

# Reversal of cisplatin resistance with a BH3 mimetic, (–)-gossypol, in head and neck cancer cells: role of wild-type p53 and Bcl-x<sub>L</sub>

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## Abstract

Organ preservation protocols in head and neck squamous cell carcinoma (HNSCC) are limited by tumors that fail to respond. We observed that larynx preservation and response to chemotherapy is significantly associated with p53 overexpression, and that most HNSCC cell lines with mutant p53 are more sensitive to cisplatin than those with wild-type p53. To investigate cisplatin resistance, we studied two HNSCC cell lines, UM-SCC-5 and UM-SCC-10B, and two resistant sublines developed by cultivation in gradually increasing concentrations of cisplatin. The cisplatin-selected cell lines, UM-SCC-5PT and UM-SCC-10BPT, are 8 and 1.5 times more resistant to cisplatin than the respective parental cell lines, respectively. The parental lines overexpress p53 and contain p53 mutations but the cisplatin-resistant cell lines do not, indicating that cells containing mutant p53 were eliminated during selection. Bcl-x<sub>L</sub> expression increased in the cisplatin-resistant lines

relative to the parental lines, whereas Bcl-2 expression was high in the parental lines and decreased in the cisplatin-resistant lines. Thus, cisplatin selected for wild-type p53 and high Bcl-x<sub>L</sub> expression in these cells. We tested a small-molecule BH3 mimetic, (–)-gossypol, which binds to the BH3 domain of Bcl-2 and Bcl-x<sub>L</sub>, for activity against the parental and cisplatin-resistant cell lines. At physiologically attainable levels, (–)-gossypol induces apoptosis in 70% to 80% of the cisplatin-resistant cells but only in 25% to 40% of the parental cells. Thus, cisplatin-resistant cells seem to depend on wild-type p53 and Bcl-x<sub>L</sub> for survival and BH3 mimetic agents, such as (–)-gossypol, may be useful adjuncts to overcome cisplatin resistance in HNSCC. [Mol Cancer Ther 2005;4(7):1096–104]

## Introduction

Cisplatin and its analogues are among the most widely used and effective agents in the treatment of various solid tumors, including those from lung, testis, bladder, ovary, and head and neck sites (1–3). Organ-sparing therapy consisting of cisplatin and 5-fluorouracil, combined with radiation for advanced head and neck squamous cell carcinoma (HNSCC), provides an alternative to conventional therapy of surgery and radiation (4). However, failure of some tumors to respond to treatment or tumor recurrence limits the overall success of these therapies. Clearly, identifying the molecular mechanisms of chemotherapy resistance and using this knowledge to develop more effective treatment strategies is an important goal.

Among the most common genetic events that occur in the genesis of tumors, including HNSCC, are mutations in the p53 tumor suppressor gene. Wild-type p53 helps maintain genomic integrity through the induction of cell growth arrest and/or apoptosis (programmed cell death) following DNA damage. p53-induced cell cycle arrest allows the cell to repair damaged DNA. If DNA damage is extensive and repair fails, p53 can induce apoptosis (5–8). Missense mutations of p53 that lead to changes in the binding properties or conformation of the protein are common. Such changes inactivate its function and enhance the half-life and stability of the p53 protein leading to overexpression. Our group showed that high expression of p53 is significantly associated with organ preservation in patients with advanced laryngeal cancer treated with induction chemotherapy and radiation (9). Additionally, *in vitro* studies showed that HNSCC cell lines with mutant p53, as a group, are more sensitive to cisplatin than cell lines containing wild-type p53 (10). These data suggest that p53 gene status is important in tumor cell response to cisplatin treatment in HNSCC.

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*In vitro* analysis of human tumor cell lines of various tumor origin, carried out by Weinstein et al. (11), showed that cell lines with *p53* mutations were generally more resistant to DNA-damaging agents than cell lines with wild-type *p53*. Consistent with these *in vitro* studies, *p53* status also has been linked to chemotherapy resistance in several tumor types, including breast cancer (12), lymphomas (13), and leukemias (14). In general, *p53* mutations in these tumor types are associated with disease progression and poor prognosis. We also noted a worse prognosis in cases with mutant *p53* (15). Thus, our findings are paradoxical in that wild-type *p53* normally functions to induce apoptosis in response to DNA-damaging agents and, thus, these tumors should be more responsive to cisplatin-based chemotherapy. In some other tumor types, findings similar to ours have been noted. For example, in bladder cancer, *p53* mutations were shown to correlate with increased chemosensitivity (16). Similarly, other *in vitro* studies are consistent with our observations and have shown that inactivation or disruption of *p53* function sensitizes tumor cells to DNA-damaging agents, specifically cisplatin (17, 18). Thus, the predictive value of *p53* status and its clinical impact seem to depend largely on the predominant effect of *p53* mutations in a specific tumor type (19). Defects in checkpoints responsive to DNA damage may enhance chemosensitivity, whereas defects in apoptosis may promote drug resistance. Because we found that *p53* mutations in HNSCC cells are generally associated with increased cisplatin sensitivity, we are investigating how *p53*-dependent pathways may confer cisplatin resistance in cells with wild-type *p53*.

Overexpression of the antiapoptotic proteins, Bcl-2 and Bcl-x<sub>L</sub>, has been shown to inhibit chemotherapy- and radiation-induced apoptosis in both hematologic and solid tumors (20, 21). In fact, exogenous coexpression of wild-type *p53* and Bcl-x<sub>L</sub> protects cells from *p53*-mediated apoptosis, whereas wild-type *p53* expression alone causes rapid cell death in breast cancer cell lines (21). Down-modulation of Bcl-x<sub>L</sub> by antisense oligonucleotide causes *p53*-dependent apoptosis induced by staurosporine treatment or serum starvation in hepatocellular carcinomas (22). We recently showed that Bcl-x<sub>L</sub> is overexpressed in 74% and Bcl-2 is overexpressed in 15% of laryngeal tumors. We also found a strong trend for a better response to chemotherapy and larynx preservation in those tumors with low Bcl-x<sub>L</sub> expression (23). Bcl-x<sub>L</sub> and/or Bcl-2 proteins are also frequently overexpressed in HNSCC cell lines. Thus, we hypothesize that *p53* gene status and expression of Bcl-2/-x<sub>L</sub> play a role in determining cisplatin response in HNSCC. Furthermore, we postulate that cisplatin resistance may be overcome by the inhibition of antiapoptotic function.

