Galectin-3 Targeted Therapy with a Small Molecule Inhibitor Activates Apoptosis and Enhances Both Chemosensitivity and Radiosensitivity in Papillary Thyroid Cancer

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Abstract
Although most patients with papillary thyroid cancer (PTC) have favorable outcomes, some have advanced PTC that is refractory to external beam radiation and systemic chemotherapy. Galectin-3 (Gal-3) is a β-galactoside–binding protein with antiapoptotic activity that is consistently overexpressed in PTC. The purpose of this study is to determine if Gal-3 inhibition promotes apoptosis, chemosensitivity, and radiosensitivity in PTC. PTC cell lines (B505-C and TPC-1) and human ex vivo PTC were treated with a highly specific small molecule inhibitor of Gal-3 (Td131_1). Apoptotic activity was determined by flow cytometric analysis as well as caspase-3 and PARP cleavage. The minimum inhibitory concentrations of Td131_1 and doxorubicin were determined, and their combined effects were measured to test for synergistic activity. The effects of Td131_1 on radiosensitivity were determined by a clonogenic assay. Td131_1 promoted apoptosis, improved radiosensitivity, and synergistically enhanced chemosensitivity to doxorubicin in PTC cell lines. In PTC ex vivo, Td131_1 treatment alone induced the cleavage of caspase-3 and PARP. Td131_1 and doxorubicin together activated apoptosis in PTC ex vivo to a greater degree than their combined individual effects. Td131_1 activated apoptosis and had synergistic activity with doxorubicin in PTC. We conclude that Gal-3 targeted therapy is a promising therapeutic strategy for advanced PTC that is refractory to surgery and radioactive iodine therapy.

Introduction
Although most patients with papillary thyroid cancer (PTC) have a favorable prognosis, a subset of patients suffers from recurrent disease that is refractory to surgical resection and radioactive iodine ablation (1). No effective systemic treatment exists for these patients. Although doxorubicin is one of the few agents with activity against PTC, the clinical effect of doxorubicin on PTC remains modest due to a high degree of intrinsic chemoresistance (1). Furthermore, external beam radiation is often used to palliate patients with locally advanced disease (2). However, external beam radiation therapy is not curative and has limited efficacy in PTC (2). Therefore, novel therapies to augment both systemic chemotherapy and radiotherapy are needed for patients with advanced PTC.

Galectin-3 (Gal-3) is a β-galactoside–binding protein that is consistently overexpressed in PTC (3). Gal-3 has potent antiapoptotic effects and promotes chemoresistance to doxorubicin in PTC (3). High Gal-3 expression is associated with unfavorable tumor characteristics in PTC, such as tumor invasion (4). Because the principal mechanism of action of doxorubicin and external beam radiation therapy involves the activation of apoptosis, Gal-3 targeted therapy is a rational strategy to improve the efficacy of both modalities. Because a prior study found that Gal-3 inhibition in vivo resulted in minimal overt toxicity (5), we believe that combination therapy which includes Gal-3 inhibition is a promising strategy for patients with advanced cancers, such as PTC.

We recently reported that the thiodigalactoside diester Td131_1 has a high affinity and specificity for Gal-3 due to the specific interactions of its two ester moieties with Arg144 and Arg186 of Gal-3 (6). Td131_1 preferentially binds and inhibits Gal-3, rather than other members of the galectin family (6). Because of the potent antiapoptotic effects of Gal-3, we hypothesized that Td131_1 would synergistically enhance chemosensitivity and radiosensitivity in PTC. This study is the first to evaluate the efficacy of a small molecule that targets Gal-3 for the treatment of PTC. Furthermore, we are the first to use thyroidectomy specimens ex vivo for preclinical efficacy testing of therapeutic agents designed to benefit patients with PTC.
Results and Discussion

**Td131_1 Activates Apoptosis in PTC In vitro**

Previously, we showed that Gal-3 knockdown activated apoptosis in PTC (3). Because small interfering RNA–based therapeutics are not currently established for thyroid disease, we used a Gal-3 inhibitor (Td131_1) that could be translated immediately into preclinical testing. To determine the effect of Td131_1 on apoptosis, we coincubated PTC cell lines with Td131_1 and found that it increased the percentage of apoptotic cells in a dose-dependent manner (Fig. 1A). Treatment of 8505-C cells with 200 μmol/L of Td131_1 increased the percentage of apoptotic cells from 0.3% to 85.4% (Fig. 1A). Treatment of the less sensitive TPC-1 cell line with 600 μmol/L of Td131_1 increased the percentage of apoptotic cells from 0.2% to 59%.

