Journal of Surgical Research **156,** 39–44 (2009)
doi:10.1016/j.jss.2009.03.070

Follicular Thyroid Cancer Cell Growth Inhibition By Proteosome Inhibitor MG132[1]

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Submitted for publication December 22, 2008

Background. **Effective therapies for the subset of follicular thyroid cancer (FTC) patients with aggressive, metastatic disease are lacking. Therefore, we sought to determine the effects of proteosome inhibition, an emerging class of chemotherapeutic agents, on metastatic FTC cells.**

Materials and Methods. **Human metastatic FTC cells (FTC236) were treated *in vitro* with the proteosome inhibitor MG132 (0 to 800 nM). Western blot analysis was performed on whole cell lysates isolated after 2 d. To measure cell growth, we performed an MTT cellular proliferation assay over 6 d.**

Results. **Treatment of FTC236 cells with MG132 led to dose-dependent cell growth inhibition. Increases in inactive, phosphorylated GSK-3β, and active β-catenin also were observed. With 800 nM MG132, growth was reduced by 87% at 6 d ($P < 0.0001$). This reduction in cellular proliferation correlated with the degree of GSK-3β inhibition. MG132 treatment also caused increased $p21^{Waf1/Cip1}$ and decreased cyclin D1 expression, suggesting that growth suppression may occur through cell cycle arrest.**

Conclusion. **Growth of metastatic human FTC cells appears to be suppressed by proteosome inhibition. Whether this effect is directly due to cell cycle arrest and inactivation of GSK-3β signaling is unclear. Nonetheless, these compounds may become novel treatments for aggressive, metastatic FTC.**

Key Words: **MG132; proteosome inhibitor; follicular thyroid cancer; glycogen synthase kinase-3β; β-catenin; FTC236.**

INTRODUCTION

Follicular thyroid cancer (FTC) accounts for approximately 10% to 15% of all thyroid cancers, and is second only to papillary thyroid cancer (PTC) in incidence [1]. While FTC does occur in people of all ages, the majority of patients are female and between the ages of 30 to 50 y [1, 2]. Thyroid cancer management has been fairly consistent over the last decade, consisting of total or near-total thyroidectomy, thyroid remnant ablation with I^{131} radioactive iodine therapy, and suppression of TSH. Most patients who receive this therapy achieve a disease-free outcome [3, 4]. Compared with PTC, FTC is often diagnosed at a later stage with a more aggressive tumor and a moderately worse prognosis [4]. Patients with FTC also are at a greater risk for the development of distant metastases due to an increased tendency to invade the vasculature [2]. In addition, patients with aggressive, metastatic FTC may be unresponsive to radioiodine treatment because the tumor cells lose their ability to concentrate radioiodine over time. The inability to respond to radioactive iodine therapy is most likely due to loss of the human sodium-iodide symporter (hNIS) function [4, 5]. As a consequence, large, aggressive FTCs are not responsive to the conventional therapies. Therefore, alternative strategies are needed for treating patients with aggressive, metastatic FTC.

The growth of cancers such as FTC is regulated *via* a complex network of signal transduction pathways. The glycogen synthase kinase-3β (GSK-3β) signaling pathway has been identified as a potential target for novel cancer therapies [6]. GSK-3β is an important regulator of several cellular processes, including development, metabolism, gene transcription, cell cycle progression, proliferation, and apoptosis through its action as a serine/threonine protein kinase [6, 7]. Inactivation of GSK-3β is thought to contribute to inhibition of

[1] This work was presented at 4th Annual Academic Surgical Congress, Fort Meyers, FL, February 3–6, 2009.

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0022-4804/09 $36.00

tumor cell growth. Expression levels of the active, unphosphorylated form of GSK-3β have been shown to be higher in tumor cells than in their normal counterparts [7]. Active GSK-3β is considered to promote tumor growth, whereas inactivate, phosphorylated GSK-3β inhibits cellular growth. GSK-3β also is responsible for constitutive phosphorylation of β-catenin, which is necessary for the ubiquitin-proteosome mediated degradation of β-catenin protein. In other tumor models, inhibition of GSK-3β by phosphorylation of a single serine residue (Ser^9) not only inhibits tumor growth, but also contributes to an accumulation of β-catenin, a key component of the Wnt signaling pathway [6–9]. When increased levels of β-catenin form, this molecule translocates into the nucleus and interacts with various transcription factors to regulate gene expression [8, 9].

