Metronomic Dosing of BH3 Mimetic Small Molecule Yields Robust Antiangiogenic and Antitumor Effects

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Abstract

Bcl-2 is an antiapoptotic protein that has also been found to function as a proangiogenic signaling molecule. Improvements in antiangiogenic therapy can be engendered by metronomic dosing. Thus, we hypothesized that BH3-mimetic drugs that antagonize Bcl-2 family proteins may exert a greater efficacy when dosed metronomically. To examine this hypothesis, we employed AT101, an orally available and well-tolerated BH3-mimetic drug that has been established as effective. In a mouse xenograft model of human squamous cell carcinomas (SCC) that includes a humanized vasculature, we explored the effects of docetaxel in combination with either daily (metronomic) or weekly (bolus) doses of AT101. In addition, we explored the effect of single or combination therapy on angiogenesis and survival of endothelial or SCC cells in vitro. Metronomic AT101 therapy increased mouse survival, decreased tumor mitotic index, and decreased tumor microvessel density, compared with bolus therapy. Therapeutic potentiation was achieved by similar overall drug exposure and without altering systemic toxicities. Combinations of AT101 and docetaxel produced additive toxicity in both endothelial and SCC tumor cells. Notably, subapoptotic concentrations of AT101 potently inhibited the angiogenic potential of endothelial cells. Taken together, our findings unveil the efficacious benefits that can be achieved by metronomic delivery of BH3-mimetic drugs, in particular suggesting that SCC patients with might benefit from low-dose continuous administration of these drugs. Cancer Res; 72(3); 1–10. ©2011 AACR.

Introduction

Induction chemotherapy with taxanes, platinum-based compounds, and 5-fluorouracil is beneficial for head and neck cancer patients (1, 2), but the prolonged use of chemotherapeutic drugs is limited by their toxicity and by the development of resistance. The combined use of molecularly targeted agents with conventional therapies has been proposed more recently for management of patients with locally advanced head and neck squamous cell carcinomas (HNSCC; ref. 3). These types of combination therapies have shown promising results, but the survival of head and neck cancer patients has not changed dramatically (4). Improvements in the survival of these patients require mechanism-based therapeutic strategies that maximize the antitumor effect of drugs while limiting their toxicities.

Metronomic chemotherapy has been proposed as a mean to potentiate the antitumor effect of chemotherapeutic drugs and to overcome drug resistance (5–7). Several independent groups have shown benefits of the metronomic regimen in preclinical and clinical studies (8–10). Although much has been learned about the use of conventional chemotherapeutic drugs in metronomic regimen over the last 10 years, little is known about this type of regimen with molecularly targeted drugs.

A significant proportion of head and neck tumors express high levels of antiapoptotic Bcl-2 proteins (11, 12). Indeed, high Bcl-2 expression correlates directly with resistance to therapy (13), and is associated with poor clinical outcomes for head and neck cancer patients (14, 15). We have shown that Bcl-2 gene expression is significantly higher (approximately 60,000-fold) in the tumor-associated endothelial cells of patients with HNSCC, as compared with endothelial cells from the normal oral mucosa (16). Collectively, these studies provide strong rationale for the investigation of the antitumor and antiangiogenic effects of drugs targeted to the Bcl-2 pathway.

BH3-mimetic compounds derived from (-)-gossypol, a natural product from cotton plant (17), inhibit the survival function of Bcl-2 family members by stimulating Noxa and Puma (18). These compounds have shown antitumor effect as a single agent or in combination with standard chemoradiotherapy in various tumor models (19–21), and seem to help to overcome resistance to therapy (22–24). We have shown the potent
antiangiogenic effect (25) and antitumor effect of a small molecule inhibitor of Bcl-2 in head and neck cancer models (26). However, we do not understand the impact of the regimen of administration of a BH3-mimetic drug (AT101) on tumor growth and angiogenesis. Here, we observed that a metronomic regimen (i.e., daily administration of low-dose AT101) has more potent antitumor and antiangiogenic effects than weekly administration without compromising the low systemic toxicity profile of the drug.

