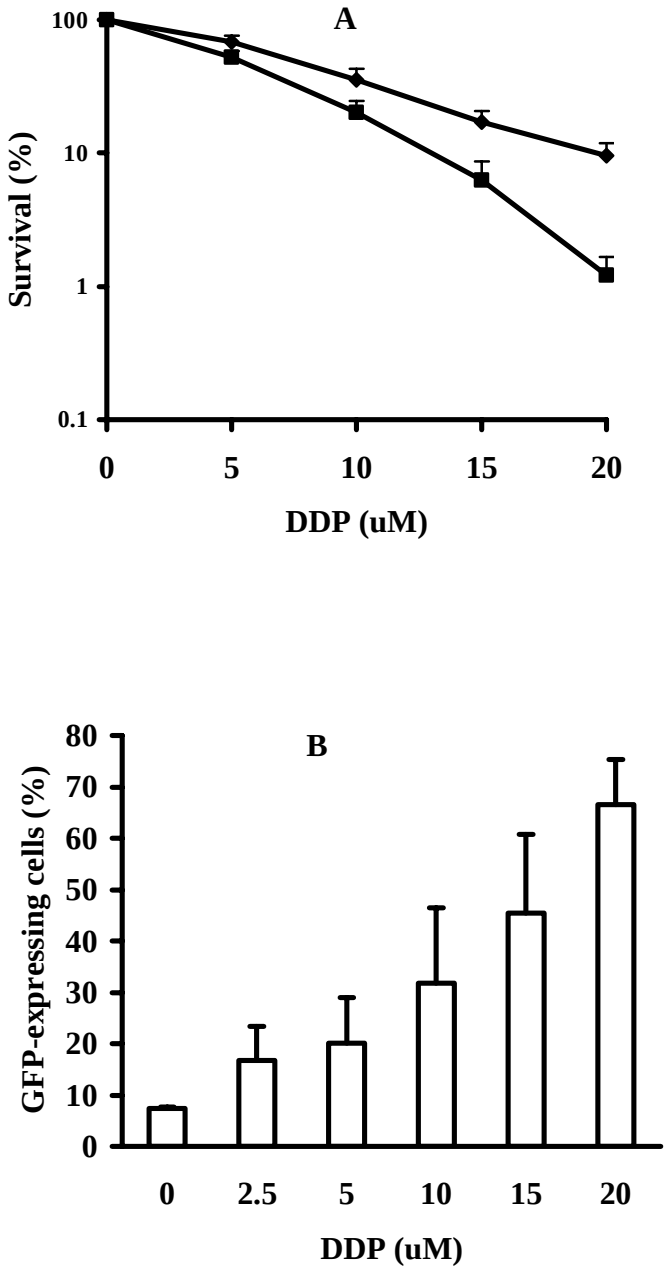


Figure 3

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

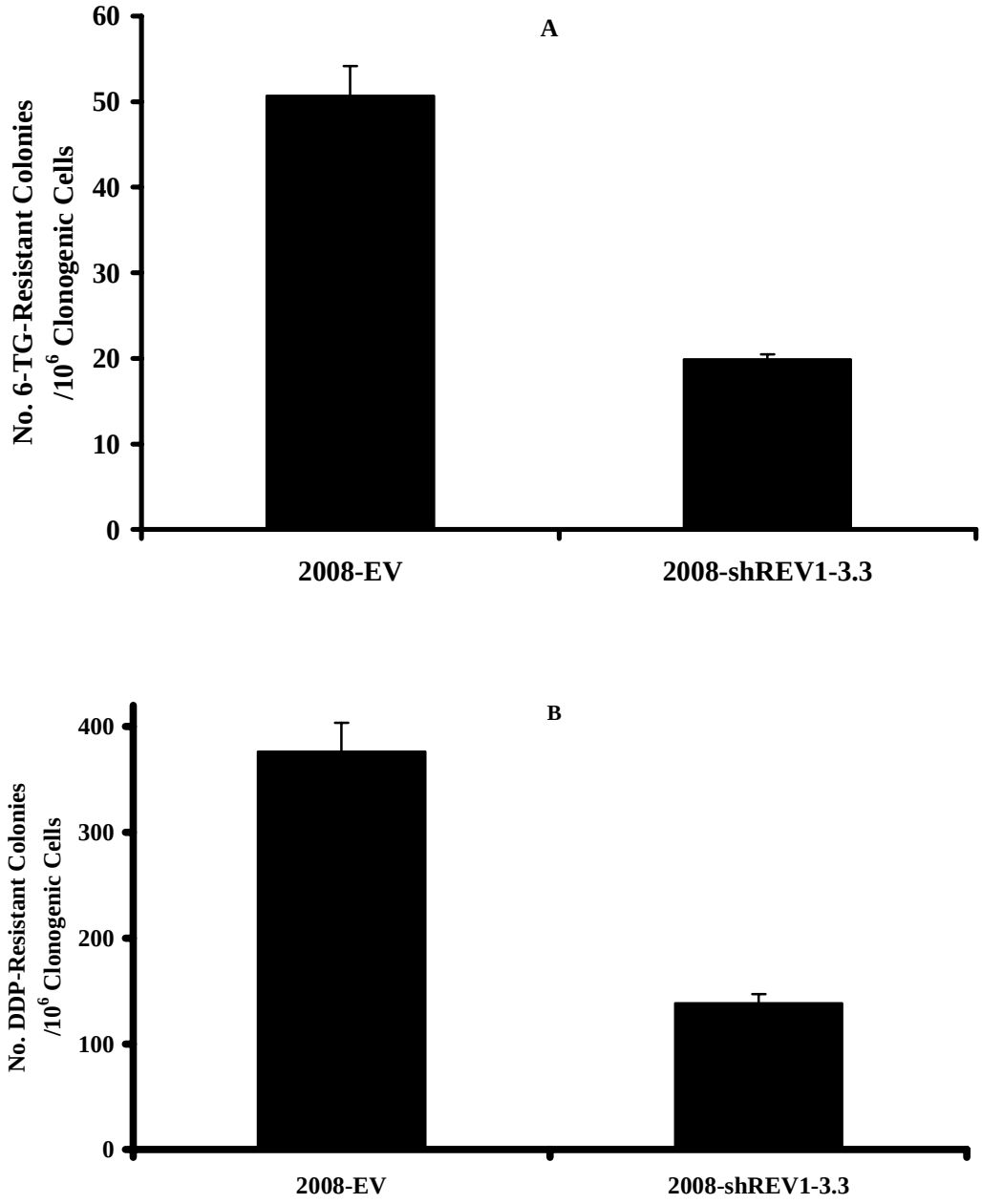


Figure 4

