In vitro Effects of the BH3 Mimetic, (−)-Gossypol, on Head and Neck Squamous Cell Carcinoma Cells

Christopher L. Oliver,1 Joshua A. Bauer,1 Keith G. Wolter,1 Mathew L. Ubell,1 Ajita Narayan,1 Kathleen M. O’Connell,1 Susan G. Fisher,2 Shaomeng Wang,3 Xihan Wu,3 Min Ji,3 Thomas E. Carey,1 and Carol R. Bradford1

1Department of Otolaryngology–Head and Neck Surgery, University of Michigan, Ann Arbor, Michigan; 2Department of Community and Preventive Medicine, University of Rochester, Rochester, New York; and 3Departments of Internal Medicine and Medicinal Chemistry, University of Michigan Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan

ABSTRACT

Purpose: Bcl-xL overexpression is common in head and neck squamous cell carcinomas (HNSCC) and correlates with resistance to chemotherapy. Thus, a nonpeptidic, cell-permeable small molecule that mimics the BH3 domain of proapoptotic proteins may inhibit Bcl-xL function and have therapeutic potential for HNSCC by overcoming drug-resistance. (−)-Gossypol, the levorotatory isomer of a natural product isolated from cottonseeds and roots, was recently discovered to bind to the BH3 binding groove of Bcl-xL and Bcl-2.

Experimental Design: We investigated the in vitro effects of (−)-gossypol on HNSCC cell lines as well as on fibroblast and keratinocyte cultures by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell survival assays and assessed the results with respect to Bcl-2 family protein expression.

Results: We observed dose-dependent growth inhibition of 10 HNSCC cell lines at biologically achievable doses (2.5–10 μmol/L). (−)-Gossypol doses required to inhibit the growth of human fibroblast cell lines by 50% were 2- to 10-fold higher than for HNSCC cell lines. To inhibit human oral keratinocyte growth by 50%, (−)-gossypol concentrations were 2- to 3-fold higher than for HNSCC cell lines.

Conclusions: There is a direct correlation between Bcl-xL-to-Bcl-xS ratios and sensitivity to (−)-gossypol. This agent induced apoptosis in a much higher proportion of cells with wild-type p53. Importantly, cell lines resistant to cisplatin were very sensitive to (−)-gossypol. These results demonstrate that (−)-gossypol has potent antitumor activity in HNSCC in vitro. This agent may be developed as a novel therapeutic agent for HNSCC, either alone or in combination with existing chemotherapeutic agents.

INTRODUCTION

An important research goal in head and neck oncology is to optimize tumor control while preserving function and maximizing quality of life. There are 40,000 new cases of head and neck squamous cell carcinoma (HNSCC) diagnosed in the United States each year, and dismal 5-year survival rates are reported for advanced disease (1). The treatment of locally advanced head and neck cancer with chemotherapy and radiation allows organ preservation, but it is limited by the emergence of resistant cancer cells. It is necessary to understand mechanisms of drug- and radioresistance to develop more effective treatments.

Abnormalities of the programmed cell death pathway play a critical role in cancer progression and outcome after chemoradiation therapy. Bcl-2 and Bcl-xL are homologous members of the Bcl-2 family of proteins that function as suppressors of mitochondrial-mediated apoptosis. A previous study from our group showed that 74% of laryngeal tumors expressed high levels of Bcl-xL and 15% overexpressed Bcl-2 (2). Overexpression of Bcl-2 or Bcl-xL has been shown by several groups to confer resistance to various therapeutic agents, presumably by their ability to inhibit chemotherapy-induced apoptosis (3–5). Thus, we hypothesized that overexpression of Bcl-2 and/or Bcl-xL plays a role in the resistance of HNSCC to chemotherapeutic agents, and that inhibition of antiapoptotic function should be a promising strategy for treatment.