In the present study, we use an *in vitro* model to investigate cisplatin resistance in HNSCC. We used two parental HNSCC cell lines and two cisplatin-resistant progeny cell lines that were selected by cultivation in gradually increasing concentrations of cisplatin. Both the parental and cisplatin-resistant progeny cell lines were

characterized for *p53* expression and genotype. We examine cisplatin sensitivity and resistance with respect to Bcl-x<sub>L</sub> and Bcl-2 protein expression. Finally, we evaluated the efficacy of a novel BH3 mimetic compound, (–)-gossypol, to target cisplatin-resistant cells in this *in vitro* model.

## Materials and Methods

### Reagents

Cisplatin (Sigma, St. Louis, MO) was prepared in 0.9% sodium chloride solution at a stock concentration of 1 mg/mL. (–)-Gossypol was synthesized starting with racemic gossypol as previously described (24). (–)-Gossypol was dissolved in DMSO at a stock concentration of 30 mmol/L.

### Cell Culture

Human HNSCC cell lines were established at the University of Michigan (UM-SCC; ref. 25). UM-SCC-5 is from a primary tumor of the supraglottis, UM-SCC-10A is from a tumor of the true vocal cord (primary site), and UM-SCC-10B is from a local recurrence that developed 1 year later in the same patient. Informed consent was obtained from all patients for the use and development of these cell lines. The two parental cell lines, UM-SCC-5 and UM-SCC-10B, were subsequently stably selected for cisplatin resistance by growth in progressively increasing concentrations of cisplatin from 20 nmol/L to 1 μmol/L over 15 passages by one of us (G. Los; ref. 26). The cisplatin-resistant sublines were given the designation UM-SCC-5PT and UM-SCC-10BPT, respectively, and were compared with the parental UM-SCC-5 and UM-SCC-10B cell lines. Cell lines were grown in complete DMEM containing 2 mmol/L L-glutamine, 1% nonessential amino acids, 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. To maintain the resistant phenotype, the cisplatin-resistant cell lines were cultured in the presence of 0.9 μmol/L cisplatin. All cell lines were tested for *Mycoplasma*, using the MycoAlert Detection kit (Cambrex, Rockland, ME) every 3 to 6 weeks.

### Cell Growth Assays

UM-SCC cell lines were seeded at  $1 \times 10^6$  cells per flask. Cells were collected, washed, stained with trypan blue (0.4%; Invitrogen), and viable (trypan blue negative) cells were counted using a hemacytometer following each time point. The natural log of the cell count was plotted versus time (hours). The doubling time ( $t_d$ ) of each cell line was calculated as the slope ( $\lambda$ ) of the linear regression line as determined by SigmaPlot Software, where  $t_d = 0.693 / \lambda$ .

### *p53* Gene Mutation Analysis

The status of the *p53* gene in UM-SCC parental and cisplatin-resistant cell lines was analyzed by single-strand conformational polymorphism analysis and confirmed by DNA sequencing as previously described (15). To confirm suspected mutations identified by single-strand conformational polymorphism, direct DNA sequencing was used. Cell lines were washed with PBS and genomic DNA was extracted using the Wizard Genomic DNA Purification kit

(Promega, Madison, WI) per the manufacturer's instructions. Single-strand conformational polymorphism analysis identified abnormally migrating bands in exon 5 of UM-SCC-5 and in exon 7 of UM-SCC-10B (10). No abnormal bands were found in UM-SCC-5PT and UM-SCC-10BPT. Exon 5 of *p53* with DNA from UM-SCC-5 and UM-SCC-5PT was amplified using the following primers: exon 5F-GTACTCCCTGCCCTCAACA, exon 5R-CTCACCA-TCGCTATCTGAGCA. Exon 7 of *p53* was amplified for UM-SCC-10B and UM-SCC-10BPT using the following primers: exon 7F-TAGGTTGGCTCTGACTGTACC, exon 7R-TGACCTGGAGTCTTCCAGTGT. PCR products were purified using the Wizard SVGEL and PCR Cleanup System (Promega). Purified PCR products were sequenced in the University of Michigan Core on an ABI 3700 (Applied Biosystems). *p53* sequences and mutations were confirmed using the National Center for Biotechnology Information website.

#### Immunohistochemistry

Cells were grown in four-well chamber slides in complete DMEM and fixed by 20-minute incubation in 4% paraformaldehyde at room temperature. Cells were rinsed in PBS (2 × 5 minutes). Slides were dehydrated using increasing concentrations of ethanol (70%, 95% and 100%; 3 minutes each), air dried, and stored at -80°C. Slides were rehydrated with decreasing grades of ethanol followed by hydration in PBS. Antigen retrieval was done using antigen retrieval buffer (DAKO, Carpinteria, CA) for 20 minutes at 90°C. Slides were allowed to cool for 20 minutes at room temperature, rinsed in PBS, and incubated with peroxidase block (DAKO) for 5 minutes at room temperature. Nonspecific binding sites were blocked with 1.5% horse serum (Vector Labs, Burlingame, CA) in PBS for 30 minutes. Cells were incubated with primary mouse monoclonal p53 antibody (Clone D01, Lab Vision, Fremont, CA) at 1:100 dilution in blocking buffer for 1 hour at room temperature. After incubation, cells were washed with PBS and incubated with biotinylated anti-mouse IgG (ABC kit, Vector Labs) for 30 minutes. Slides were washed and incubated with avidin/biotin-conjugated peroxidase for 30 minutes at room temperature. Cells were washed, developed with diaminobenzidine tetrahydrochloride (Sigma), counterstained with hematoxylin, dehydrated, mounted, and visualized. Affinity-purified mouse IgG2a (Sigma) was used as a negative control.

