TW-37, a small-molecule inhibitor of Bcl-2, mediates S-phase cell cycle arrest and suppresses head and neck tumor angiogenesis

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Abstract
Members of the Bcl-2 family play a major role in the pathobiology of head and neck cancer. We have shown that Bcl-2 orchestrates a cross talk between tumor cells and endothelial cells that have a direct effect on the progression of head and neck squamous cell carcinoma (HNSCC). Notably, Bcl-2 is significantly up-regulated in the tumor-associated endothelial cells compared with the endothelial cells of normal oral mucosa in patients with HNSCC. Here, we evaluated the effect of TW-37, a small-molecule inhibitor of Bcl-2, on the cell cycle and survival of endothelial cells and HNSCC and on the progression of xenografted tumors. TW-37 has an IC50 of 1.1 μmol/L for primary human endothelial cells and averaged 0.3 μmol/L for head and neck cancer cells (OSCC3, UM-SCC-1, and UM-SCC-74A). The combination of TW-37 and cisplatin showed enhanced cytotoxic effects for endothelial cells and HNSCC in vitro, compared with single drug treatment. Notably, whereas cisplatin led to an expected G2-M cell cycle arrest, TW-37 mediated an S-phase cell cycle arrest in endothelial cells and in HNSCC. In vivo, TW-37 inhibited tumor angiogenesis and induced tumor apoptosis without significant systemic toxicities. Combination of TW-37 and cisplatin enhanced the time to tumor failure (i.e., 4-fold increase in tumor volume), compared with either drug given separately. Collectively, these data reveal that therapeutic inhibition of Bcl-2 function with TW-37 is sufficient to arrest endothelial cells and HNSCC in the S phase of the cell cycle and to inhibit head and neck tumor angiogenesis. [Mol Cancer Ther 2009;8(4):893–903]

Introduction
The long-term prognosis of patients with advanced head and neck squamous cell carcinoma (HNSCC) has shown modest improvement over the last three decades (1, 2). The treatment of choice for these patients depends on the stage and the site of the tumor, but in general it consists of a combination of surgery, chemotherapy, and radiation therapy (3). Cisplatin is the most commonly used conventional chemotherapeutic drug for the treatment of locally advanced head and neck cancer (4, 5). The contribution of chemotherapeutic agents in the clinical outcome of patients with advanced HNSCC is becoming increasingly well understood. Studies have shown that chemotherapy improves larynx preservation rates when combined with radiation (6–9). Intensification of combination chemotherapy regimens with taxanes, platinum-based compounds, and 5-fluorouracil has shown improvement of survival of HNSCC patients (10–15). These results suggest that the combination of drugs might yield better results than single drug therapies. However, these combination regimens have increased normal tissue toxicities shown by weight loss requiring feeding tube placement, failure to complete the treatment course, and even deaths due to therapy. Combination therapies involving cisplatin and molecularly targeted agents, particularly inhibitors of epidermal growth factor signaling, have been used to reduce the toxicity of combined regimens described above but have also shown modest results (16). Considering the critical role of Bcl-2 family proteins in the pathobiology of squamous cell carcinomas (17), the therapeutic inhibition of Bcl-2 function might improve the survival of patients with head and neck cancer.

Bcl-2 family proteins are key regulators of cell survival (18). Interestingly, whereas germline Bcl-2 knockout is lethal (19), conditional knockout mice seem to be healthy and have normal survival upon Bcl-2 down-regulation (20). These data show that Bcl-2 is required during development, but does not seem to play a critical role in the homeostasis of adult tissues. Together, these studies may explain the lack of significant systemic toxicities observed when Bcl-2 is inhibited systemically with a small-molecule inhibitor (21).
Prosurvival proteins, such as Bcl-xL and Bcl-2, are upregulated in many cancers and contribute to resistance to therapy (18, 22). The use of adjuvant agents that target antiapoptotic proteins in HNSCC may overcome chemotherapeutic resistance. Notably, (-)-gossypol was shown to decrease cisplatin resistance in head and neck cancer cells (23–25). TW-37 belongs to a novel class of targeted drugs that has been developed by structure-based design (26). TW-37 binds to the Bcl-2 homology domain 3 (BH3) binding groove of Bcl-2 and competes with proapoptotic proteins (such as Bid, Bim, and Bad), preventing their heterodimerization with Bcl-2 and therefore allowing these proteins to induce apoptosis (26). TW-37 binds to Bcl-2 with a $K_i$ of 290 nmol/L (26, 27). In addition, TW-37 also binds to Bcl-xL and Mcl-1 with a $K_i$ of 1,110 and 260 nmol/L, respectively (26, 27). This small molecule has shown antitumor effects in lymphoma and pancreatic cancer models as monotherapy (27, 28). In addition, we have shown that inhibition of Bcl-2 function with subapoptotic concentrations of TW-37 are sufficient to induce a significant decrease the angiogenic phenotype of endothelial cells in vitro (21). Here, we performed experiments to test the hypothesis that TW-37 inhibits head and neck tumor angiogenesis and slows down tumor progression.

