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MG-132 Inhibits Carcinoid Growth and Alters the Neuroendocrine Phenotype

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Background. **Carcinoid cancers are the most common neuroendocrine (NE) tumors, and limited treatment options exist. The inhibition of glycogen synthase kinase-3β (GSK-3β) has been shown to be a potential therapeutic target for the treatment of carcinoid disease. In this study, we investigate the ability of MG-132, a proteasome inhibitor, to inhibit carcinoid growth, the neuroendocrine phenotype, and its association with GSK-3β.**

Materials and Methods. **Human pulmonary (NCI-H727) and gastrointestinal (BON) carcinoid cells were treated with MG-132 (0–4μM). Cellular growth was measured by the 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Levels of total and phosphorylated GSK-3β and the NE markers chromogranin A (CgA), Achaete-Scute complex-like 1 (ASCL1), as well as the apoptotic markers poly (ADP-ribose), polymerase (PARP), and cleaved caspase-3 were determined by Western blot.**

Results. **Treating carcinoid cells with MG-132 resulted in growth inhibition, a dose-dependent inhibition of CgA and ASCL1, as well as an increase in the levels of cleaved PARP and cleaved caspase-3. Additionally, an increase in the level of phosphorylated GSK-3β was observed.**

Conclusion. **MG-132 inhibits cellular growth and the neuroendocrine phenotype. This proteasome inhibitor warrants further preclinical investigation as a possible therapeutic strategy for intractable carcinoid disease.**

Key Words: **MG-132; proteasome inhibitor; carcinoid; glycogen synthase kinase-3β; ASCL1; apoptosis.**

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INTRODUCTION

Carcinoid tumors are the most common type of neuroendocrine (NE) tumor and may be found in the lungs, gastrointestinal (GI) tract, and other organs. A relatively uncommon malignancy of the digestive tract, the incidence is estimated at approximately 1 to 5 per 100,000 people in the United States, and is the second most common cause of isolated hepatic metastases [1, 2]. Metastatic disease, present in 75% at the time of diagnosis, is often associated with the debilitating carcinoid syndrome and surgical resection, while potentially curative for early disease, is often not an option for widespread metastases [3]. Chemotherapy and radiation have shown limited efficacy and, thus, novel treatments are necessary [3].

The 26S proteasome is a large, ATP-dependent, multi-subunit complex that degrades ubiquitinylated proteins. Critical in the elimination of damaged cellular proteins as well as in the proteolysis of short-lived functional proteins, the ubiquitin-proteasome system (UPS) has been the target of recent cancer therapeutic research [4]. Modulation of this system has been shown to regulate the level of proteins important in the regulation of apoptosis, cell-cycle progression, and gene transcription including: the caspase and BCL-2 families, cyclin dependent kinases, and nuclear factor κB (NFκB) [5, 6].

The UPS has been investigated as a novel therapeutic target for various hematologic and solid malignancies. Proteasome inhibition has been shown to be an effective therapeutic strategy in human malignancy, most notably in phase III trials using bortezomib for salvage therapy of multiple myeloma. While toxicity was noted in these trials, grade 4 toxicity was

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indistinguishable when bortezomib and dexamethasone arms were compared [7]. Additionally preclinical investigation in a number of other solid and hematologic malignancies has stimulated interest in a variety of tumors [7]. MG-132 (Z-Leu-Leu-Leu-aldehyde), a member of the peptide aldehyde proteasome inhibitor class, has been recently shown to inhibit osteosarcoma and colon cancer cells [8, 9]. These data suggest a possible role for this compound in anti-cancer therapy.

The Raf-1/mitogen-regulated extracellular kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway has been shown to play an important role in regulating tumor cell growth. Interestingly, recent data suggest that proteasome inhibitors are capable of inducing the Raf-1/MEK/ERK pathway, contributing to growth inhibition and apoptosis in breast cancer cells [10]. Downstream targets of this pathway, namely glycogen synthase kinase-3β (GSK-3β), have been shown to regulate numerous cellular processes, such as metabolism, proliferation, and survival [11–13]. GSK-3β, a multifunctional serine/threonine protein kinase, is highly active in carcinoid cells and is inhibited by phosphorylation of a single serine residue (Ser^9). Phosphorylation of GSK-3β has been shown to inhibit NE tumor growth and the carcinoid phenotype [11, 14].