Material and Methods

Cells and reagents

Primary human dermal microvascular endothelial cells (HDMEC; Lonza) were cultured in endothelial cell growth medium (EGM2-MV; Lonza). HN-SCC cell lines UM-SCC-17B and UM-SCC-74A (gift from T. Carey, University of Michigan) were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 200 mmol/L L-glutamine, penicillin, and streptomycin at 37°C with 5% CO₂. The identity of all tumor cell lines was confirmed by genotyping at the University of Michigan DNA sequencing core facility in May 2010. For in vitro studies, the small molecule inhibitor of Bcl-2 (AT101; ref. 27) and taxotere (TXT; LC laboratories) were dissolved in DMSO. For in vivo studies, AT101 was resuspended in carboxymethyl cellulose and sonicated for 30 minutes, whereas TXT was dissolved in 5% ethanol.

SRB assay

Sulforhodamine B (SRB) cytotoxicity assays were done as described (25). Briefly, cells were seeded at 2 x 10⁵ cells per well of 96-well plates, allowed to attach overnight, and treated with AT101 and/or taxotere for 72 to 96 hours. Cells were fixed with 10% trichloroacetic acid, stained with 0.4% SRB (Sigma-Aldrich) in 1% acetic acid, and plates were read in 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 is fixed with 10% buffered formalin at 4°C, and processed for histology. The histopathology was assessed by a trained pathologist unaware of treatment conditions. The mitotic index (29) was determined by counting mitotic figures in 5 high-power fields per tumor section. Mice were euthanized whenever the tumor volume reached 2,000 mm³ following the University of Michigan Guidelines for Use and Care of Animals. Tumor size was calculated using the formula: volume (mm³) = L x W²/2 (L, length; W, width).

Immunohistochemistry

Paraffin-embedded tissue sections were incubated in antigen retrieval solution (Dako) for 20 minutes at 90°C to 95°C. Tissues were exposed to 1:500 dilution of the anti-human factor VIII antibody (NeoMarkers) overnight at 4°C, as described (28). Blood vessels were counted in 6 random fields per tumor, from 8 tumors per group.

Statistical analysis

Time-to-failure data was analyzed using the Kaplan–Meier method and the log-rank test. Where multiple fields were measured, the average was taken for each mouse. Continuous measurements, such as tumor volume, average mitosis per mm² and average microvessel density were analyzed using nonparametric rank-based Wilcoxon tests and Kruskal–Wallis tests. The growth curve of tumor volume (mm³) was transformed by log₂ to improve linearity assumptions of residuals for longitudinal modeling. We refer to this transformation as log₂ tumor volume and can be interpreted as tumor doubling. A longitudinal mixed model was used to test the difference in growth patterns based on treatment group. Kaplan–Meier analysis, longitudinal analysis, and Wilcoxon tests were carried out using SAS v9.2 software. In vitro statistical analysis was carried out using ANOVA followed by Tukey’s test (SigmaStat 2.0 software; SPSS). The synergism or additivity was calculated using the combinatorial index (CI), a mathematical and quantitative evaluation of a 2-drug pharmacologic interaction (30). Using Calcusyn ver. 2.0 software (Biosoft), CI values were generated over a range of fractional cell kill levels (Fa) from 0.05 to 0.95 (5%–95% cell kill). Accordingly, a synergistic effect is represented by CI < 0.9; an additive effect by 0.9 ≤ CI ≤ 1.1; and absence of combinatorial effect CI > 1.1. Statistical significance was defined as P ≤ 0.05, unless otherwise specified.