**Materials and Methods**

**Cell Culture**

Primary human dermal microvascular endothelial cells (HDMEC; Lonza) were cultured in endothelial cell growth medium (EGM2-MV; Lonza). Oral squamous cell carcinoma-3 (OSCC3; gift from M. Lingen, University of Chicago, Chicago, IL) and UM-SCC-1, UM-SCC-74A (gift from T. Carey, University of Michigan, Ann Arbor, MI) were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 200 mmol/L L-glutamine, 125 units/mL penicillin, and 125 μg/mL streptomycin in a humidified CO₂ incubator at 37°C.

**Cytotoxicity Assays**

Sulforhodamine B (SRB) cytotoxicity assays were done as described (21). Briefly, optimal cell density for cytotoxicity assays was determined by growth curve analysis. HDMEC were seeded at $2 \times 10^5$ cells per well of 96-well plates and allowed to adhere overnight. Drug or vehicle control was diluted in EGM2-MV and used to treat cells for 72 or 96 h. Cells were fixed onto the plates by addition of 10% cold trichloroacetic acid (final concentration) for 1 h at 4°C. Cellular protein was stained by addition of 0.4% SRB (Sigma) in 1% acetic acid and incubation at room temperature for 30 min. Unbound SRB was removed by washing with 1% acetic acid and plates were air dried. Bound SRB was resolubilized in 10 mmol/L unbuffered Tris base and absorbance was determined on a microplate reader at 560 nm (Genios Tecan). Test results were normalized against initial plating density and drug-free controls. Data were obtained from triplicate wells per condition and representative of at least three independent experiments.

**Western Blot Analysis**

HDMEC, UM-SCC-1, or OSCC3 were exposed to 0 to 2.2 μmol/L TW-37 and/or 0 to 4 μmol/L cisplatin. Whole-cell lysates were resolved by electrophoresis and membranes were probed overnight at 4°C with a 1:1,000 dilution of hamster anti-human Bcl-2 monoclonal antibody (BD Biosciences) for 1 h at room temperature. Alternatively, HDMEC were exposed to 0 to 10 μmol/L TW-37 for 24 h. Whole-cell lysates were resolved by electrophoresis and membranes were probed overnight at 4°C with a 1:1,000 dilution of rabbit anti-human Chk1 and phosphor-Chk1 (Cell Signaling), rabbit anti-human cyclin D1, or mouse anti-human CDK4 antibody (Santa Cruz Biotechnology). Then, membranes were exposed to appropriate peroxidase-coupled secondary antibodies and proteins were visualized with enhanced chemiluminescence (Amersham).

**Flow Cytometry**

Cells were seeded at $5 \times 10^4$ per well in a six-well plate and allowed to adhere overnight. Medium was aspirated, and drug or controls were diluted in EGM2-MV medium and added to the cells. Cells were incubated for 72 h and assessed for apoptosis by hypotonic lysis and staining of DNA with propidium iodide, as described (29). Apoptotic levels were determined by flow cytometry and cell cycle analysis of sub-G₀-G₁ fractions. Data were obtained from triplicate wells per condition and are representative of at least three independent experiments.