#### Chemoresensitivity [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay

Logarithmically growing cells were plated at 5,000 to 10,000 cells per well in five replicate wells in 96-well plates and allowed to attach and grow in complete DMEM at 37°C overnight. The next day, cisplatin (0, 0.5, 1, 5, 10, and 25 μmol/L) or (-)-gossypol (0, 1, 5, 10, and 25 μmol/L) was added to five replicate wells. Cells were incubated with drug or vehicle control in 300 μL of complete DMEM for 5 days, after which the media was removed from the wells and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany)

as previously described (10). Percent absorbance value relative to vehicle control (Y axis) was plotted as a function of drug concentration (X axis). The concentration of drug required for a 50% reduction in absorbance relative to control was taken as the IC<sub>50</sub>. All experiments were done in triplicate.

#### Western Blot Analysis

Exponentially growing (60–90% confluence) cells were washed in PBS and lysed in a PBS buffer containing 1% NP40 (Sigma), 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), and a cocktail of protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Total protein from cell extracts was quantified using the Bradford assay (Bradford Reagent; Bio-Rad, Hercules, CA). For Western blotting, protein (25–50 μg) was electrophoresed on 12% Tris-glycine SDS-polyacrylamide gels under denaturing conditions, and transferred to Hybond-P polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). Membranes were blocked in Tris-buffered saline plus 0.05% Tween containing 5% nonfat dry milk at room temperature for 1 hour followed by incubation for 3 hours with primary antibody; mouse anti-p53 monoclonal antibody, 2 μg/mL (Oncogene, San Diego, CA); mouse anti-Bcl-x<sub>L</sub> monoclonal antibody, 2 μg/mL (Trevigen, Gaithersburg, MD); mouse anti-Bcl-2 monoclonal antibody, 2 μg/mL (Calbiochem, La Jolla, CA); or mouse anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody, 1:10,000 (Chemicon International, Temecula, CA). Membranes were then incubated for 45 minutes with a secondary horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham) and analyzed using the Enhanced Chemiluminescence Plus reagent (Amersham) by exposing membranes to X-ray film (X-Omat, Kodak, Rochester, NY). Protein expression was quantified using densitometry and fold change was assessed using NIH ImageJ software.

#### Apoptosis (Terminal Deoxynucleotidyl Transferase dUTP Nick End – Labeling) Assay

Apoptosis was determined by enzymatic labeling of DNA strand breaks using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay and analyzed by flow cytometry to detect staining of the Alexa-Fluor-488-labeled antibromodeoxyuridine antibody (Molecular Probes, Inc., Eugene, OR). UM-SCC cell lines were plated and treated at 60% to 80% confluence with 10 μmol/L (-)-gossypol, 10 μmol/L cisplatin, or vehicle control for 48 hours, harvested, fixed, and stained according to the manufacturer's protocol (Molecular Probes). Cells were analyzed in the Flow Cytometry Core at the University of Michigan Comprehensive Cancer Center. Apoptotic cells are those that show higher fluorescence intensity above that of the gated untreated control cells. Apoptotic cells are represented as a percent of total number of cells in each population (untreated or treated).

#### Statistical Methods

For each drug-cell line pair, a cubic polynomial was fitted to the relationship between concentration and the log

percentage of viable cells. The fitted models were then evaluated at 50% viable cells to produce point estimates and 95% confidence intervals for  $IC_{50}$  values. Regression  $t$  tests were used to compare the  $IC_{50}$  estimates for two cell lines. The probabilities of apoptosis between cell lines was evaluated through Cochran-Mantel-Haenszel statistics. All statistical analyses are done using SAS v8.2 (SAS, Inc., Cary, NC). A two-tailed  $P$  value  $\leq 0.05$  is considered to be statistically significant.

## Results

### Parental and Cisplatin-Resistant Cell Lines

After selection in cisplatin (as described above), the resistant cell lines UM-SCC-5PT and UM-SCC-10BPT were noted to have similar but slightly altered morphology when compared with the respective parental lines. The cisplatin-resistant cells are predominantly smaller, somewhat more rounded cells with increased nuclear-to-cytoplasmic ratios when compared with the larger, polygonal cells with lower nuclear-to-cytoplasmic ratios in the parental lines (Fig. 1A). The cisplatin-resistant cells also seem to grow more rapidly than parental cells as confirmed by cell growth experiments (Fig. 1B). The parental UM-SCC-5 and UM-SCC-10B cells both grow relatively slowly ( $t_d = 28.4$  and 28.0 hours, respectively), whereas the UM-SCC-5PT ( $t_d = 17.8$  hours) and UM-SCC-10BPT ( $t_d = 22.1$  hours) cells proliferate faster.