These observations show that PTC cell lines may vary somewhat in their sensitivity to Td131_1. These cell type–specific differences in Td131_1 response may reflect the variation in the relative abundance of galectins. Of the seven thyroid cancer cell lines that we tested, the endogenous Gal-3 expression level is lowest in human follicular thyroid carcinoma (FTC) cell lines, FTC-236 and FTC-238 (Fig. 1B). In contrast to the PTC cell lines with abundant Gal-3 expression, treatment with Td131_1 showed limited effects on apoptosis in FTC-236 and FTC-238 cell lines (Supplementary Fig. S1).

Therefore, micromolar concentrations of Td131_1 activated apoptosis in PTC cell lines. Consistent with our Gal-3 knockdown results, the antiapoptotic effects of Gal-3 were reversible with Td131_1.

**Td131_1 Activates Apoptosis in PTC Ex vivo**

Standard models used for testing the efficacy of anticancer therapies suffer from many potential limitations. Cell lines are subjected to selective pressures from multiple passages and may not meaningfully reflect the phenotype and genotype of the parental tumor (7). Although primary cell culture–based models may more closely reflect native cancers, they are difficult to establish and they lack the complex stromal elements which are known to influence tumor phenotype (8, 9). Furthermore, in vivo animal models, such as nude mice, do not accurately reflect the specific tumor-host interactions seen in immunocompetent humans (10).

Therefore, to investigate the effects of Td131_1 on human PTC without the extrapolations associated with in vitro models or animal studies, we used a human ex vivo model of freshly harvested PTC. To confirm the presence of ongoing DNA synthesis under ex vivo maintenance conditions, we coincubated the specimens with bromodeoxyuridine (BrdU) at different time points and found that BrdU uptake continued up to 24 hours ex vivo (Fig. 2A).

We next tested if Gal-3 inhibition activated apoptosis in human PTC ex vivo. Coincubation of the freshly harvested tumors with Td131_1 for 16 hours resulted in a substantial increase of activated caspase-3 and PARP cleavage in a dose-dependent fashion (Fig. 2B). Immunohistochemical analysis showed increased cleaved caspase-3 expression in PTC specimens treated with Td131_1 (Fig. 2B). These results indicate that Td131_1 activates apoptosis in human PTC under conditions that more closely resemble the true tumor environment than in vitro studies.
Td131_1 Enhances Doxorubicin Activity in a Synergistic Manner

We next sought to determine if Gal-3 targeted therapy could enhance the effects of systemic chemotherapy. Doxorubicin is one of the few agents with any known activity against PTC. However, doxorubicin is largely ineffective in advanced PTC due to a high degree of intrinsic chemoresistance. The principal mechanism of action of doxorubicin involves DNA damage and p53 activation, with induction of apoptosis (11). A recent study found that p53-induced apoptosis is associated with the repression of Gal-3 (12); we recently showed that Gal-3 knockdown enhances PTC chemosensitivity to doxorubicin (3). Thus, Td131_1 is a rational adjuvant therapy to enhance PTC chemosensitivity to doxorubicin.

For this reason, we tested the effect of Td131_1 on chemosensitivity to doxorubicin in PTC cell lines. Because the greatest degree of apoptosis activation induced by Td131_1 was observed at a concentration of 600 μmol/L (Fig. 1A), we used this concentration in our initial experiments. Administration of 600 μmol/L of Td131_1 decreased the IC_{50} of doxorubicin by 43% and 46% in 8505-C and TPC-1 cells, respectively (Supplementary Fig. S2).

To test if Td131_1 and doxorubicin synergistically activate apoptosis in PTC, we determined the lowest concentration of each agent individually that resulted in a statistically significant increase in apoptotic cell fraction (IC_{min}). The IC_{min} of Td131_1 and doxorubicin were 10 μmol/L and 6 nmol/L, respectively (Figs. 3 and 4). At the IC_{min} of each agent, there were no detectable changes in caspase-3 or PARP cleavage by immunoblotting (Fig. 5A). However, the combination of agents, each at their respective IC_{min}, dramatically increased cellular apoptosis fraction, caspase-3 activation, and PARP cleavage (Fig. 5A).