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

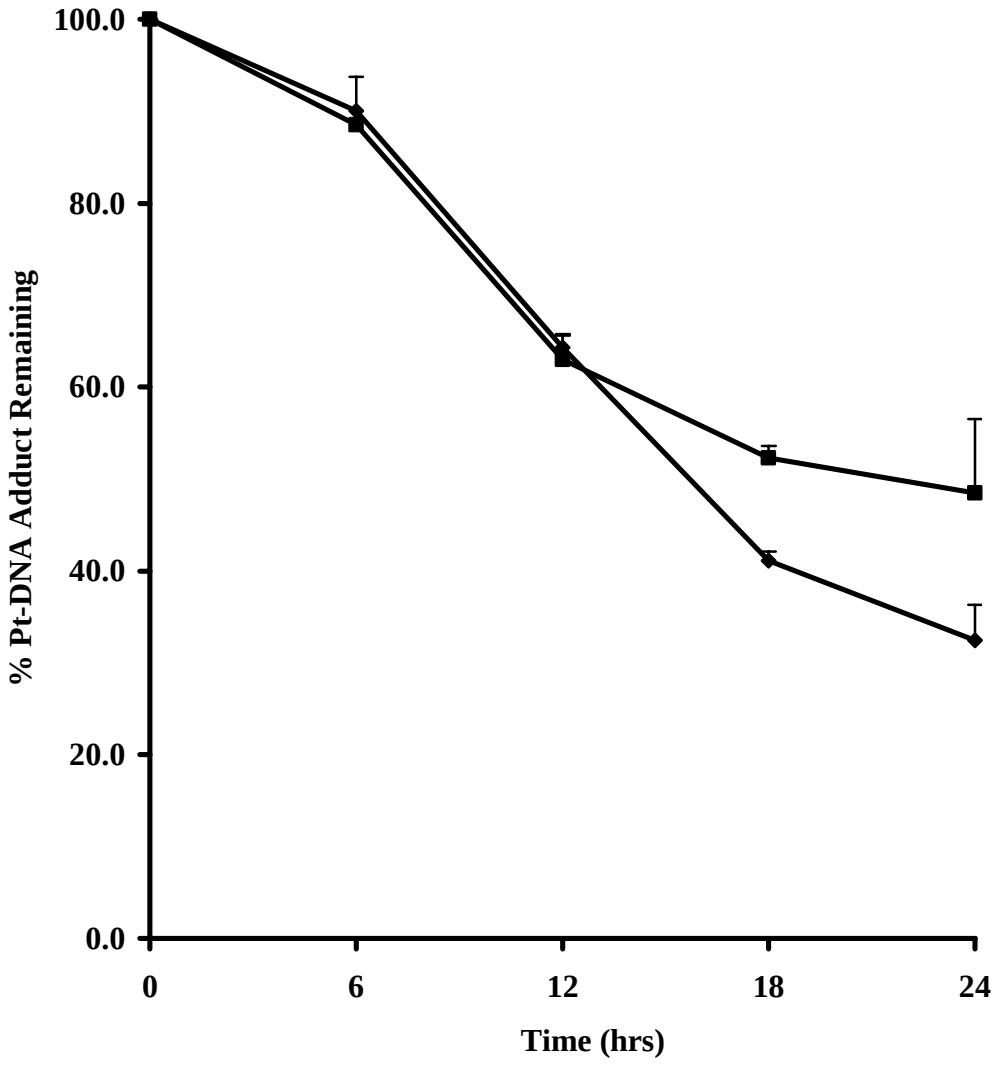


Figure 5

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