### Cisplatin Selects against Mutant $p53$

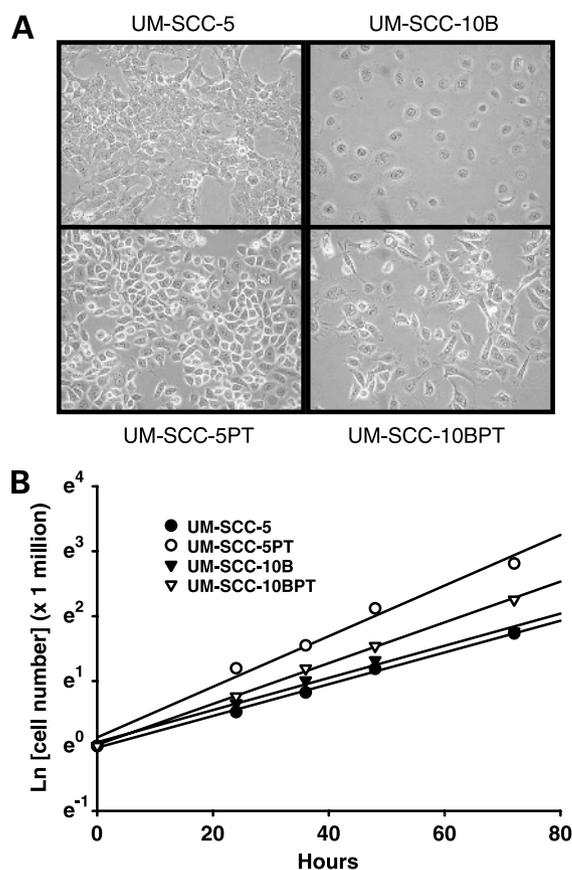
Based on our previous findings, we postulated that  $p53$  status might be a factor in cisplatin sensitivity. The parental cell lines UM-SCC-5 and UM-SCC-10B overexpress  $p53$ , whereas  $p53$  is barely detectable in the cisplatin-resistant (UM-SCC-5PT and UM-SCC-10BPT) cell lines (Fig. 2A).  $p53$  mutations were predicted by single-strand conformational polymorphism analysis of exons 5 and 7 of UM-SCC-5 and UM-SCC-10B, respectively (10), and were confirmed in both parental lines by DNA sequencing (Fig. 2B). Both cisplatin-resistant lines contain only wild-type alleles. The parental UM-SCC-5 cell line contains a valine to phenylalanine, G to T, transversion mutation at codon 157 in exon 5 and UM-SCC-10B has a G to T transversion mutation resulting in a glycine to cysteine change at codon 245 in exon 7 (Fig. 2B). The UM-SCC-5 population contains both mutant and wild-type  $p53$  alleles (minor peak in Fig. 2B), whereas in UM-SCC-5PT, following cisplatin selection, only the wild-type  $p53$  population was detected. In UM-SCC-10B, the population containing wild-type  $p53$  is not detectable in the chromatogram, but following cisplatin selection only wild-type  $p53$  was found in UM-SCC-10BPT (Fig. 2B).

Immunohistochemistry shows strong nuclear  $p53$  staining in the majority of cells in the parental lines UM-SCC-5 and UM-SCC-10B, consistent with a predominantly mutant  $p53$  population (Fig. 2C). A minority of cells without  $p53$  staining are also present in each population. Following cisplatin selection, the cisplatin-resistant lines UM-SCC-5PT and UM-SCC-10BPT show only a small fraction of cells

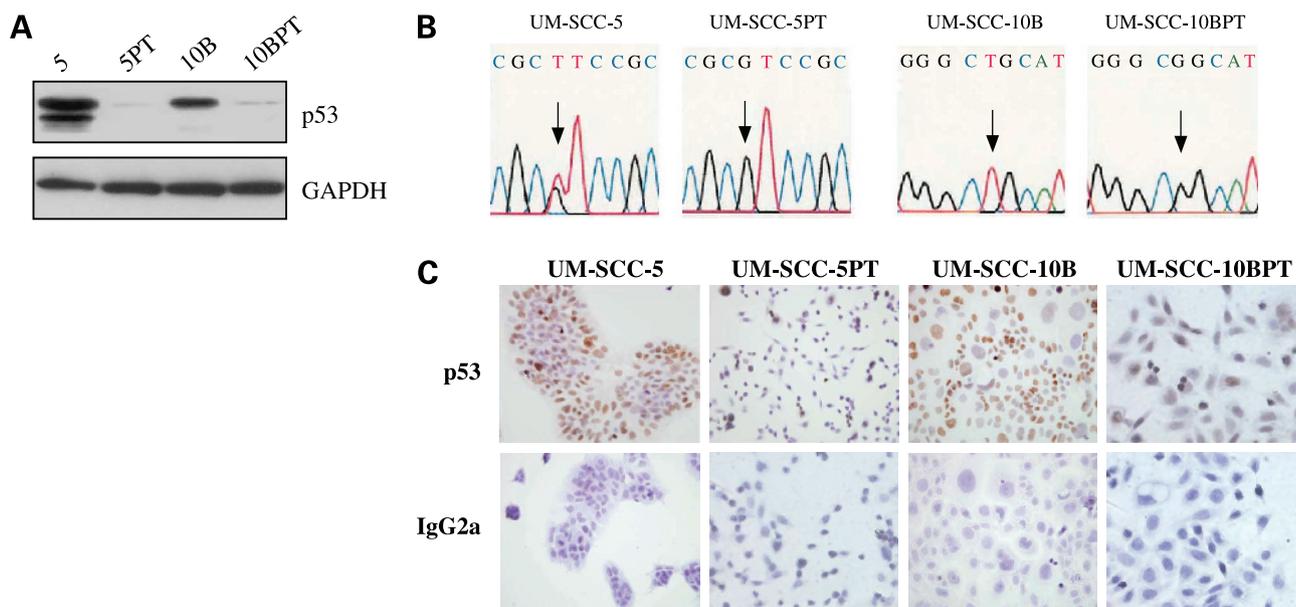
with  $p53$  nuclear staining, which is consistent with a predominantly wild-type  $p53$  population of cells (Fig. 2C). The isotype control, IgG2a, shows no nonspecific staining (Fig. 2C).

### Cisplatin Selects for High Bcl- $x_L$ Expression

Expression of the anti-apoptotic proteins, Bcl-2 and Bcl- $x_L$ , was examined in the parental and cisplatin-selected cell lines. We observed that elevated expression of Bcl- $x_L$ , but not Bcl-2, correlates with cisplatin resistance. As shown in Fig. 3, Bcl- $x_L$  is expressed at a 4.4-fold higher level in UM-SCC-5PT and at a relatively low level in UM-SCC-5. Similarly, although UM-SCC-10B has moderately high levels of Bcl- $x_L$  expression, the expression of Bcl- $x_L$  is further increased 1.5-fold in UM-SCC-10BPT. Thus, Bcl- $x_L$  expression increased in both cisplatin-resistant lines relative to the parental lines. Bcl-2 expression also changed with cisplatin selection, but in the opposite direction. In both cases, the parental lines have 3-fold higher Bcl-2



**Figure 1.** **A**, morphology of parental and cisplatin-resistant UM-SCC cell lines. Parental cell lines UM-SCC-5 and UM-SCC-10B (*top*) and corresponding cisplatin-resistant cell lines UM-SCC-5PT and UM-SCC-10BPT (*bottom*) are shown at  $\times 20$  magnification by phase-contrast microscopy. **B**, cell proliferation in parental and cisplatin-resistant cell lines. Cell counts using trypan blue exclusion to assess viable cells were used to determine the doubling time (proliferation rate) for each cell line. Doubling times were calculated as described in Materials and Methods. Data are from one representative experiment of three that showed the same results.