Bcl-2 and Bcl-xL proteins have been previously targeted for therapeutic strategies. Song and Grandis (6) have shown that targeting Stat3 leads to down-modulation of Bcl-xL expression and increased apoptosis in head and neck tumors and is a novel strategy to explore. A Bcl-2 antisense oligonucleotide, designed
to inhibit Bcl-2 expression, is currently in advanced clinical trials for the treatment of several forms of cancer (7). Single-chain anti-Bcl-2 antibodies induce apoptosis in cancer cells that express high levels of Bcl-2 (8). Cell permeable peptides, designed to mimic the Bcl-2 homology 3 (BH3) domain of Bad, bind to Bcl-2 and induce apoptosis in Bcl-2-overexpressing cancer cells (3, 9). However, certain limitations are associated with the delivery of antisense strategies, and poor oral availability, and/or poor in vivo stability and high cost are associated with large molecule and peptide approaches. A tumor-specific small-molecule inhibitor that can be administered orally, displays appropriate distribution kinetics, and has antitumor activity at biologically achievable plasma levels would be ideal to exploit the Bcl-2/xL cell-survival pathway.

It is hypothesized that small molecules with BH3-binding pocket affinity would function as Bcl-2/xL antagonists by blocking the heterodimerization of Bcl-2 or Bcl-xL with proapoptotic proteins such as Bax, Bad, and Bcl-xL (10). Although it is difficult to identify small molecules that block protein–protein interactions, there have been several reports of nonpeptide small-molecular inhibitors that bind the BH3 binding pocket of Bcl-2/xL, inhibit antiapoptotic function and induce apoptosis in cells with high Bcl-2/xL expression (11–13). One of us (S. Wang) developed a computerized screening program to identify molecules that bind to the BH3 binding pocket of Bcl-2 family proteins (BH3 mimetics; ref. 14). Such molecules could block the antiapoptotic function of these prosurvival proteins. Candidate compounds detected by the virtual docking software are tested in competitive fluorescence-polarization–based binding assays for the ability to displace BH3 peptides from purified Bcl-2 or Bcl-xL proteins. One such candidate is gossypol, a natural product isolated from cottonseeds and roots that has been studied as an anticancer agent since the 1980s in both in vitro and in vivo models. The racemic form of gossypol [(±)-gossypol] was tested in several clinical trials and was well tolerated. It demonstrated a low but measurable response in patients who had failed conventional therapy (15–18). Dr. Wang and his colleagues (University of Michigan) determined that the racemic form gossypol [(±)-gossypol] binds to Bcl-xL protein with a Kd of 0.5 to 0.6 μmol/L (19). They further determined that (±)-gossypol also potently binds to Bcl-2 protein with a Kd value of 0.2–0.3 mmol/L. The binding of (±)-gossypol has been independently confirmed (20). The natural racemic gossypol has two enantiomers, namely the (−)-gossypol and (+)-gossypol enantiomers. Dr. Wang’s laboratory has determined that although (−)-gossypol and (+)-gossypol binds to Bcl-xL with similar binding affinities, (−)-gossypol is more potent than (+)-gossypol in inhibition of cell growth and induction of apoptosis, possibly due to the influence of serum in the cell culture experiments.

Because the majority of HNSCC express high levels of Bcl-xL, we hypothesized that this protein is an important survival mechanism in this cancer type, and, therefore, (−)-gossypol should be an effective agent in this tumor type, possibly through the inhibition of Bcl-xL activity. In this study, we report the in vitro growth-inhibitory activity of (−)-gossypol in a panel of HNSCC cell lines, and the relationship between the expression of a Bcl-2 family of proteins and the anticancer activity of (−)-gossypol in vitro. We demonstrate greater activity of (−)-gossypol against HNSCC cell lines than against normal fibroblast and oral keratinocyte cultures. We further demonstrate the ability of (−)-gossypol to induce apoptosis in tumor cells and its relationship to p53 status.

MATERIALS AND METHODS

Cell Lines. Ten squamous cell carcinoma cell lines established at the University of Michigan (UM-SCC) and three human fibroblast lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. UM-SCC cell lines originated from the larynx (n = 2; UM-SCC-12, -23), oral cavity/oropharynx (n = 5; UM-SCC-1, -6, -14A, -74B, -81B), hypopharynx (n = 1; UM-SCC-22A), and metastases from laryngeal cancers (n = 2; UM-SCC-17B, -25). Human fibroblast cells originated from surgical specimens (fibroblast cells 2 and 3) and neonatal foreskin samples (fibroblast cells 1). For the keratinocyte experiments, human oral keratinocytes were cultured from surgical specimens and grown in keratinocyte growth media (KGM, Cambrex, East Rutherford, NJ). After passing the cells three times, a uniform population of undifferentiated keratinocytes was achieved, and was used for subsequent experiments. Informed consent was obtained from all patients and the University of Michigan Institutional Review Board reviewed and approved the study.