**Severe Combined Immunodeficient Mouse Model of Human Tumor Angiogenesis**

Xenograft human tumors vascularized with human blood vessels were generated, as described (30, 31). Briefly, highly porous poly-L (lactic) acid scaffolds were prepared and seeded with $9 \times 10^5$ HDMEC plus $1 \times 10^5$ OSCC3 cells. Male 5- to 7-wk-old severe combined immunodeficient (SCID) mice (CB.17.SCID; Taconic) were anesthetized with ketamine and xylazine, and two scaffolds were implanted in the subcutaneous space of the dorsal region of each mouse. Eighteen days after implantation, mice were randomized into four groups (seven mice per group) and adjusted to equalize the mean tumor volume in each group. For tumor progression studies, mice were injected i.p. with either two doses of 5 mg/kg cisplatin (5 d apart); or with 15 mg/kg TW-37 for 10 consecutive days, or with the combination of the two drug regimens. Vehicle alone (i.e., PBS/Tween 80/ethanol) was injected i.p. for 10 consecutive days in the control group. Tumor volume was calculated using the following formula: volume ($mm^3) = L \times W^2 / 2$ ($L$, length, mm; $W$, width, mm). At the termination of the experiment, mice were euthanized and tumors were retrieved and fixed overnight in 10% buffered formalin at 4°C. Kaplan-Meier curves were generated using as criteria for failure the time when tumor volume reached a 4-fold increase compared with pre-treatment volume. Alternatively, for tumor angiogenesis studies, mice either received two doses of 5 mg/kg cisplatin 5 d apart or 15 mg/kg TW-37 for 6 consecutive days or combination treatment for 6 d. The following day, mice were euthanized and tumors were harvested, fixed, and processed for standard immunohistochemistry. Histologic sections...
were incubated in antigen retrieval solution (Dako) for 30 min at 90°C, followed by incubation with polyclonal anti-human factor VIII antibody (1:500 dilution; Lab Vision) overnight at 4°C, as previously described (32). The number of microvessels in six random fields per scaffold was counted in eight scaffolds per experimental condition under a light microscope at ×200 magnification. The care and treatment of experimental animals was in accordance with University of Michigan institutional guidelines. At least three independent experiments were done to verify reproducibility of results.

**In situ Terminal Deoxyribonucleotide Transferase-Mediated Nick-End Labeling**

Tissues were permeabilized by incubation with 0.1% Triton X-100, 0.1% sodium citrate solution for 8 min. Subsequently, tissues were incubated with terminal deoxyribonucleotide transferase and fluorescein-dUTP (In Situ Cell Death Detection Kit Fluorescein; Roche), according to manufacturer's instructions. The number of terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL)-positive cells was quantified under fluorescence microscopy (Leica DM 5,000B) with the Image J software (NIH). Confocal images were done using a Zeiss 510 META laser scanning confocal microscope. Laser excitation was 364 for 4',6-diamidino-2-phenylindole and 488 for FITC. Zeiss software provided the scanned images, which were incorporated into Photoshop CS2 (Adobe) for producing the final configurations presented here.

**Statistical Analyses**

Statistical significance was determined by one-way ANOVA followed by post hoc tests, using the SigmaStat 2.0 software (SPSS). The analysis of the data from the Kaplan-Meier curves was done with the Gehan-Breslow-Wilcoxon test using the GraphPad software (GraphPad Software). The combinatorial index (CI) was calculated by CalcuSyn software (Biosoft).

**Results**

**Comparative Analysis of the Cytotoxicity of TW-37 and Cisplatin in Endothelial Cells and Head and Neck Cancer Cells**

The initial screening of the effect of cisplatin and TW-37 on primary human endothelial cells and several HNSCC cell lines was done using the SRB cytotoxicity assay. Pilot studies showed that seeding 2,000 endothelial cells per well or 2,000 tumor cells per well for 72 hours allows for evaluation of the effect of the drugs while cells were still in linear phase of proliferation (data not shown). The IC50 for TW-37 was 1.1 μmol/L in HDMEC (Fig. 1A) and ~0.3 μmol/L...
in the three head and neck cancer cell lines (i.e., OSCC3, UM-SCC-1, and UM-SCC-74A) evaluated here (Fig. 1B–D). For cisplatin, the IC₅₀ for all cell lines tested here was \( \sim 2.0 \) μmol/L (Fig. 1A–D). Together, these results show that TW-37 is more cytotoxic on an equimolar basis than cisplatin in endothelial cells and head and neck cancer cells in vitro.