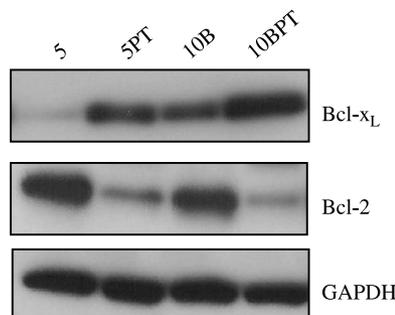
**Figure 2.** **A**, Western blot analysis of p53 expression in parental and cisplatin-resistant cell lines. High p53 expression in the parental lines corresponds to mutant *p53* and low expression in the resistant lines corresponds to wild-type *p53*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) shows equal loading. **B**, DNA sequencing confirms that parental cell line UM-SCC-5 contains a transition mutation at Val<sup>157</sup> of exon 5 and UM-SCC-10B contains a transversion mutation at Gly<sup>245</sup> of exon 7. Cisplatin-selected cell lines UM-SCC-5PT and UM-SCC-10BPT contain only the wild-type *p53* allele in each case. **C**, immunohistochemical staining for p53 (*top*) demonstrating a nuclear staining pattern in a heterogeneous population of cells in the parental lines and low staining in cisplatin-resistant lines. IgG2a isotype control (*bottom*) staining indicates no nonspecific antibody staining. Magnification,  $\times 40$ .

expression than the cisplatin-resistant cells, suggesting that Bcl-2 does not protect against cisplatin and that there may be a reciprocal relationship between Bcl-x<sub>L</sub> and Bcl-2 expression. These observations strongly support the hypothesis that Bcl-x<sub>L</sub> expression protects tumor cells from cisplatin toxicity, but that Bcl-2 does not. The altered expression of these proteins does not seem to be due to a direct inductive effect of cisplatin on Bcl-2 family gene expression because Bcl-x<sub>L</sub> and Bcl-2 expression do not significantly change in these cell lines directly following (i.e., within 72 hours) exposure to cisplatin. Thus, the effect of cisplatin is to select against cells with high Bcl-2 and low Bcl-x<sub>L</sub> and for cells with high Bcl-x<sub>L</sub> and low Bcl-2 expression.

#### Cisplatin-Resistant Cells Are More Sensitive to (-)-Gossypol than the Parental Cisplatin-Sensitive Cells

(-)-Gossypol, an isomer of a natural product found in cottonseed extract, was identified by a computational structure-based search as a compound that binds to the BH3 binding site of Bcl-2 and Bcl-x<sub>L</sub>. *In vitro* assays confirmed high-affinity binding of (-)-gossypol to the BH3 pocket of Bcl-x<sub>L</sub> and Bcl-2 as assessed by the ability of this small molecule to displace fluorescently labeled BH3 peptides (24). We also reported that (-)-gossypol inhibited cell survival of a wide range of HNSCC cell lines in MTT assays and that this agent induced apoptosis in a much higher proportion of cells in HNSCC with wild-type *p53* than in HNSCC cells with mutant *p53* (23). This, together with the observation that cisplatin selects for cells with high Bcl-x<sub>L</sub> expression, suggested that (-)-gossypol might

be an effective agent against cisplatin-resistant cells. We first assessed the sensitivity of the parental and cisplatin-resistant cell lines to cisplatin and (-)-gossypol in MTT cell survival assays (Fig. 4). As expected, the cisplatin-resistant cell lines are significantly more resistant to cisplatin than the parental cell lines (UM-SCC-5 versus UM-SCC-5PT,  $P < 0.0001$ ; UM-SCC-10 versus UM-SCC-10BPT,  $P = 0.0042$ ). UM-SCC-5PT is ~8-fold more resistant to cisplatin than its parental line, UM-SCC-5 (Fig. 4A, *top*). UM-SCC-10B is relatively resistant to



**Figure 3.** Western blot analysis of Bcl-2 and Bcl-x<sub>L</sub> in parental and cisplatin-resistant cell lines. Bcl-x<sub>L</sub> is expressed at higher levels in the cisplatin-resistant cell lines than in the parental lines. Bcl-2 expression is inversely correlated with cisplatin resistance and is higher in parental lines than the cisplatin-resistant cell lines. Glyceraldehyde-3-phosphate dehydrogenase shows equal loading. Western blots were repeated a minimum of thrice. Bands were quantified for fold change using ImageJ software ( $n = 3$ ).

cisplatin; nevertheless, after selection, there was 1.5-fold further increase in cisplatin resistance in UM-SCC-10BPT (Fig. 4A, bottom). All four cell lines exhibit growth inhibition at physiologically achievable (–)-gossypol concentrations (i.e., < 10  $\mu\text{mol/L}$ ; Fig. 4B). The cisplatin-resistant lines are significantly more sensitive to (–)-gossypol than the parental cell lines (estimated  $\text{IC}_{50}$  values: 3.7  $\mu\text{mol/L}$  for UM-SCC-5 versus 1.7  $\mu\text{mol/L}$  for UM-SCC-5PT,  $P = 0.0091$ ; 5.5  $\mu\text{mol/L}$  for UM-SCC-10B versus 1.5  $\mu\text{mol/L}$  for UM-SCC-10BPT,  $P < 0.0001$ ; Fig. 4B). Furthermore, when the response of the cisplatin-resistant lines to cisplatin and (–)-gossypol are compared (dotted lines in each panel), the cisplatin-resistant lines are more sensitive to (–)-gossypol than they are to cisplatin (estimated  $\text{IC}_{50}$  values of 1.0  $\mu\text{mol/L}$  for UM-SCC-5 versus 8.9  $\mu\text{mol/L}$  for UM-SCC-5PT,  $P < 0.0001$ ; 4.2  $\mu\text{mol/L}$  for UM-SCC-10 versus 7.5  $\mu\text{mol/L}$  for UM-SCC-10BPT,  $P = 0.0042$ ; Fig. 4A).