Resolution of (−)-Gossypol. Racemic gossypol acetic acid purchased from Aldrich Chemicals was dissolved in diethyl ether and washed twice with water to remove the acid, and then the ether layer was concentrated by rotary evaporation. To a solution of 1.04 g of gossypol (2 mmol) in 50 mL of CH3Cl2, was added 1 g of 4-phenylalanine methyl ester hydrochloride (4.6 mmol), 0.4 g of NaHCO3 (4.7 mmol), 3 g of 4Å molecular sieves, and 1 mL of 2-propanol; the resulting mixture was stirred at room temperature overnight under N2 in the dark and was filtered. The 4-phenylalanine methyl ester reacted with the aldehyde groups of gossypol to form a Schiff’s base with two diastereoisomers, which were then resolved on a normal silica flash chromatography column. The filtrate was concentrated, and the residue was purified by chromatography on silica gel eluting with hexanes and EtOAc (3:1) to give two fractions. Acid hydrolysis of the two fractions in +5N HCl and tetrahydrofuran (THF; 1:5; room temperature, overnight) regenerated the individual gossypol enantiomers, respectively. The first fraction with a higher Rf value contained (−)-gossypol, and the second fraction with a lower Rf value contained (+)-gossypol. The crude gossypol fractions were extracted into ether from the residue after removing THF from the reaction mixture. The gossypol fractions were then purified by chromatography on silica gel and were eluted with hexanes and EtOAc (3:1) to give optically pure gossypol, with a yield of 30 to 40% in two steps. The optical rotatory dispersion values for our products were αT = −352° (c = 0.65, CHCl3) for (−)-gossypol, and αT = +341° (c = 0.53, CHCl3), in excellent agreement with literature values (19).

Chemosensitivity Assay (MTT Method). Logarithmically growing cell lines were cultured, washed, counted, and plated at 5,000 to 15,000 cells per well in duplicate wells of 96-well plates and were incubated overnight. The following day, serial dilutions were made from stock solutions of racemic,
(+)-, or (−)-gossypol to achieve the desired concentrations. All of the experimental conditions were done with 5 replicates. The sample plates were incubated for 6 days in 300 μL of DMEM containing gossypol or solvent controls, except for the keratinocyte experiments, in which a 1:1 ratio of DMEM to KGM was used. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were then performed according to the manufacturer’s protocol (Roche Diagnostics, Mannheim, Germany). The MTT assay measures cell survival based on mitochondrial conversion of MTT from a soluble tetrazolium salt into an insoluble colored formazan precipitate, which is dissolved in dimethyl sulfoxide and quantitated by spectrophotometry (21). Percent absorbance relative to control was plotted as a linear function of drug concentration. The 50% inhibitory concentrations (IC_{50}) were identified as the concentration of drug required to achieve 50% growth inhibition relative to untreated control populations.

Western Blot Analysis. Proteins were harvested during log phase growth by lysing cells in the flask with a solution of PBS (BioWhittaker, Walkersville, MD) containing 1 mL NP40 (Sigma; St. Louis, MO), 1 mM phenylmethylsulfonyl fluoride (Sigma) and 1 tablet of a mixture of protease inhibitors (Boehringer Mannheim, Germany) per 100 mL of PBS. The protein extracts were quantified with a colorimetric assay (Bradford Reagent, Bio-Rad, Hercules, CA). Thirty micrograms of protein extracts were solved in dimethyl sulfoxide and quantitated by spectrophotometry (21). Percent absorbance relative to control was plotted as a linear function of drug concentration. The 50% inhibitory concentrations (IC_{50}) were identified as the concentration of drug required to achieve 50% growth inhibition relative to untreated control populations.