The ubiquitin-proteosome system (UPS) has recently been the focus of several studies involved in the development of new cancer therapies [10–12]. The 26S proteosome is a large, multicatalytic threonine protease that provides the major pathway for degradation of ubiquitinylated intracellular proteins [10]. This 26S proteosome is involved in the targeted degradation of key regulatory proteins necessary for cell-cycle progression and apoptosis in normal and malignant cells. Therefore, the 26S proteosome has become a potentially important therapeutic target in diseases of cell proliferation, including cancer [11, 12]. Many findings conclude that actively proliferating malignant cells are more sensitive to proteosome inhibition than noncancerous cells. Consequently, novel cancer therapies are exploring the use of proteosome inhibitors against several kinds of tumors [12]. MG132 is an example of one of the first described synthetic peptide aldehyde proteosome inhibitors that is both potent and reversible [11].

In this study, we investigated the effects of the proteosome inhibitor MG132 on a human metastatic FTC cell line, FTC236. An increase in inactive, phosphorylated GSK-3β was observed in FTC236 cells treated with MG132. β-Catenin, a downstream molecule of GSK-3β, also increased in expression. FTC cell growth was significantly reduced by MG132 treatment in a manner that was proportional to the degree of GSK-3β phosphorylation. In addition, MG132 treatment led to a decrease in $p21^{Waf1/Cip1}$ and an increase in cyclin D1, suggesting that cellular proliferation was inhibited through cell cycle arrest. As a result, proteosome inhibition may be an acceptable novel therapeutic approach for treating aggressive, metastatic FTC.

MATERIALS AND METHODS

Cell Culture

FTC236 cells (a human metastatic FTC cell line isolated from a lymph node) provided by Dr. Fiemu Nwariaku (UT Southwestern, Dallas, TX) were maintained in Dulbecco's modified Eagle/Ham's F-12 (1:1; Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum, 0.01 IU/mL thyroid-stimulating hormone, 40 μg/mL insulin (Sigma-Aldrich, St. Louis, MO), and 50 μg/mL penicillin/streptomycin (Invitrogen).

Cell Proliferation Assay

Cellular growth was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) rapid colorimetric assay (Sigma-Aldrich) as previously described [13]. Briefly, cells were seeded in quadruplicate on 24-well plates and incubated for 24 h under standard conditions to allow cell attachment. FTC236 cells were then treated with varying concentrations of MG132 (0 to 800 nM) and incubated for up to 6 d. The media was changed every 2 d with the appropriate concentration of MG132. The MTT assay was performed by replacing the standard medium with 250 μL of serum-free medium containing 0.5 mg/mL MTT and incubating for 3 h at 37°C. After incubation, 750 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to each well and mixed thoroughly. The plates were then measured at 540 nM using a spectrophotometer (μQuant; Bio-Tek Instruments, Winooski, VT). To confirm results, the experiment was repeated.

Western Blot Analysis

FTC236 cells were treated with varying concentrations of MG132 (0 to 800 nM) for 2 d, and whole-cell lysates were prepared as previously described [14]. Total protein concentrations were quantified using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Denatured cellular extracts (30 μg) were resolved using either 10% or 4% to 12% NuPAGE Novex Bis-Tris Mini Gels (Invitrogen) and transferred onto nitrocellulose membranes (BioRad Laboratories, Hercules, CA). The membranes were then blocked in milk (5% nonfat dry milk and 0.05% Tween 20 in 1 × phosphate-buffered saline) and incubated with the appropriate antibodies. Primary antibody dilutions were as follows: 1:500 for β-catenin, 1:1,000 for GSK-3β, phosphorylated GSK-3β (pGSK-3β), and phosphorylated β-catenin (pβ-catenin), 1:2,000 for cyclin D1 and $p21^{Waf1/Cip1}$ (Cell Signaling Technology, Beverly, MA), and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen, Gaithersburg, MD). Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Pierce Biotechnology) were used depending on the source of the primary antibody. Protein signal visualization was performed using membranes developed by Immunstar (BioRad Laboratories) for GSK-3β, pGSK-3β, β-catenin, cyclin D1, and GAPDH or by SuperSignal West Femto chemiluminescence substrate (Pierce Biotechnology) for pβ-catenin and $p21^{Waf1/Cip1}$ per the manufacturers' instructions.

Statistical Analysis

ANOVA was performed using a statistical analysis software package (SPSS version 10.0; SPSS, Chicago, IL). Statistical significance was defined as a P value < 0.05.

RESULTS

MG132 Treatment Caused Inactivation of GSK-3β in FTC236 Cells

MG132 treatment of FTC236 cells resulted in progressive phosphorylation of GSK-3β, suggesting a dose-dependent inhibition of the GSK-3β signaling pathway (Fig. 1). Phosphorylation and, thus, inactivation of GSK-3β caused by MG132 treatment were