**Combination of TW-37 and Cisplatin Showed Enhanced Cytotoxic Effects for Endothelial Cells and Head and Neck Cancer Cells Compared with Single Drug Treatment**

TW-37 was discovered using structure-based database screening for molecules that interacted with Bcl-2 with high affinity and prevented its interaction with proteins of the Bcl-2 family, such as Bax, Bim, Bad, and Bid (26, 27). Therefore, it is not expected that TW-37 would affect Bcl-2 expression levels. However, the effect of the combination of TW-37 and cisplatin on Bcl-2 expression in endothelial cells and head and neck cancer cells is not known. Here, we observed that concentrations of TW-37 and/or cisplatin that inhibit cell growth do not affect the expression of Bcl-2 in the endothelial cells or in the head and neck tumor cells (Supplementary Fig. S1).

For combination therapy studies in endothelial cells, we selected three concentrations of cisplatin (1, 2, and 3 μmol/L) and three concentrations of TW-37 (0.7, 1.1, and 1.5 μmol/L). These concentrations were selected as representatives of the IC₅₀ in endothelial cells for each drug (Fig. 1) plus one concentration above and one below the IC₅₀. Combination treatment with TW-37 at IC₅₀, or at a higher concentration, resulted in higher cytotoxicity to endothelial cells than single drug treatment (Fig. 2A). When cisplatin was combined with 1.1 μmol/L TW-37, the CI was between 0.9 and 1.1, which indicated an additive effect of the drugs. However, when cisplatin was combined with higher concentrations of TW-37 (i.e., 1.5 μmol/L), the CI was less than 0.9, which showed synergy between the two drugs (Fig. 2A).

We also investigated the effect of combination treatment on three head and neck cancer cell lines. Here, the concentrations of TW-37 were lowered to 0.1, 0.3, and 0.5 μmol/L to reflect a range above and below the average IC₅₀ of this drug (i.e., 0.3 μmol/L) for the head and neck cancer cell lines tested here. The concentrations of cisplatin were maintained at 1, 2, and 3 μmol/L (as above) because these values remained within the active range of cisplatin in the head and neck tumor cell lines. Combination treatment had significantly higher cytotoxic effect than exposure to a single drug in the three cancer cell lines evaluated here (Fig. 2B–D). The CI trends for both drugs in these cell

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7 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
lines were similar to the CI for endothelial cells. The CI was below 0.9 when higher concentrations of TW-37 (i.e., 0.5 μmol/L) were used in combination with cisplatin in OSCC3 and UM-SCC-74A cells, indicating synergism between drugs (Fig. 2B–D). In contrast, the CI was between 0.9 and 1.1 at the IC_{50} concentration of TW-37, which shows additive effects of the drug combination. The CI was >1.1 with most conditions using the lowest concentration of TW-37, which shows lack of additive or synergistic effect of the combination.

**Treatment Sequence Has a Significant Impact on the Cytotoxicity of TW-37 and Cisplatin In vitro**

Next, we investigated the impact of treatment sequence on the effect of the combination cisplatin and TW-37. We either initiated treatment simultaneously with both drugs or pretreated with one drug for 24 hours and then completed the experimental period with both drugs together. The concentration of drugs was fixed at the 72-hour IC_{50} for both endothelial cells and head and neck cancer cells. The highest effect of combination treatment was observed when we started treatment with both drugs at the same time (Fig. 3A–D). Notably, pretreatment with either drug essentially eliminated the benefit of combination therapy, when compared with single drug therapy (Fig. 3A–D). Together, these data show the critical impact of the treatment sequence on the cytotoxic effect of these drugs in vitro.

**Combination Treatment Enhances Apoptotic Level of Endothelial Cells and Cancer Cells**

SRB assays are useful for the initial screen of cytotoxic effects of drugs, but they do not allow for the discrimination between the effects of drugs on cell survival versus effects on cell cycle. Therefore, we performed flow cytometric studies with propidium iodide to determine the effects of the drugs in the sub-G_0-G_1 fraction (i.e., apoptotic cells) as well as in the distribution of cells in different phases of cell cycle. We did not observe an increase in the percentage of apoptotic endothelial cells when 1.1 μmol/L TW-37 was given by itself or in combination treatments (Fig. 4A). However, a significant increase in the proportion of apoptotic endothelial cells was observed when 2.2 μmol/L TW-37 (i.e., 2× IC_{50}) was used in combination with cisplatin (Fig. 4A), compared with single drug treatment (cisplatin or TW-37). In contrast, 0.6 μmol/L TW-37 was sufficient to induce a significant increase in the percentage of apoptotic head and neck tumor cells (Fig. 4A). In general, the combination of 0.6 μmol/L TW-37 with cisplatin was sufficient to mediate higher apoptotic indexes compared with single drug treatment with either drug (Fig. 4A).