In this study, we explore the effects of MG-132 on carcinoid cells. We observed significant growth inhibition, an alteration in the cancerous phenotype, as well as an induction of apoptosis and inhibition of GSK-3β in carcinoid cells.

MATERIALS AND METHODS

Cell Culture and Reagents

Human GI carcinoid cancer cells (BON), graciously provided by Dr. B. Mark Evers and Dr. Courtney M. Townsend Jr. (University of Texas Medical Branch, Galveston, TX), and NCI-H727 human bronchopulmonary carcinoid tumor cells (H727) (American Type Culture Collection, Manassas, VA), were maintained in RPMI 1640 and DMEM/F12 (Life Technologies, Rockville, MD), respectively, supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin and 100 μg/mL streptomycin (Life Technologies) in a humidified atmosphere of 5% CO_2 in air at 37°C [15]. MG-132 (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a stock concentration 5 mmol/L and stored at –20°C. Fresh dilutions in medium were made for each experiment.

Cellular Proliferation Assay

Carcinoid tumor cell proliferation was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) rapid colorimetric assay (Sigma-Aldrich) as previously described [15]. Briefly, cells were seeded in quadruplicate on 24-well plates and incubated 24 h under standard conditions to allow cell attachment. The cells were then treated with MG-132 at concentrations of 0–4 μM and incubated for up to 6 d. 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 at 37°C for 3 h. After incubation, 750 μL of DMSO was added to each well and mixed thoroughly. The plates were then measured at 540 nm using a spectrophotometer (μQuant; BioTek Instruments, Winooski, VT). Experiments were performed at least twice.

Immunoblot Analysis

Whole-cell lysates of human carcinoid tumor cells treated with MG-132 for 48 h were prepared as previously described [16]. Total protein concentrations were quantified with a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). Denatured cellular extracts (20–40 μg) were resolved by 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), blocked in milk (5% nonfat dry milk and 0.05% Tween-20 in phosphate-buffered saline), and incubated with appropriate antibodies. Antibodies were diluted as follows: 1:1,000 for GSK-3β, pGSK-3β, cleaved caspase-3, poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA), and mammalian achaete-scute homolog-1 (MASH1) for achaete-scute complex-like 1 (ASCL1) (BD Pharmingen, San Diego, CA); 1:2,000 for chromogranin A (CgA) (Zymed Laboratories, San Francisco, CA); 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.

For visualization of protein signal, membranes were developed by Immunstar (Bio-Rad Laboratories) for GSK-3β, pGSK-3β, CgA, PARP, and GAPDH, or by SuperSignal West Femto chemiluminescence substrate (Pierce Biotechnology) for cleaved caspase-3, and ASCL1.

Statistical Analysis

Statistical analyses were performed utilizing analysis of variance testing (SPSS software version 10.0, SPSS; Chicago, IL). A P value < 0.05 was considered significant. Unless specifically noted, all data are represented as mean ± SE.

RESULTS

MG-132 Inhibits Carcinoid Cells Proliferation

We first investigated the ability of MG-132 to inhibit carcinoid cell growth by performing a MTT proliferation assay with H727 and BON cells. Cells were treated for up to 6 d with doses ranging from 0 to 4 μM of MG-132. Significant growth inhibition was seen (Fig. 1) and, importantly, after 2 d of treatment with 0.5 μM MG-132, H727 cells were inhibited by 49% and BON cells were inhibited by 63%, compared with vehicle control (Fig. 1A and B, respectively).

MG-132 is Associated With an Alteration of the Neuroendocrine Phenotype

It has been previously described that ASCL1 and CgA are associated with the neuroendocrine phenotype as well as with the production of various bioactive substances [16, 17]. Therefore, we performed Western blot analysis for ASCL1 and CgA to investigate whether