Results

Metronomic AT101 enhances the time-to-failure of xenograft head and neck tumors

To investigate the effect of metronomic (low dose, high frequency) versus bolus (high dose, low frequency) administration of a BH3-mimetic drug on tumor growth, we generated
xenografts with humanized vasculature (31, 32). All experimental conditions included low-dose taxotere (5 mg/kg taxotere, once a week) to mimic the most probable clinical scenario for the use of the targeted drug in combination with a conventional chemotherapeutic drug. The maximum-tolerated dose (MTD) for BH3-mimetic was estimated to be around 40 mg/kg for 3 days (20) and for taxotere around 50 mg/kg weekly (33). In pilot experiments, we observed that oral administration of 105 mg/kg AT101 plus 5 mg/kg taxotere weekly caused 15% to 20% weight loss, and was considered to be the MTD for the combination therapies (data not shown). These studies also showed that weekly 70 mg/kg AT101 plus 5 mg/kg taxotere was well tolerated by mice (<15% weight loss).

The criterion to determine failure for the Kaplan–Meier analysis was a 5-fold increase in tumor volume as compared with baseline (i.e., immediately prior to start of treatment). These experiments were carried out with an aggressive squamous cell carcinoma (UM-SCC-17B), and therefore the time-to-failure was as low as 28 days (vehicle control) after transplantation of 100,000 tumor cells/mouse. The average time-to-failure was 30.6 days (vehicle), 33.3 days (taxotere), 37.3 days (weekly AT101+taxotere), and 48.5 days (daily AT101+taxotere). The distribution of time-to-failure was significantly different across the 4 experimental groups ($P < 0.0001$; Fig. 1A). Daily AT101+taxotere mediated a significant delay in time-to-failure as compared with all other groups.

![Graph of survival analysis](image)

**Figure 1.** Metronomic AT101 enhances the time-to-failure of xenograft head and neck tumors. To generate human xenografts vascularized with human vessels, mice received a scaffold seeded with human HNSCC cells (UM-SCC-17B) and human endothelial cells. When tumors reached 200 mm$^3$, mice were randomized into the different treatment regimens, that is, vehicle, taxotere, weekly AT101+taxotere, or daily AT101+taxotere ($n = 8$). Daily administration of low-dose AT101 (10 mg/kg) was designated as “metronomic” regimen, whereas weekly administration of high-dose AT101 (70 mg/kg) was designated as “bolus” regimen. In both cases, AT101 was delivered via oral gavage and treatment was carried out for 3 weeks. In all conditions, 5 mg/kg TXT was administered weekly via intraperitoneal injection. A, Kaplan–Meier analysis using as criterion for failure a 5-fold increase in tumor volume as compared with baseline. $P$ value indicates a significant difference across the 4 groups ($P < 0.0001$). Asterisk (*) depicts a statistical difference between weekly AT101+taxotere and daily AT101+taxotere ($P = 0.0290$). B, graph depicting tumor volume from the first day of treatment through the day in which the first mouse had to be euthanized (tumor volume reached the cutoff size of 2,000 mm$^3$) in each group. C, distribution of Wilcoxon scores for log$_2$ tumor volume at 30 days of treatment. $P$ value indicates a significant difference across the 4 groups ($P = 0.0006$). Different letters depict statistically significant differences based on pairwise comparisons ($P < 0.050$). D, graph depicting average body weight during treatment, normalized against pretreatment weight.
other experimental conditions (Fig. 1A). In pairwise comparisons, survival was prolonged by daily AT101+taxotere treatment as compared with weekly AT101+taxotere ($P = 0.0290$), to taxotere ($P < 0.0001$), or to vehicle control ($P < 0.0001$). Weekly AT101+taxotere did not improve survival when compared with vehicle control ($P = 0.1542$) or with taxotere alone ($P = 0.6535$). We observed an overall delay in tumor growth when taxotere and AT101 were used in combination, particularly with the daily AT101 regimen (Fig. 1B). Figure 1C depicts a detailed analysis of tumor volume after 30 days of treatment, the day when we had to begin to euthanize animals from the untreated control group. Tumor volume was
reduced in the daily AT101+taxotere as compared with the weekly AT101+taxotere group at 30 days, but did not reach statistical significance ($P = 0.0660$). However, starting at day 31 of treatment and continuing thereafter, the tumor volume in mice that received daily AT101+taxotere was consistently lower ($P < 0.050$) than the tumor volume in mice treated with weekly AT101+taxotere (Fig. 1B). Notably, we did not observe deaths attributed to drug toxicity with the regimens used here, and the average weight loss was consistently less than 15% (Fig. 1D). Moreover, no signs of toxicity were observed in vital organs including liver and lung (Supplementary Fig. S1).