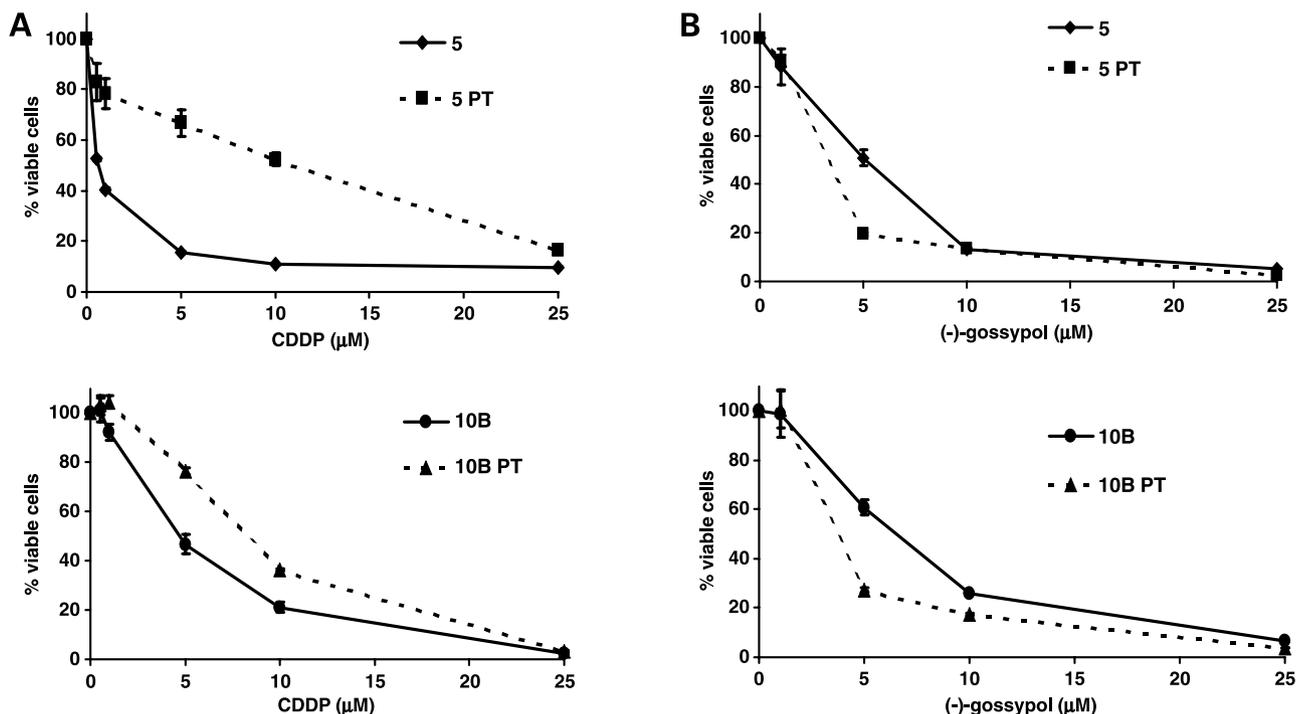
#### (–)-Gossypol Efficiently Induces Apoptosis in Cisplatin-Resistant Cell Lines

The growth inhibition/cell survival (MTT) assays do not indicate a mechanism of action for (–)-gossypol. However, (–)-gossypol is predicted to induce apoptosis by binding to the BH3 binding pocket of Bcl-x<sub>L</sub> and releasing proapoptotic proteins (23). Because, from our data, cisplatin resistance seems to be dependent on Bcl-x<sub>L</sub> and wild-type *p53*, we postulated that the cisplatin-resistant cells would be sensitive to induction of apoptosis by (–)-gossypol. As

shown in Fig. 5, (–)-gossypol induces apoptosis with high efficiency in the cisplatin-resistant cell lines that express high levels of Bcl-x<sub>L</sub>. When compared with the parental cell lines, the difference in (–)-gossypol-induced apoptosis at 48 hours is highly significant (74.5% in UM-SCC-5PT versus 25.8% in UM-SCC-5,  $P < 0.0001$ ; 80.8% in UM-SCC-10BPT versus 42.4% in UM-SCC-10B,  $P < 0.0001$ ; Fig. 5). Thus, the degree of apoptosis induction by (–)-gossypol seems to correlate with both high Bcl-x<sub>L</sub> expression and with the presence of wild-type *p53*. In contrast, when all four cell lines were assessed for cisplatin-induced apoptosis, only UM-SCC-5 exhibited significant apoptosis (45.6%; Fig. 5) and only UM-SCC-5 has very low Bcl-x<sub>L</sub> expression. The other cell lines, UM-SCC-5PT, UM-SCC-10B, and UM-SCC-10BPT, all have high Bcl-x<sub>L</sub> expression and all exhibit low levels of cisplatin induced apoptosis (<10%). This observation is consistent with our hypothesis that Bcl-x<sub>L</sub> protein plays an important role in cisplatin resistance.