**Figure 3.** Effect of treatment sequence on the cytotoxicity of TW-37 and cisplatin. A to D, cells were treated either with single drug alone or both drugs simultaneously for 72 or 96 h. Alternatively, cells were pretreated for 24 h with either cisplatin or TW-37. This pretreatment was followed by a 48- or 72-h treatment with both drugs together. *, P < 0.05, significant difference of the experimental condition against all the other conditions in the study. Each panel is representative of at least three independent experiments, done in triplicate wells per condition.
Whereas the effects of cisplatin in the cell cycle are very well known, i.e., it mediates G2 cell cycle arrest (33), the effects of a small-molecule inhibitor of Bcl-2 are unclear. As expected, cisplatin treatment resulted in dose-dependent increase in the proportion of HDMEC and cancer cells in the G2 phase of the cell cycle (Fig. 4B; Supplementary Fig. S2). In contrast, treatment of HDMEC or UM-SCC-1 with 2.2 μmol/L TW-37 alone was associated with an

Figure 4. Effect of cisplatin and TW-37 on apoptosis and cell cycle of endothelial or head and neck tumor cells. A, cells were exposed to cisplatin and/or TW-37. After 72 h, cells were stained with propidium iodide and subjected to flow cytometry for the analysis of the proportion of apoptotic cells (sub-G1-G0). *, P < 0.05, the combination regimen is significantly higher than any of the single-drug (cisplatin or TW-37) regimen by itself. B, cells were treated as in A, stained with propidium iodide, and the percentage of cells in each cell cycle phase was determined by flow cytometry. Representative of at least three independent experiments done in triplicate wells per condition.
increase in the proportion of cells in the S phase of the cell cycle (Fig. 4B; Supplementary Fig. S2). Interestingly, when cisplatin was combined with lower concentrations of TW-37, it resulted in an increase in the proportion of endothelial cells in the G2 phase (Fig. 4B; Supplementary Fig. S2). This is consistent with a dominant effect of cisplatin on the cell cycle. However, when cisplatin was combined with higher TW-37 concentrations, the combination resulted in a marked increase in endothelial cells and tumor cell in the S phase of the cell cycle (Fig. 4B; Supplementary Fig. S2). Because TW-37 alone or in combination with cisplatin caused markedly lower cell numbers (Figs. 1 and 2), these data show that TW-37 is causing an S-phase cell cycle arrest in endothelial and head and neck tumor cells. Notably, it is well known that phosphorylation of Chk1 triggers a signaling cascade that results in proteolysis of CDC25A, which in turn inhibits the replication machinery causing S-phase cell cycle arrest (34). Here, we observed that TW-37-induced S-phase cell cycle arrest correlates with increase in Chk1 phosphorylation and a decrease in cyclin D1 and CDK4 expression in endothelial cells (Supplementary Fig. S3).^7

**Combination with TW-37 Potentiates the Antitumor Effect of Cisplatin**

We have previously shown that xenografted human tumors vascularized with human functional microvessels can be engineered in SCID mice (30, 31). Using this approach, we investigated the effect of cisplatin and TW-37 on tumor progression and tumor angiogenesis. We implanted HDMEC along with human oral squamous cell carcinoma (OSCC3) in SCID mice and observed the development of tumors (Fig. 5A). At the end of the

![Graph](image-url)