**Tumors treated with metronomic AT101 combined with taxotere were less aggressive and less vascularized**

In general, xenografts showed tumor cells with basophile cytoplasm, increased nuclear-to-cytoplasm diameter ratio, and marked cellular pleomorphism (Fig. 2A). In contrast, the metronomic AT101+taxotere group presented a distinct pattern, with a large proportion of clear cells (Fig. 2A). The tumor cells presented clear cytoplasm and smaller nucleus with higher condensation of chromatin. In addition, larger necrotic areas were observed (Fig. 2A). We observed a significant overall difference in mitotic index (Fig. 2B, $P < 0.0001$) and tumor microvessel density (Fig. 2C, $P = 0.0003$). Pairwise analysis revealed that the mitotic index of daily AT101+taxotere was lower than the mitotic index of each one of the other experimental conditions, as follows ($P = 0.0047$, compared with vehicle; $P = 0.0044$, compared with taxotere; and $P = 0.0046$, compared with weekly AT101+taxotere; Fig. 2B). We also observed that the microvessel density in the daily AT101+taxotere group was lower than in the vehicle control ($P = 0.0047$), taxotere only ($P = 0.0046$), and weekly AT101+taxotere ($P = 0.0046$; Fig. 2C). Moreover, qualitative assessment revealed that the daily AT101+taxotere regimen induced more tumor cell apoptosis, as compared with the other experimental conditions (Supplementary Fig. S2).

**In vitro analyses of the cytotoxic effects of AT101 and taxotere**

The IC$_{25}$ for AT101 was 0.80 µmol/L (HDMEC) and ranged from 2.13 to 2.54 µmol/L for head neck tumor cells (UM-SCC-17B, UM-SCC-74A; Fig. 3). The IC$_{25}$ for taxotere was 0.37 nmol/L (HDMEC), and ranged form 0.21 to 0.53 nmol/L for the tumor cells evaluated (Fig. 3). The IC$_{25}$ and IC$_{75}$ of these 2 drugs were also calculated in a separate set of experiments with narrower range of drug concentrations (data not shown).

To investigate whether AT101 sensitizes HDMEC and tumor cells to chemotherapy, we examined the cytotoxic effects of the IC$_{25}$, IC$_{50}$, IC$_{75}$ concentrations for each drug by itself or in combination. Additive effects were observed primarily when higher concentrations of AT101 were combined with taxotere for all cell types examined here (Fig. 4). Interestingly, the overall trends for the CI analysis were similar for endothelial cells and head and neck tumor cells (Fig. 4).

Next, we investigated the effect of treatment sequence on the cytotoxicity of these drugs in HDMEC and tumor cells. For each cell type, we either pretreated cells with one drug for 24 hours, or started treatment together with the 2 drugs. The drug concentration was set at the IC$_{50}$ and 2 time periods were evaluated to factor-in the overall time of treatment on the effects observed. We observed that starting the 2 drugs at the same time provided the highest cytotoxic effect for all cell types evaluated here ($P < 0.01$; Fig. 5). Propidium iodide staining followed by flow cytometry was carried out to observe the effects of different treatment sequence on apoptosis and cell cycle of UM-SCC-74A. The use of both drugs at the same time induced more apoptosis than any other experimental condition tested here ($P < 0.05$; Supplementary Fig. S3A). Cell-cycle analysis showed that drug sequence had no detectable effect on the proportion of UM-SCC-74A cells in S-phase (Supplementary Fig. S3B and S3C).
Effect of AT101 regimen on the angiogenic potential of endothelial cells in 3-D matrices