Cell Survival Assay. UM-SCC-1, -6, -12, and –14A were treated with 10 μmol/L (−)-gossypol for 48 h, trypsinized, washed, stained with 0.4% trypsin blue, and counted with a hemocytometer. trypan blue-excluding (viable) cells are reported as a percentage relative to untreated controls.

Apoptosis Assays (TUNEL Method). Apoptosis of UM-SCC cell lines after (−)-gossypol treatment was quantitatively detected by enzymatic labeling of DNA strand breaks with terminal deoxynucleotidyltransferase (TdT) and Alexa Fluor-BrduU, known as the TdT-UTP nick end-labeling (TUNEL) assay by flow cytometry. Cells were exposed to (−)-gossypol for 48 h, harvested, fixed and TUNEL-stained according to the manufacturer’s protocol (Molecular Probes Inc., Eugene, OR). Ten thousand cells were analyzed per sample with excitation of Alexa Fluor at 488 nm. Apoptotic index (AI) was defined as percentage of apoptotic cells in the treatment population minus that in the vehicle control population.

Statistical Analysis. Univariate analyses were conducted including Student’s t tests for continuous measures between independent groups and Spearman correlation coefficients for continuous independent and dependent variables. Two-sided α levels of less than 0.05 were considered statistically significant.

RESULTS

Growth Inhibition by Racemic, (+)-, and (−)-Gossypol in UM-SCC Cell Lines. The racemic form and each of the enantiomers of gossypol were tested against UM-SCC-6 and UM-SCC-14A in 6-day MTT assays (Fig. 1). (−)-Gossypol exhibited greater growth inhibition relative to (±)-gossypol than (+)-gossypol in both cell lines tested (P < 0.001). An interme-
Immediate growth inhibitory effect was observed with (+)-gossypol but this effect was only observed at the higher dose of gossypol (10 μmol/L, \( P < 0.0001 \)).

\((-\)\)-Gossypol Selectively Inhibits UM-SCC Cell Growth. \((-\)\)-Gossypol caused dose-dependent inhibition of cell growth in ten UM-SCC cell lines over a range from 0.5 to 10 μmol/L in a 6-day MTT assay. The relative sensitivity of the cell lines varied from a very sensitive group with an \( IC_{50} \) of 2–5 μmol/L and a less sensitive group with \( IC_{50} \) clustered around 10 μmol/L. In contrast, fibroblast cultures showed significantly higher resistance with mean \( IC_{50} \) of 20.31 ± 9.20 μmol/L (\( P = 0.0142 \)) compared with the mean \( IC_{50} \) of 5.57 ± 2.57 μmol/L in HNSCC cell lines. To ensure that the difference in sensitivity to \((-\)\)-gossypol was not due to cell type, we also tested human oral keratinocyte cultures. The mean \( IC_{50} \) for the keratinocytes was 15.36 ± 0.28 μmol/L, a statistically significant difference (\( P = 0.001 \); Fig. 2B).

Bcl-2 Protein Family Expression. Protein expression levels of Bcl-2, -xL, and -xS in UM-SCC cell lines were determined by Western blot as shown in Fig. 3A. Bcl-xL is expressed in the majority of UM-SCC cell lines (9 of 10) in this panel, with only UM-SCC-74B lacking detectable expression. Bcl-2 is expressed in UM-SCC-74B, -17B, and -6. All of the cell lines expressed Bcl-xS at some level. Previous work has demonstrated that human keratinocytes express little or no Bcl-2, moderate Bcl-xL, and no Bcl-xS (22).

Bcl-xL to Bcl-xS Protein Expression Ratio and Sensitivity to \((-\)\)-Gossypol. Levels of Bcl-2 family protein expression were compared with the in vitro growth-inhibitory activity of \((-\)\)-gossypol. The relative levels of expression for Bcl-xL and Bcl-xS were evaluated by densitometry, but no statistically significant correlation with sensitivity to \((-\)\)-gossypol was identified (\( r = -0.14, P = 0.70 \) and \( r = 0.20, P = 0.58 \); data not shown). Because both Bcl-xL and –xS expression are common and vary among cell lines, we investigated the possibility that...
the Bcl-xL-to-Bcl-xS ratio was associated with sensitivity to (-)-gossypol. As shown in Fig. 3B, there is an inverse correlation between the Bcl-xL to Bcl-xS expression ratios and the IC\textsubscript{50} of (-)-gossypol (r = -0.83; P = 0.0029). When the fibroblast cultures are excluded from the analysis, the correlation between Bcl-xL/Bcl-xS expression and cell line sensitivity to (-)-gossypol remains statistically significant (r = -0.77; P = 0.016).