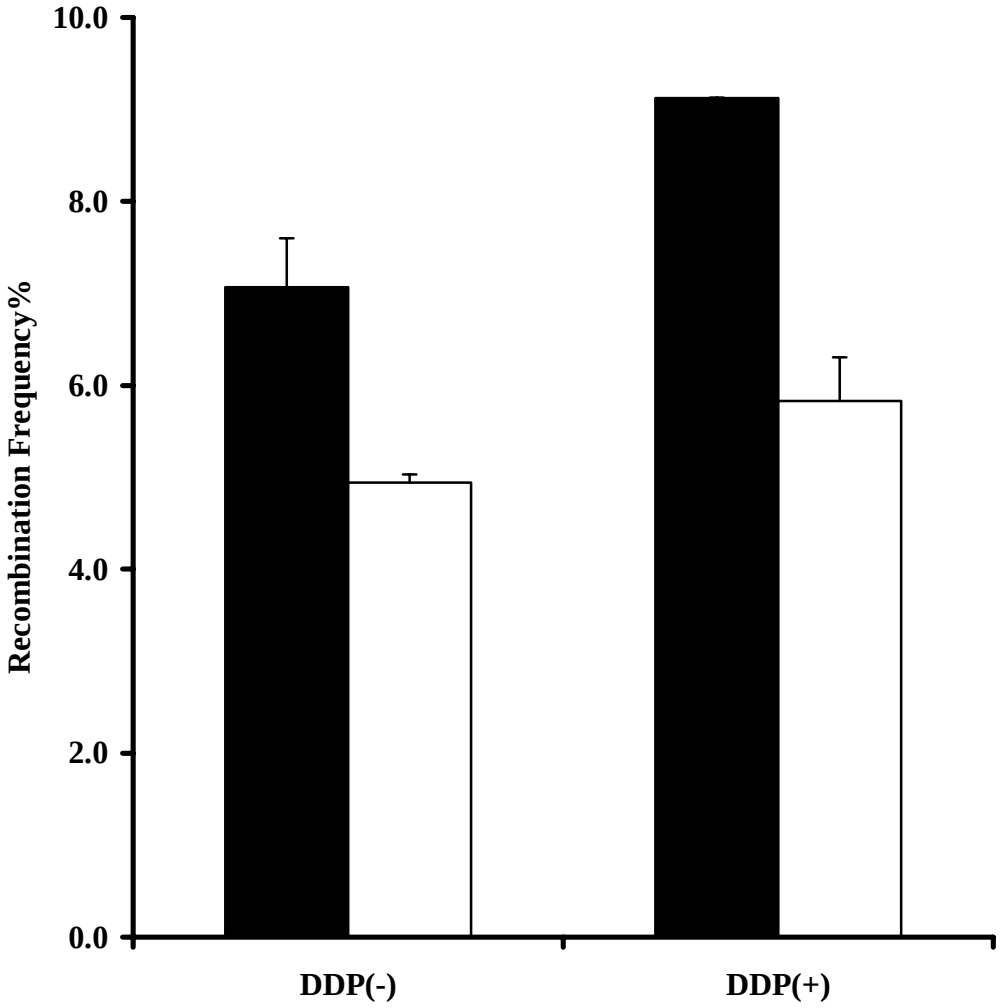


Figure 6

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

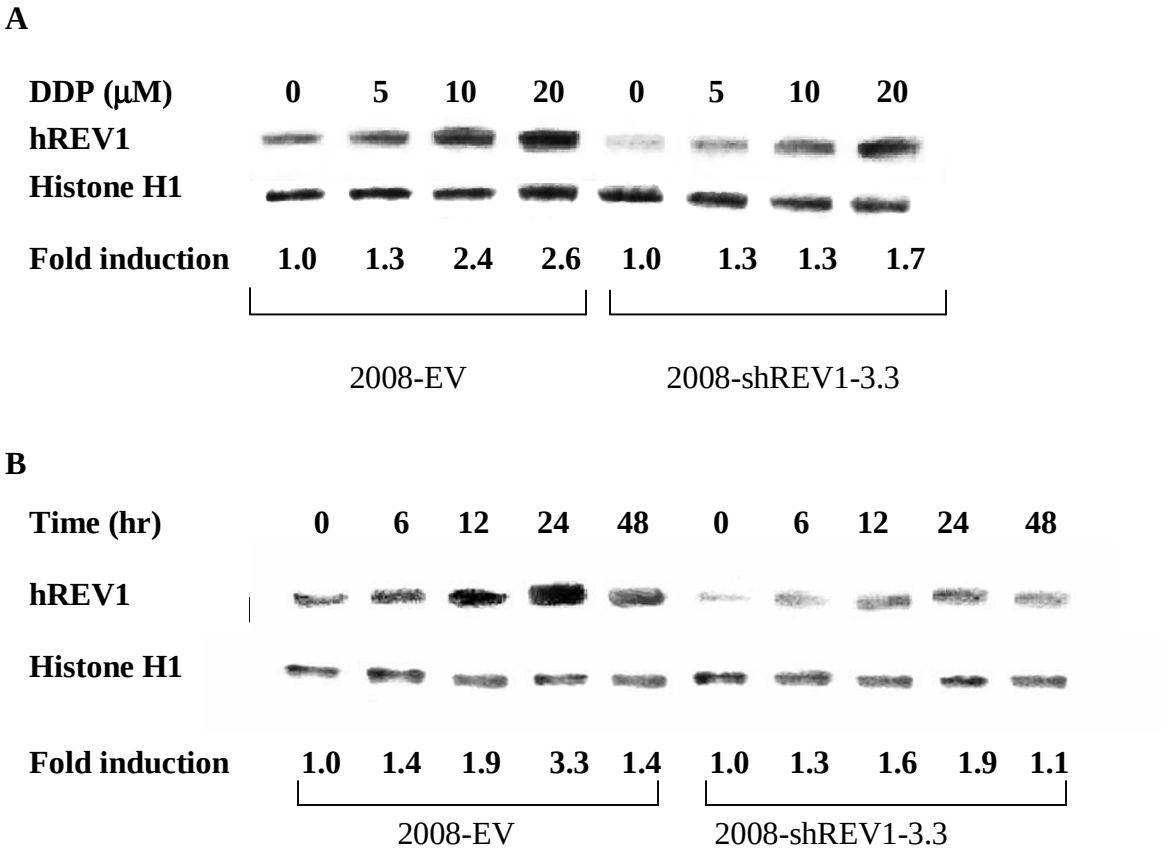


Figure 7

Molecular Pharmacology Fast Forward. Published on March 9, 2005 as DOI: 10.1124/mol.104.010579
This article has not been copyedited and formatted. The final version may differ from this version.