**Figure 5.** Effect of cisplatin and/or TW-37 on time to tumor failure. Each SCID mouse was implanted with two scaffolds seeded with 1.0 x 10^5 OSCC-3 and 9.0 x 10^5 HDMEC. Eighteen days after implantation, mice were randomly assigned to four groups (n = 14 tumors per experimental group) as follows: 5 mg/kg cisplatin on day 0 and day 5 via i.p. injection; 15 mg/kg TW-37 daily for 10 d via i.p. injection; combination of the regimens above for cisplatin and TW-37 for 10 d; or vehicle-injected controls. A, tumor progression curve, starting on day 0 and finishing when the average tumor volume reached 1,000 mm^3. Columns, mean; bars, SE. B, tumor volume 4 d after the beginning of the treatment. *, P < 0.05, significant difference of one experimental condition against all the other conditions. C, mouse weight during treatment. Data were normalized against initial mice weight. D, Kaplan-Meier end point analysis using a 4-fold increase of tumor volume compared with pretreatment volume was used as the criterion for tumor failure. In the graph, survival depicts the percentage of mice bearing tumors that did not reach the 4-fold increase in tumor volume. *, P < 0.05, significant difference against all the other experimental conditions, using the Gehan-Breslow-Wilcoxon test.
The average volume of the nontreated group mice increased by 5.7-fold (Fig. 5A). Four days after the beginning of the treatment, the combination of TW-37 and cisplatin already showed an inhibition of tumor growth compared with the other conditions (Fig. 5B). A significant loss of weight was observed in the groups treated with cisplatin or both drugs (Fig. 5C). The degree of weight loss was more pronounced in the drug combination group than in the cisplatin monotherapy group, and mice treated with both drugs required more time to return to the original weight after completion of treatment (Fig. 5C). Minimal weight loss was observed with the regimen of TW-37 alone. Mice treated with TW-37 recovered weight quickly and surpassed baseline weight 9 to 10 days after completion of treatment (Fig. 5C). A Kaplan-Meier analysis was done using as the criteria for failure a 4-fold increase in tumor volume compared with pretreatment tumor volume (Fig. 5D). The average times to failure were 8.5 days (control group), 17.5 days (cisplatin), 11.5 days (TW-37), and 21 days (combination treatment; Fig. 5D). The combination treatment extended the time to failure of tumor significantly compared with single treatment with either TW-37 or with cisplatin (Fig. 5D).

We have previously shown that TW-37 decreases the angiogenic potential of human endothelial cells in vitro and mediates a significant decrease in microvessel density in vivo (21). To evaluate the effect of the drugs on xenografted head

Figure 6. Effect of cisplatin and/or TW-37 on tumor angiogenesis and apoptosis. Each SCID mouse was implanted with two scaffolds seeded with 1.0 × 10⁶ OSCC3 and 9.0 × 10⁵ HDMEC. Eighteen days after implantation, mice were randomly assigned to four groups (n = 14 tumors per experimental group) as follows: 5 mg/kg cisplatin on day 0 and day 5 via i.p. injection; 15 mg/kg TW-37 daily for 5 d via i.p. injection; combination of the regimens above for cisplatin and TW-37 for 5 d; or vehicle-injected controls. Mice were euthanized on day 6, i.e., 1 d after the end of treatment. A and B, tissue sections were stained for Factor VIII (red) and counterstained with hematoxylin. Factor VIII-positive vessels were counted under light microscopy at ×200 magnification. C and D, tissue sections were stained with in situ TUNEL and with 4′,6-diamidino-2-phenylindole (DAPI). Images were prepared at ×200 magnification and TUNEL-positive cells were counted using the Image J software. Statistical significance (P < 0.05) is depicted by lowercase letters, as follows: (a) blood vessel density is significantly lower in cisplatin- or TW-37–treated tumors than in control tumors; (b) blood vessel density is significantly lower in combination treatment than in any other condition; (c) number of apoptotic cells is significantly higher in cisplatin- or TW-37–treated tumors than in control tumors; (d) number of apoptotic cells is significantly higher in combination treatment than in any other condition.
and neck tumor angiogenesis, we shortened the duration of treatment to 5 days and euthanized the mice the day after completion of treatment. Here, we observed a modest (but statistically significant) decrease in tumor microvessel density when TW-37 alone is compared with vehicle-treated controls (Fig. 6A). Combination treatment further inhibited tumor microvessel density, and a significant decrease of blood vessel numbers was observed when compared with the single drug therapies evaluated here (Fig. 6B).