Cell Survival After (-)-Gossypol Treatment. To investigate whether the growth-inhibitory effects were due to the inhibition of cell growth or cell kill, we used vital dye exclusion assays to measure the loss of membrane integrity after (-)-gossypol treatment. Four cell lines, UM-SCC-1, -6, -12, and -14A, all exhibited significant trypan blue staining after 48 hours’ exposure to 10 \( \mu \text{mol/L} \) (-)-gossypol, with surviving (trypan blue excluding) fractions of only 20, 9, 14, and 16%, respectively, relative to untreated controls (P = <0.0001; data not shown).

(-)-Gossypol Induces Apoptosis More Efficiently in UM-SCC Cell Lines with Wild-Type p53. The TUNEL assay for apoptosis (Fig. 4) was done on fibroblasts and a subset of four UM-SCC cell lines that span the spectrum of sensitivity to (-)-gossypol (see above). UM-SCC-1, which is very sensitive to (-)-gossypol, displayed the highest AI after (-)-gossypol treatment (AI = 90.1%). In contrast, fibroblast cell lines showed no induction of apoptosis. Of the four tumor cell lines, UM-SCC-1 (IC\textsubscript{50} = 2 \( \mu \text{mol/L} \)) and UM-SCC-6 (IC\textsubscript{50} = 8 \( \mu \text{mol/L} \)) cell lines have wild-type p53, whereas UM-SCC-12 (IC\textsubscript{50} = 4 \( \mu \text{mol/L} \)) and UM-SCC-14A (IC\textsubscript{50} = 11 \( \mu \text{mol/L} \)) contain p53 mutations (23). The two wild-type p53 tumor cell lines displayed a mean AI of 85.2 ± 6.9 × (UM-SCC-1, AI = 90.1%; UM-SCC-6, AI = 80.3%). In contrast, the two cell lines with mutant p53 had a mean AI of 20.7 ± 9.3% (UM-SCC-12, AI = 27.2%; UM-SCC-14A, AI = 14.1%). This difference is statistically significant with a P value of 0.0157.

**DISCUSSION**

In the present study, we show that (-)-gossypol, a natural product that binds to the BH3-binding groove of Bcl-xL and Bcl-2 proteins with fairly high affinity, has potent activity against HNSCC cell lines in vitro at physiologically attainable drug concentrations. Furthermore, it induces apoptosis with high efficiency in HNSCC tumor cells that express functional p53 and that also kills tumor cells with mutant p53 by a different mechanism. However, normal cells are relatively resistant to (-)-gossypol under the same conditions. Thus, (-)-gossypol has potential as a novel agent for HNSCC treatment.

Although there was no correlation between the expression...
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of Bcl-xL and the response to (-)-gossypol, there appeared to be a correlation between (-)-gossypol response and Bcl-xL expression ratios among the cell lines examined. These data suggest, in agreement with others, that the antiapoptotic-to-proapoptotic protein expression ratio may represent a predictive measure of sensitivity to (-)-gossypol (24, 25). It seems that Bcl-xL is an important antiapoptotic factor in HNSCC and that Bcl-xL may be the corresponding positive regulator of cell death in HNSCCs. In fact, we observed increased expression of Bcl-xL after transfection of Bcl-xS in the UM-SCC-6 cell line (data not shown). Similarly, Bcl-xL expression increased in the same cell line after transfection with a Bcl-xL expression vector (data not shown).