AT101 [(-) gossypol] has been developed and characterized as a functional inhibitor of Bcl-2 via binding to BH3-binding domain and interference with the interaction of Bcl-2 with other BH3-containing proapoptotic proteins (34, 35). As expected, Bcl-2 expression was not affected by AT101 treatment (Supplementary Fig. S4), confirming previous observations (23). To evaluate the antiangiogenic effect of AT101 in vitro, we created three dimensional (3-D) droplets of endothelial cells and plated them in collagen matrices. Endothelial cells were induced to sprout with VEGF, and $0.1\times IC_{50}$, $IC_{50}$, and $10\times IC_{50}$ AT101 were used. We either administered these dosages at once (bolus), or mimicked the metronomic regimen by dividing them in 4 daily administrations. The highest concentration of AT101 eliminated capillary sprouts by 4 days in both regimens (Fig. 6). For both $0.1\times IC_{50}$ and $IC_{50}$ conditions, the daily administration of the drug led to more potent reduction in capillary sprouting than the administration of the same overall dosage at once ($0.08 \mu mol/L$ daily vs. $0.08 \mu mol/L$ bolus; $P<0.001$, $0.8 \mu mol/L$ daily vs. $0.8 \mu mol/L$ bolus; $P=0.023$; Fig. 6B). Interestingly, the lowest concentration of AT101 used here ($0.08 \mu mol/L$ AT101) induced a potent antiangiogenic effect ($P<0.001$), despite the fact that this concentration is subapoptotic (Fig. 6B). These data corroborated our previous reports that Bcl-2 functions as a proangiogenic signaling molecule (36), in addition to its well-known effects on the regulation of endothelial cell survival (28).

Discussion

Improvements in the survival of head and neck cancer patients will most likely come from the development and characterization of mechanism-based therapies that combine the potency of cytotoxic drugs with the specificity of molecularly targeted agents. Evidence clearly shows the role of Bcl-2
in the poor outcomes of patients with head and neck cancer (37). We have also shown that Bcl-2 plays a dominant role in head and neck tumor angiogenesis and tumor growth (16, 25, 31, 38). Therefore, targeting the Bcl-2 system is appealing because it has 2 complimentary effects mediated by the direct induction of tumor cell apoptosis and the selective disruption of tumor blood vessels (35).

The combination of the BH3-mimetic drug AT101 with taxotere, a microtubule-binding drug, was selected for these experiments. Although taxotere is widely used in many tumor types including head and neck cancer, strong side effects such as myelosuppression have suggested that combination therapies that allow for a reduction of its dosage are highly desirable (1, 39, 40). Likewise, although single-drug therapy with BH3 mimetics partially inhibits tumor growth in some cancer models (41, 42), combination therapies tend to enhance the efficacy of the BH3 mimetics (43, 44).

Notably, it has been recently reported that the combination of BH3 mimetic with taxotere inhibits tumor cell growth and affects expression of several genes involved in drug resistance, drug metabolism, DNA repair, cell cycle, and oncogenes in a prostate tumor cell line (PC3; ref. 22). Here, we observed that the combination of AT101 with taxotere augmented the antitumor cell and antiangiogenic effects of each one of these drugs used individually. Notably, best results were achieved with higher doses of AT101, a drug with minimal systemic cytotoxicities, combined with lower doses of taxotere. Collectively, these data unveil a drug combination that is highly effective in vivo while showing modest systemic cytotoxic effects.