We next analyzed the effect of combination treatment on apoptosis in the xenografted tumors by in situ TUNEL. TUNEL-positive cells were only counted in regions of intact tumor in such a way that the central core necrosis typically observed in xenografts did not interfere with quantification of apoptotic cells. Tumor samples from animals treated with TW-37 or cisplatin alone showed higher TUNEL-positive cells compared with untreated group (Fig. 6D). Notably, combination of the drugs caused ∼3.5-fold increase in the number of apoptotic cells compared with untreated controls, and 1.5- to 2-fold increase when compared with single drug therapy (Fig. 6D).

Discussion
Organ preservation approaches incorporating chemotherapy minimize the need for radical surgery and tend to improve the quality of life of patients with head and neck cancer (35). However, current drugs have shown relatively modest improvements in the survival of patients with head and neck cancer. Bcl-2 is up-regulated in poorly differentiated head and neck carcinomas, and its expression correlates with positive nodal status (36, 37). A closely related member of the Bcl-2 family, Bcl-xL, is up-regulated in laryngeal cancer and is associated with poor response to chemotherapy and radiation (17). We have shown that Bcl-2 gene expression is ∼60,000-fold higher in the endothelial cells lining tumor blood vessels, compared with the endothelial cells of adjacent normal oral mucosa in patients with head and neck tumors (38). Notably, Bcl-2 down-regulation in tumor-associated endothelial cells by gene silencing is sufficient to inhibit the growth of xenografted head and neck tumors (38). Therefore, Bcl-2 seems to be a compelling target for treatment of patients with head and neck cancer.

TW-37 has an antitumor effect on lymphoma and pancreatic tumor models (27, 28). We hypothesize that the antitumor action of TW-37 is due to a combination of a proapoptotic effect on the tumor cells, as well as a specific antiangiogenic effect. This hypothesis is based on the following observations made by our research group: (a) Bcl-2 initiates a proangiogenic signaling pathway that is mediated by nuclear factor-κB transcriptional activity and result in up-regulated expression of the proangiogenic chemokines CXCL1 and CXCL8 in endothelial cells (39); (b) subapoptotic concentrations of TW-37 inhibited the angiogenic potential of endothelial cells in vitro (21); and (c) subapoptotic concentrations of both the BH3 mimetics (−)-gossypol (BL193) and TW-37 inhibit the expression of the proangiogenic chemokines CXCL1 and CXCL8 in endothelial cells (21, 39). Notably, we have recently shown that Bcl-2 functions as the orchestrator of a cross talk between neovascular endothelial cells and tumor cells, which has a direct effect on head and neck tumor progression (38). Indeed, inhibition of Bcl-2 function in endothelial cells by gene silencing was sufficient to inhibit tumor cell proliferation in cocultures in vitro, as well as to slow down tumor progression in vivo (38). These observations provided the rationale for the current investigation where we designed a detailed study of the effect of TW-37 alone or in combination with cisplatin in both endothelial cells and head and neck tumor cells.

The use of multiple drugs with different mechanisms of action may increase the efficacy of the therapeutic effect, minimizing or slowing down the development of drug resistance and providing selective synergism against target versus host (40). We chose cisplatin for combination studies with TW-37 because this drug is widely used in the treatment of head and neck cancer (5, 41) and because it has clearly a different mechanism of action. Cisplatin causes DNA damage by making platinum-DNA adducts, which leads to cell cycle arrest, inhibition of transcription, and initiation of the apoptotic cascade (33). The effects of cisplatin are expected to be primarily in highly proliferative cells, such as tumor cells (4). The putative functions of TW-37 in a combined therapy with cisplatin are as follows: (a) TW-37 may sensitize the tumor cells to cisplatin by blocking the function of a critical prosurvival pathway; (b) TW-37 will have an antiangiogenic effect by inducing apoptosis of endothelial cells and by inhibiting the secretion of proangiogenic chemokines by resistant endothelial cells; and (c) TW-37 will block endothelial cell-initiated cross talk with tumor cells that lead to enhanced tumor progression. Here, we used cisplatin at maximum tolerated dose for the mice in this study, as shown by a decrease in ∼15% in weight by the end of treatment. In contrast, we used a suboptimal dose of TW-37 for the in vivo studies, i.e., 15 mg/kg TW-37 daily. The maximum tolerated dose for this drug was determined to be 40 mg/kg daily (27). Nevertheless, combination of TW-37 and cisplatin at maximum tolerated dose was significantly more efficient in slowing down tumor progression when compared with single drug treatment with cisplatin. Likewise, combination treatment resulted in a significant decrease in tumor microvessel density and increase in the tumor apoptotic index when compared with treatment with cisplatin alone. Together, these results suggest that TW-37 may sensitize xenografted head and neck tumors to cisplatin.