In our panel of 10 HNSCC cell lines, only one, UM-SCC-74B, has no detectable expression of Bcl-xL. Interestingly, UM-SCC-74B has intermediate sensitivity to (-)-gossypol with an IC_{50} value of ~4 μmol/L. Because (-)-gossypol also binds to Bcl-2 protein, we have examined the expression of Bcl-2 in this cell line. Indeed, UM-SCC-74B does express Bcl-2 at moderate levels. These results suggest that overexpression of Bcl-2 protein also protects cancer cells from undergoing apoptosis, and that cell lines with low levels of Bcl-xL protein but relatively high levels of Bcl-2 protein may also be sensitive to (-)-gossypol, presumably by the inhibition of the antiapoptotic activity of Bcl-2. All of the tumor cell lines are much more sensitive to (-)-gossypol than are the normal fibroblast cells and the human oral keratinocytes. We speculate that this specificity is related to the absence of a deregulated cell survival pathway in normal cells. Bcl-xL and Bcl-xS are expressed in fibroblasts in low levels, and human keratinocytes express no detectable level of Bcl-xS. Consistent with this expression pattern, (-)-gossypol does not induce either apoptosis or growth inhibition in normal cells until high concentrations are reached. This in vitro observation is consistent with earlier reports of favorable toxicity profiles of racemic gossypol in previous human clinical trials (15–18).

Another novel finding in our study is the observation that p53 status plays an important role in (-)-gossypol-induced apoptosis. Cell lines with wild-type p53 exhibited much more robust induction of apoptosis in response to (-)-gossypol treatment relative to tumor cells with mutant p53. Of note, cells with wild-type p53 were only slightly more sensitive to cell killing by (-)-gossypol. This suggests that cell-killing by (-)-gossypol as determined in the MTT assay is not simply through apoptosis alone, especially in high concentrations. Interestingly, the cell lines with wild-type p53 that we tested for apoptosis also expressed higher levels of Bcl-xL protein than the lines with mutant p53, indicating that both factors may be involved in sensitivity to apoptosis. This is an area that deserves further investigation.

We have previously demonstrated that cisplatin resistance in HNSCC is correlated with the presence of wild-type p53 (23), and the present work also implicates high Bcl-xL expression in in vitro cisplatin resistance (26). Furthermore, studies of specimens from completed clinical trials demonstrate that tumors with high expression of p53, which correlates closely with p53 mutation, are more likely to be controlled by chemoradiation treatment protocols (27). Additionally, low expression of Bcl-xL correlates with response to induction chemotherapy with cisplatin and 5-fluorouracil (2). Thus, our work indicates that a subset of tumors and cell lines with wild-type p53 and high expression of Bcl-xL are highly resistant to cisplatin-based therapeutic regimens. In this investigation, we show that (-)-gossypol displays significant activity against cell lines with wild-type p53 and high levels of Bcl-xL that are unlikely to respond to agents such as cisplatin (e.g., UM-SCC-1 and –6; both have high levels of Bcl-xL expression and wild-type p53 and are resistant to cisplatin). As such, strategies combining cisplatin with (-)-gossypol may be useful to use in preclinical and clinical studies dedicated to optimizing antitumor effect and to overcoming cisplatin resistance.

On the basis of this study, we conclude that (-)-gossypol is an agent with therapeutic potential for the treatment of HNSCCs. Although the precise molecular mechanism of the...
anticancer activity of (−)-gossypol has not been unequivocally established, our data are consistent with the hypothesis that the inhibition of the antiapoptotic activity of Bcl-xL and Bcl-2 is likely one of the major mechanisms of action. Presumably, (−)-gossypol interacts with the BH3-binding groove in Bcl-2 and Bcl-xL in which proapoptotic proteins such as Bad and Bid bind. Our group has preliminary data suggesting that (−)-gossypol is effective in an orthotopic murine model (data not shown). Experiments have been done with UM-SCC-1, a tumor cell line that is very sensitive to (−)-gossypol in vitro, and with UM-SCC-17B, a cell line that shows moderate sensitivity in vitro. In these xenografts, (−)-gossypol inhibits tumor cell growth very effectively at both 5 and 15 mg/kg (data not shown). Additional investigations on the specific mechanisms of action of (−)-gossypol and the most effective combination strategies with cisplatin are areas of active investigation in our laboratory.

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