We next sought to determine if Td131_1 and doxorubicin had synergistic activity in freshly excised human PTC ex vivo. The IC_{min} of Td131_1 and doxorubicin in our ex vivo model were 100 μmol/L and 10 nmol/L, respectively (Supplementary Fig. S3). Treatment of ex vivo tumors with Td131_1 and doxorubicin at their IC_{min} doses significantly enhanced activated caspase-3 expression and PARP cleavage in tumor cells (Fig. 5B). Thus, we also observed the synergistic effects of Td131_1 and doxorubicin in PTC ex vivo. Ultimately, these
results suggest that Gal-3 inhibition in combination with traditional systemic chemotherapy agents have synergistic antitumor effects in PTC.

**Td131_1 Exhibited Minimal Effects on Apoptosis in Normal Thyrocytes**

To determine the effects of Gal-3 inhibition on normal thyroid cells, we treated PCCL3 cells and human *ex vivo* specimens with Td131_1. In contrast to the PTC cell lines, Td131_1 in a concentration range of 10 to 200 μmol/L showed no significant activation of apoptosis in PCCL3 cells (Fig. 6A).

Furthermore, Gal-3 expression level is low in normal thyroid tissue relative to PTC. We treated three consecutive normal thyroid tissue specimens with 100 and 200 μmol/L of Td131_1. This caused no detectable effects on caspase-3 and PARP cleavage *ex vivo* (Fig. 6B). These results suggest that Td131_1 has no significant activity in normal thyrocytes which have intrinsically low Gal-3 expression.

**Td131_1 Improves Radiosensitivity in PTC**

Because apoptosis is a prominent mode of cell death in cancer cells treated with ionizing radiation and because Gal-3 has potent antiapoptotic effects in PTC (13), we hypothesized that Td131_1 would also improve radiosensitivity. To explore this possibility, we exposed PTC cell lines to external γ-radiation in the absence or presence of Td131_1. We found that Td131_1 significantly enhances radiosensitivity in a dose-dependent fashion in both 8505-C and TPC-1 cell lines (Fig. 7). Gal-3 inhibition is a promising adjuvant therapy to enhance PTC response to ionizing radiation, which is relevant for patients with locally advanced PTC with compressive symptoms that require palliation. Sensitizing PTC to ionizing radiation would also benefit patients with postsurgical microscopic residual disease amenable to adjuvant external beam radiation therapy. Future studies should also determine if Gal-3 targeted therapy could enhance the efficacy of adjuvant radioactive iodine.
ablation, which has the potential to improve the care of tens of thousands of patients with PTC every year.

The Effects of Td131_1 on Akt, ERK, Bad, and Protein Kinase C Are Consistent with a Mechanism of Gal-3 Inhibition

Previous studies have shown several downstream effectors of Gal-3: Td131_1 should selectively affect these downstream effectors. Gal-3 overexpression stimulates the phosphorylation of Akt, ERK, and Bad in various cancer cell lines (3, 14-16). We found that treatment of Td131_1 downregulates the phosphorylation levels of Akt, ERK, and Bad but not protein kinase C (PKC; Fig. 8). Together with our other observations, these findings suggest that the proapoptotic effects of Td131_1 are mediated through its inhibition of Gal-3.

Td131_1 is a promising agent for advanced PTC that is refractory to conventional therapies. Td131_1 activates apoptosis and improves the sensitivity of PTC to radiotherapy and to chemotherapy in a synergistic fashion. Our study is the first to use a small molecule inhibitor to target Gal-3 in PTC and the first to use an ex vivo model of PTC. Because Gal-3 is overexpressed in many cancers, further Td131_1 studies should be extended to other cancer types and for combination therapy with systemic agents that use a mechanism of action that involves the induction of apoptosis. Finally, these results support the further development of Gal-3 inhibitors with improved affinity, selectivity, and pharmacokinetics.

Materials and Methods

Reagents

The following antibodies were purchased for use: cleaved caspase-3 Asp175, PARP, phosphorylated Akt Ser473, phosphorylated Bad Ser112, Bad, and phosphorylated PKC (Cell Signaling Technology), PKC and phosphorylated ERK1/2 (Upstate), Akt1 and ERK1 (Santa Cruz Biotechnology), Gal-3 (Sigma-Aldrich), actin (Neomarker), and secondary horseradish peroxidase-conjugated antibodies (Vector). Doxorubicin was obtained from Fluka.