#### Discussion

Until recently, the molecular mechanisms of resistance to chemotherapy or radiation therapy in HNSCC were poorly understood. As our knowledge of these mechanisms increases, the ability to predict which HNSCC patients will respond to chemotherapy and those that will fail should allow for better treatment decisions. Thus, identification of markers that can predict response is an area of intense



**Figure 4.** MTT cell survival assays of parental and cisplatin-resistant cell lines. **A**, response to cisplatin.  $\text{IC}_{50}$  values were obtained for each cell line. Fold change in sensitivity was determined by comparing  $\text{IC}_{50}$  values ( $P = 0.0091$ , 5 versus 5PT;  $P < 0.0001$ , 10 versus 10BPT). **B**, response to (–)-gossypol (estimated  $\text{IC}_{50}$  values of 1.5–2  $\mu\text{mol/L}$  versus 3.7–5.5  $\mu\text{mol/L}$ ,  $P = 0.0091$ , 5 versus 5PT;  $P < 0.0001$ , 10 versus 10BPT). Data represent five replicate wells. Experiments were repeated thrice.

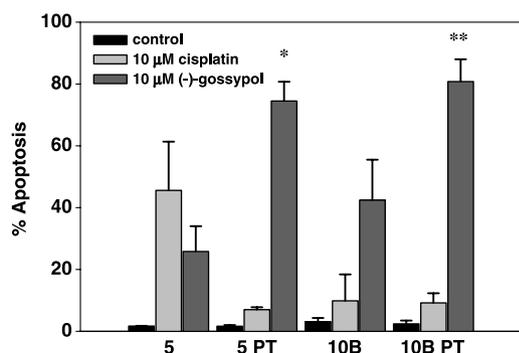
research. We showed previously that p53 overexpression is significantly associated with response to organ-sparing therapy in patients with advanced laryngeal cancer (15). Similarly, we found a strong trend for response to chemotherapy in tumors with low Bcl-x<sub>L</sub> expression (23). Therefore, these markers may also serve as targets for novel treatments to overcome resistance. Here, we attempt to elucidate the mechanisms of cisplatin resistance using an *in vitro* model. Additionally, we show that a BH3 mimetic compound efficiently and rapidly induces apoptosis in the cisplatin-resistant phenotype characterized by overexpression of Bcl-x<sub>L</sub> and wild-type p53.

Several mechanisms have been identified as contributing to cisplatin resistance, including reduced drug accumulation, increased inactivation by thiol-containing molecules, increased DNA repair, loss of DNA mismatch repair, and reduced drug accumulation (27–29). Cisplatin resistance arises in the heterogeneous tumor cell population either by clonal expansion of tumor cells with inherent resistance to cisplatin or by clonal expansion of a small population that acquires a resistance mechanism in the course of treatment. We observed that continuous cisplatin exposure selected for cells within the parental UM-SCC tumor cell populations that have a survival and proliferative advantage in the presence of cisplatin. The resistance to cisplatin may be explained partly by the loss of DNA damage-induced apoptosis and partly by enhanced repair of cisplatin-induced DNA lesions. This is consistent with the observation that the UM-SCC cells that survive *in vitro* cisplatin selection are those with wild-type p53 (Fig. 2) and high Bcl-x<sub>L</sub> expression (Fig. 3), factors that are conducive to cell-cycle arrest, induction of DNA repair mechanisms, and inhibition of apoptosis.

We show in two cell lines that the cisplatin-resistant cells selected by long-term exposure to low levels of cisplatin contain wild-type p53, whereas the predominant cell populations in the corresponding parental cell lines contain mutant p53 (Fig. 2). These results are consistent with our observations in clinical specimens that p53 overexpression

in HNSCC tumors is an independent predictor of successful organ preservation in patients with advanced laryngeal squamous cell carcinoma treated with chemotherapy (cisplatin/5-fluorouracil) followed by radiation (30). This association of wild-type p53 with cisplatin resistance was also shown in a panel of HNSCC cell lines. We found that the majority of HNSCC cell lines with mutations and overexpression of p53 are relatively sensitive to cisplatin, whereas most HNSCC cell lines with wild-type p53 are relatively resistant (10). These results were at first somewhat surprising in that a major mechanism of cisplatin activity is known to involve activation of wild-type p53 and subsequent induction of apoptosis (28). However, of the four cell lines used in this study, the two that contained wild-type p53 were the cisplatin-selected cells and these do not undergo significant apoptosis in response to cisplatin (Fig. 5). These cells also express very high levels of Bcl-x<sub>L</sub> and we postulate that this blocks cisplatin-induced apoptosis. Indeed, we found significant induction of apoptosis by cisplatin only in UM-SCC-5, a cell line with a mixed population of cells, some with wild-type p53, and some with mutant p53. In fact, by immunohistochemistry (Fig. 2C), there is a subpopulation of 20% to 40% of cells in UM-SCC-5 that do not overexpress p53 and presumably contain wild-type p53 (as indicated in the exon 5 chromatogram; Fig. 2B). This cell line expresses very low levels of Bcl-x<sub>L</sub>; thus, we speculate that the subpopulation of cells with wild-type p53 and low Bcl-x<sub>L</sub> in UM-SCC-5 are susceptible to cisplatin-induced apoptosis (Fig. 5). We postulate further that when tumor cells with mutant p53 are treated with cisplatin, they develop cisplatin-DNA adducts but continue to cycle, ultimately leading to errors in DNA replication resulting in genomic instability and cell death. Thus, together these two pathways can contribute to the high sensitivity of UM-SCC-5 to cisplatin. In contrast, although UM-SCC-5PT and UM-SCC-10BPT have wild-type p53, cisplatin is not effective in inducing apoptosis because these cells express high levels of Bcl-x<sub>L</sub>. Thus, it seems that when the apoptotic pathway is blocked by high Bcl-x<sub>L</sub> levels, the tumor cells are able to avoid cisplatin-induced apoptosis. Presumably, the tumor cells with wild-type p53 and high Bcl-x<sub>L</sub> most likely survive the cisplatin-induced DNA damage by activation of other p53 pathways, such as the cell cycle arrest and DNA repair mechanisms.