Metronomic therapy of DNA-damaging agents or microtubule inhibitors has shown more efficiency than MTD therapy in randomized phase III clinical trials (45, 46). Metronomic therapy of certain drugs induces sustained suppression of circulating endothelial progenitor cells, whereas MTD schedules seem to allow for rebound vessel repair responses mediated primarily by bone marrow cell recruitment (43). Here, we examined the approach of using the metronomic regimen for the molecularly targeted drug in combination with conventional regimen for chemotherapy drug. Remarkably, combination of metronomic AT101 with taxotere showed potent inhibition of tumor growth, and prolongation of the survival of mice with established tumors (as compared with bolus treatment of AT101 and taxotere) despite the fact that the total amount of drug administered in both conditions was exactly the same. We believe that this difference in response was mediated by the potentiation of both the antiangiogenic and the tumor cell effect of AT101 when used in a metronomic regimen.

It is well known that vessel rebound rapidly occurs during the breaks in treatment (47). We postulate that by keeping constant the antiangiogenic pressure, achieved even when very low concentrations of BH3-mimetic are used (25, 36), one does not allow for the vascular rebound and therefore the overall antitumor growth effect is potentiated. This hypothesis is currently under investigation in our laboratory. In addition, based on our results we speculate that low-dose BH3-mimetic prevents the development of resistance of tumor cells to therapy, by interfering with the prosurvival effect of Bcl-2 family proteins. A closer look at the histopathology of tumors treated with metronomic AT101 combined with taxotere supports this hypothesis. We observed that this treatment regimen results in lower mitotic index as compared with all other treatment groups of this study. Notably, recent studies showed that the mitotic index is a potent indicator of patient survival with melanoma or breast carcinoma (48, 49). Moreover, in the metronomic AT101 group the tumor cells displayed a clear cytoplasm, suggesting glycogen accumulation compatible with chronic hypoxia and glucose deprivation. This might be related to the fact that this treatment regimen resulted in lower microvessel density.

**Figure 5.** Effect of drug sequence on endothelial cells and head and neck tumor cells. A-C, cells were exposed to the IC50 concentration of each drug, and cytotoxicity was determined by the SRB assay. Cells were pretreated either with AT101 or with TXT for 24 hours. Then, cells were exposed to AT101 or TXT for additional 48 or 72 hours. Alternatively, treatment was done with both drugs administered at the same time, or carried out with single drug as controls. Asterisk (\*) depicts statistically significant differences at \( P < 0.010 \). Data were normalized against initial plating density. Experiments were done in triplicate wells per condition. Each graph is representative of 3 independent experiments.
In summary, we showed that metronomic administration of the BH3-mimetic drug AT101 in combination with low-dose taxotere improves the survival of mice-bearing HNSCC xenografts. These results were correlated with lower mitotic index and tumor microvessel density. Notably, we observed that subapoptotic concentrations of AT101 potently inhibited the angiogenic potential of endothelial cells. The fact that AT101 can be administered orally raises the possibility that patients could take low does of this drug in both therapeutic and chemopreventive regimen. Collectively, this work unveils the...

Figure 6. Effect of metronomic versus bolus AT101 on the angiogenic potential of human endothelial cells. The effect of AT101 on the angiogenic potential was evaluated in primary HDMEC plated in 3-D collagen matrices. A, graph depicting the number of capillary sprouts in VEGF-treated HDMEC exposed to AT101 for 4 days. AT101 was used at the following concentrations: 0.1 × IC50 (0.08 μmol/L), IC50 (0.8 μmol/L), 10 × IC50 (8 μmol/L). In the metronomic group, 1/4 of the IC50 was administered daily (from day 0 to 3); whereas in the bolus treatment group, the full dose was administered on day 0. The total amount of drug was the same in the metronomic and bolus treatment group. B, graph depicting the number of sprouts at the end of the experimental period. Different letters depict statistically significant differences at *P* < 0.050. C, representative photomicrographs of capillary sprouts (×150) at the end of the experimental period. Results were normalized against initial number of sprouts. Experiments were done in triplicate wells per condition. Data are the representative of 3 independent experiments.
antitumor potential of metronomic administration of a molecularly targeted drug.

Disclosure of Potential Conflicts of Interest

S. Wang owns stocks, stock options, and serves as a consultant for Ascenta Therapeutics that has licensed technologies related to AT101 from the University of Michigan.

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