We observed enhanced cytotoxic effects of the two drugs in endothelial cells when cells were exposed to higher concentrations of TW-37. In parallel experiments, we observed that the efficacy of the treatment with TW-37 or cisplatin presented an inverse relation with cell density, i.e., more cells correlated with lower efficacy of the drugs (data not shown). These results suggest that combination treatment might have a predominant effect in the highly proliferative endothelial cells of tumor neovessels, while sparing the more mature endothelial cells of physiologic vessels.
Indeed, here we observed that whereas there was a significant decrease in tumor microvessel density in mice treated with TW-37 and cisplatin, these animals did not show signs of overt toxicity.

Before the in vivo experiments, we performed a detailed study of the effect treatment sequence in the overall response to combination of TW-37 and cisplatin. Others have shown that treatment schedule may have a profound effect on the antitumor effect of drugs. For example, pretreatment with paclitaxel before coadministration of paclitaxel and A-385358 (inhibitor of Bcl-xL) potentiated the activity of combination treatment (42). Here, we observed that pretreatment with TW-37 or with cisplatin abrogated the beneficial effect of combination treatment. Indeed, there was no observable advantage of the combination therapy when pretreatment with one of the drugs was done, compared with the use of a single drug. These results were somewhat unexpected. However, the trends observed here were highly reproducible in four independent experiments. We are currently performing experiments to evaluate in more depth possible mechanistic explanations for these results. Nevertheless, these results guided our decision to start both drugs at the same time in our in vivo studies.

Interestingly, TW-37 in the low to mid nanomolar range markedly reduced head and neck tumor cell density in vitro without an equivalent increase in cell apoptosis. This apparent conundrum was resolved, in part, when we performed cell cycle analysis. TW-37 treatment is accompanied by a marked accumulation of cells in the S phase of the cell cycle. This was distinctively different than the effect of cisplatin, which resulted in the accumulation of cells in the G2 phase, as expected. Indeed, combination treatment showed a preponderant effect of TW-37 over cisplatin in tumor cells, because accumulation of cells in the S phase was observed in most experimental conditions involving both drugs. Others have shown that inhibition of the signal transducer and activator of transcription 3 signaling pathway lead to S-phase cell cycle arrest by inhibiting signal transducer and activator of transcription 3 transcriptional activity (38). Therefore, we hypothesize that the therapeutic blockade of Bcl-2 function with TW-37 leads to an S-phase cell cycle arrest by inhibiting signal transducer and activator of transcription 3 transcriptional activity. These results suggest a novel function for Bcl-2 in the regulation of cell cycle and explain the marked reduction in cell numbers observed here with subapoptotic concentrations of TW-37.

This study showed that TW-37, a small-molecule inhibitor of Bcl-2, is a potent inhibitor of endothelial cell and head and neck tumor cell growth in vitro. In vivo, single therapy with daily administration of 15 mg/kg TW-37 showed modest antitumor effects. These results were somewhat expected, considering that the dosage used here was significantly below the maximum tolerated dose for single-agent TW-37 that was determined to be 120 mg/kg given in three divided daily dosages of 40 mg/kg per injection i.v. (27). Notably, combination of TW-37 and cisplatin suppressed xenografted head and neck tumor angiogenesis and tumor progression. The small-molecule inhibitors of Bcl-2 are emerging as a new class of molecularly targeted drugs that have both a direct antitumor cell cytotoxic effect and an antiangiogenic effect (44). The evidence presented here suggests that therapeutic inhibition of Bcl-2 with a small-molecule inhibitor such as TW-37 might benefit patients with HNSCC.

Disclosure of Potential Conflicts of Interest

S. Wang: commercial research grant, ownership interest, and consultant, Ascenta Therapeutics. No other potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

TW-37, a small-molecule inhibitor of Bcl-2, mediates S-phase cell cycle arrest and suppresses head and neck tumor angiogenesis

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