High expression of Bcl-2 and Bcl-x<sub>L</sub> has been observed in HNSCC and found to contribute to drug resistance. However, in our prior study of tissue specimens, high Bcl-x<sub>L</sub> expression, which is found in the majority of advanced laryngeal tumors, correlated with poor response to chemotherapy and reduced organ preservation, whereas Bcl-2 expression showed no correlation with resistance to chemotherapy and five of seven tumors with high Bcl-2 expression had complete responses to treatment (23). Consistent with these observations, high Bcl-x<sub>L</sub> expression was observed in the cisplatin-resistant lines, whereas high Bcl-2 expression was found in the parental, cisplatin-sensitive cell lines. The significance of the overexpression of Bcl-x<sub>L</sub> and its relationship to Bcl-2 expression in HNSCC,



**Figure 5.** TUNEL assay of parental and cisplatin-resistant cell lines following 48-h treatment with vehicle, cisplatin, or (-)-gossypol. The level of TUNEL-positive cells is determined as a percent of total cells above the gated untreated control cells. Data represents three independent experiments. Significant difference between cisplatin-resistant and parental cell lines, (\*, \*\*  $P < 0.0001$ ).

as well as how it contributes to cisplatin resistance, warrants further investigation in a larger panel of tumor types and cell lines. Antiapoptotic proteins are promising targets for novel therapeutics in cancer (31, 32) because of their common role in promoting cancer cell survival. We and others have shown that (–)-gossypol, a naturally occurring polyphenolic compound, binds with high affinity to the BH3 binding site of Bcl-2 and Bcl-x<sub>L</sub> allowing for the induction of apoptosis (24, 32–34). More recently, Oliver et al. (35) has shown that (–)-gossypol acts directly on the mitochondria to induce cytochrome *c* release, caspase activation, and induction of apoptosis in Jurkat cells and to a lesser extent in Jurkat cells that overexpress Bcl-2 or Bcl-x<sub>L</sub>. We previously showed that (–)-gossypol effectively inhibits the growth of HNSCC cells, most of which express Bcl-x<sub>L</sub> and or Bcl-2, as well as the proapoptotic protein Bcl-x<sub>S</sub> (24). In that study, we noted that (–)-gossypol was an efficient inducer of apoptosis, but that the degree of apoptosis was considerably higher in tumor cells with wild-type *p53* and high levels of Bcl-x<sub>L</sub>, suggesting a *p53*-dependent mechanism of apoptosis. We had previously shown that many of the cell lines with wild-type *p53* were more resistant to cisplatin than those with mutant *p53* (10). In the current study, we show that tumor (cisplatin-resistant) cells that survive cisplatin selection *in vitro* are those with both wild-type *p53* and high Bcl-x<sub>L</sub> expression. We also show that these cisplatin-resistant cells are more sensitive to (–)-gossypol-induced apoptosis than are their parental lines of origin (Fig. 5), suggesting that these cells depend on Bcl-x<sub>L</sub> for survival. However, it should be noted that despite similar dose-response curves to (–)-gossypol in 6-day cell survival assays (Fig. 4B), there is a highly significant difference in induction of apoptosis shown in the 48-hour TUNEL assay (Fig. 5) between parental and cisplatin-resistant cell lines. Curiously, although (–)-gossypol has similar binding characteristics for Bcl-2 and Bcl-x<sub>L</sub>, the high expression of Bcl-2 and relatively lower levels of Bcl-x<sub>L</sub> in parental cell lines led to only moderate sensitivity to (–)-gossypol-induced apoptosis (Figs. 4 and 5). This suggests that (–)-gossypol may have other nonapoptotic, *p53*-independent mechanisms of growth inhibition or cell kill. Indeed, several groups have reported that racemic (+/–) gossypol has other mechanisms of action, including protein kinase C inhibition (36), blockade of cellular metabolism (37), modulation of Rb and cyclin D1 in cell cycle regulation (38), induction of transforming growth factor-β (39, 40), and other mitochondrial toxicities including production of reactive oxygen species (41–43). These other modes of action may contribute to the antiproliferative and cytotoxic effects observed in HNSCC cells with low Bcl-x<sub>L</sub> and mutant *p53*. Nonetheless, the very high efficiency of apoptosis induction by (–)-gossypol in cells resistant to cisplatin (cisplatin-resistant) suggests that this may be a useful agent in combination with cisplatin in the clinic to prevent or overcome cisplatin resistance. Whereas no BH3 mimetic compounds have been reported

in clinical trials, Bcl-2 antisense oligonucleotides (G3139), targeted to overcome Bcl-2 overexpression, have shown significant promise in phase I trials in patients with both advanced-stage solid and hematologic tumors (44–47).

Our new *in vitro* data support our clinical findings that indicate both *p53* gene status and Bcl-x<sub>L</sub> contribute to cisplatin resistance in HNSCC. Because cisplatin is a mainstay of HNSCC chemotherapy, finding agents that kill resistant tumor cells is an important goal for improving tumor response and patient survival. We postulate that a better understanding of the mechanisms of cisplatin resistance in HNSCC using both *in vitro* cell lines and *in vivo* xenograft mouse models should help to identify new targets to overcome resistance. We believe that one promising area is the use of BH3 mimetic compounds [e.g., (–)-gossypol] that target survival pathways linked to cisplatin resistance. The findings established with these four cell lines are also true of other HNSCC lines that have not been selected for cisplatin resistance but have mutant or wild-type *p53* and either high or low Bcl-x<sub>L</sub> expression.<sup>7</sup> Testing BH3 mimetic compounds *in vivo* for safety and efficacy will be crucial for their approval for use in clinic. It will be important to develop effective combination strategies for use of cisplatin-based therapy in concert with BH3 mimetics. Our preliminary *in vitro* results suggest that sequential use will be more effective than simultaneous administration. In addition, modified BH3 mimetics with improved stability are becoming available; however, to our knowledge, none are yet being used in a clinical setting. We expect these compounds to be used in conjunction with conventional chemotherapy to overcome resistance, improve organ preservation rates, and, most important, increase survival for advanced head and neck cancer patients.

<sup>7</sup> Unpublished observations.

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## Reversal of cisplatin resistance with a BH3 mimetic, (–)-gossypol, in head and neck cancer cells: role of wild-type p53 and Bcl-x<sub>L</sub>

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