Td131_1 Treatment

The thiodigalactoside diester bis-[3-O-(3-methoxybenzoyl)-β-D-galactopyranosyl]sulfane (Fig. 3), a small molecule with a 100-fold enhanced binding affinity for Gal-3 in comparison to unmodified thiodigalactosides, was synthesized as previously described (compound 16) and was named Td131_1 (6). The

FIGURE 5. Synergistic effects of Td131_1 and doxorubicin in PTC cells and PTC tissue. A, 8505-C and TPC-1 cells were treated with DMSO, Td131_1 (10 μmol/L), doxorubicin (6 nmol/L), or Td131_1 (10 μmol/L) plus doxorubicin (6 nmol/L) for 24 h. Treated cells were trypsinized and then subjected to Annexin-V and propidium iodine flow cytometry analysis. Cell lysates were isolated and activated caspase-3 expression or PARP cleavage levels were determined by Western blotting. B, Human PTC specimens were treated with DMSO, Td131_1 (100 μmol/L), doxorubicin (10 nmol/L), or Td131_1 (100 μmol/L) plus doxorubicin (10 nmol/L) for 16 h. Activated caspase-3 expression, PARP cleavage and endogenous Gal-3 levels were also determined by Western blotting.
Td131_1 solution was prepared by dissolving in DMSO solution at 65°C for 5 min. The final concentration of stock Td131_1 solution is 20 mmol/L. Stock Td131_1 solution was stored at 4°C. Cells or tissues were grown in six-well dishes or culture inserts containing 800 or 600 μL of cultured medium, respectively. Stock Td131_1 solution was diluted with culture medium to final concentrations.

Quantitation of Apoptosis by Annexin-V and Propidium Iodine Flow Cytometry

Apoptotic cells were assayed by the ApopNexin ApoAlert Annexin V-FITC apoptosis detection kit (Clontech) as previously described (3).

Cell Culture and Radiation Exposure

TPC-1 and 8505-C PTC cells were cultured as described previously (3). The human FTC cell lines FTC-133, FTC-236, and FTC-238, human thyroid medullary carcinoma cell lines MTC 1.1, and human Hurthle thyroid cancer cell line XTC-1 were cultured as described previously (17). Rat thyroid PCCL3 cells were maintained in HI medium consisting of Coon’s medium/F-12 high zinc supplemented with 5% fetal bovine serum (Invitrogen), 0.3 mg/mL l-glutamine (Invitrogen), 1 mIU/mL TSH (Sigma-Aldrich), 10 μg/mL insulin (Sigma-Aldrich), 5 μg/mL apotransferrin (Sigma-Aldrich), 10 nmol/L hydrocortisone (Sigma-Aldrich), and penicillin-streptomycin (100 IU/mL-100 μg/mL; Invitrogen), 0.25 mg/mL Amphotericin B (Invitrogen). To assay cell survival following γ-radiation exposure, cells were plated at the appropriate dilution, irradiated, and surviving colonies were stained with crystal violet 10 d later (18).

Western Blotting

Cell lysates were prepared and analyzed by Western blotting as described previously (3).

Ex vivo PTC Tumor Specimen Culture and Immunohistochemistry Staining

This study was approved by the Committee for Human Research at Brigham and Women’s Hospital. Human normal
thyroid tissue and PTC tumor specimens were obtained from patients undergoing total thyroidectomy. The specimens were evaluated by a pathologist, who confirmed the histologic diagnosis of PTC. A piece of each tissue specimen was excised for our studies using sterile technique. Vital specimens (0.5 mm³) were cultured in Millicell cell culture inserts (Millipore) containing 600 μL of DMEM/F12 medium with 15 mmol/L of HEPES (Sigma-Aldrich) supplemented with GlutaMAX I (Invitrogen) and 10% fetal bovine serum (Invitrogen) at 37°C and 5% CO₂ for varying time periods in the absence or presence of Td131_1 or doxorubicin. For detection of BrdUrd incorporation, 10 μmol/L of BrdUrd (Sigma-Aldrich) were added to each specimen for 1 h before harvest. Treated tumor specimens were harvested and subjected to sectioning and immunohistochemical analysis of BrdUrd or activated caspase-3 by standard procedures, as described previously (19). Endogenous Gal-3 expression levels were determined by Western blotting.

Statistical Analysis

Differences between treatment groups were evaluated with a two-tailed independent Student’s t test. Each assay was done in triplicate, and P < 0.05 was considered statistically significant. We defined positive treatment “synergy” when the interaction of the two agents had a combined effect on the activation of apoptosis that is greater than the sum of their individual effects. To display such activity, we determined the minimum concentration of each agent to achieve a significant effect (ICₘᵢₙ) and determined the combined activity at the ICₘᵢₙ concentrations of both agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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