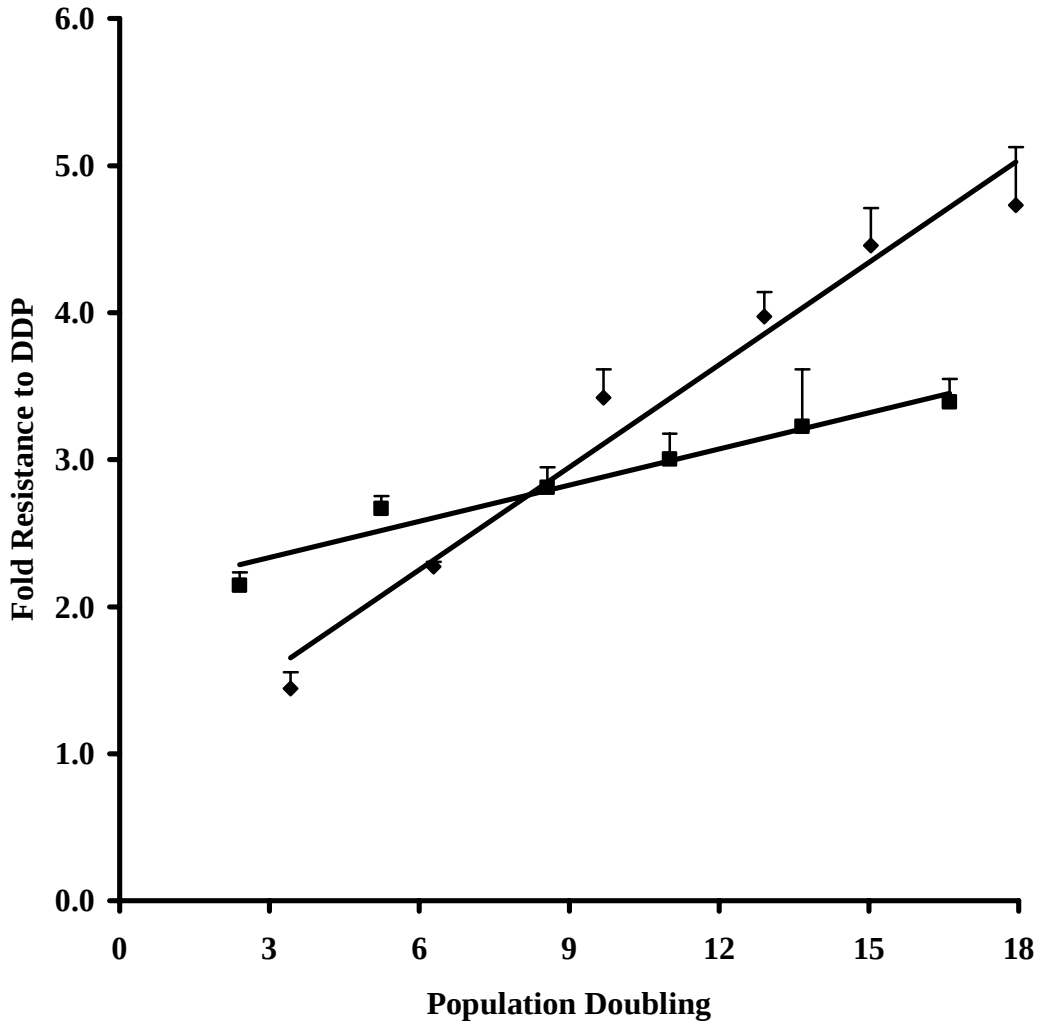


Figure 8