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MG-132 Inhibits Carcinoid Growth and Alters the Neuroendocrine Phenotype

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Abstract

Background—Carcinoid cancers are the most common neuroendocrine (NE) tumors and limited treatment options exist. The inhibition of Glycogen Synthase Kinase-3 β (GSK-3 β) has been shown to be a potential therapeutic target to 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-3 β .

Materials and methods—Human pulmonary (NCI-H727) and gastrointestinal (BON) carcinoid cells were treated with MG-132 (0 to 4 μ M). 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-3 β 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-3 β 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.

Keywords

MG-132; proteasome inhibitor; carcinoid; glycogen synthase kinase-3beta; ASCL1; apoptosis

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

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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 which degrades ubiquitinated 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 κ B (NF κ B) (5,6).

The UPS has been investigated as a novel therapeutic target for various hematologic and solid malignancies, as it has been. 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 indistinguishable when bortezomib and dexamethasone arms were compared (7). Additionally pre-clinical investigation in a number of other solid and hematological 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 suggests 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-3 β (GSK-3 β), have been shown to regulate numerous cellular processes, such as metabolism, proliferation and survival (11–13). GSK-3 β , a multifunctional serine/threonine protein kinase, is highly active in carcinoid cells and is inhibited by phosphorylation of a single serine residue (Ser⁹). Phosphorylation of GSK-3 β 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-3 β in carcinoid cells.

MATERIALS AND METHODS

Cell Culture and Reagents

Human GI carcinoid cancer cells (BON), graciously provided by Drs. B. Mark Evers and Courtney M. Townsend, Jr. (University of Texas Medical Branch, Galveston, TX, USA), and NCI-H727 human bronchopulmonary carcinoid tumor cells (H727) (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 and DMEM/F12 (Life Technologies, Rockville, MD, USA), respectively, supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/mL penicillin and 100 μ g/mL streptomycin (Life Technologies) in a humidified atmosphere of 5% CO₂ in air at 37°C(15). MG-132 (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a stock

concentration 5mmol/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 hours 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 days. The MTT assay was performed by replacing the standard medium with 250 μl of serum-free medium containing 0.5mg/ml MTT and incubating at 37°C for 3 hours. After incubation, 750 μl of DMSO was added to each well and mixed thoroughly. The plates were then measured at 540nm using a spectrophotometer (μQuant ; Bio-Tek Instruments, Winooski, VT, USA). Experiments were performed at least twice.

Immunoblot Analysis

Whole-cell lysates of human carcinoid tumor cells treated with MG-132 for 48 hours were prepared as previously described (16). Total protein concentrations were quantified with a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL, USA). 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, USA), 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-3 β , pGSK-3 β cleaved caspase-3, poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA, USA), and mammalian achaete scute homolog-1 (MASH1) for Achaete-Scute complexlike 1 (ASCL1) (BD Pharmingen, San Diego, CA); 1:2,000 for chromogranin A (CgA) (Zymed Laboratories, San Francisco, CA, USA); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen, Gaithersburg, MD, USA). 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 Immunstarr (Bio-Rad Laboratories) for GSK-3 β pGSK-3 β 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 of <0.05 was considered significant. Unless specifically noted, all data are represented as mean $\pm$ 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 days with doses ranging from 0–4 μM of MG-132. Significant growth inhibition was seen (Fig. 1) and, importantly, after 2 days of treatment with 0.5 μM MG-132, H727 cells were inhibited by 49% and BON cells were inhibited by 63%, compared to vehicle control (Fig. 1A and 1B 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 MG-132 could alter the NE phenotype of carcinoid cancer cells.

We observed a dose dependent reduction of ASCL1 in H727 and BON cells after 48 hours of treatment (Fig. 2A and B respectively). A similar reduction in CgA was seen at 48 hours (Fig. 2A and B respectively). Taken together, the observed changes in ASCL1 and CgA expression indicate that MG-132 is associated with an alteration of the NE phenotype in carcinoid cancer cells.

MG-132 is associated with the induction of apoptosis

Following the MTT proliferation assay, we explored the mechanism of MG-132-induced growth inhibition. Western blot analysis for markers of apoptosis was carried out on H727 and BON cells treated for 48 hours with varying concentrations of MG-132. PARP, a well known marker of apoptosis, and caspase-3, the final executioner of extrinsic and intrinsic apoptotic pathways, were examined(18).

Cleaved caspase-3 as well as cleaved PARP were noted above 1.5 μ M MG-132 in H727 cells (Fig. 3A) while as little as 0.5 μ M MG-132 resulted in the same effect in BON cells (Fig. 3B). Cleavage of both PARP and caspase-3 indicate that at least part of the cellular growth suppression seen was a result of induction of apoptosis.

MG-132 is associated with phosphorylation of GSK-3 β in carcinoid cells

Western blot analysis was performed on whole cell lysates of H727 and BON to demonstrate the phosphorylation of GSK-3 β after the treatment with MG-132. Untreated pulmonary and GI carcinoid cancer cells have no phosphorylated GSK-3 β , demonstrating mostly active GSK-3 β . Treatment with MG-132 for 48 hours resulted in a dose dependent increase, thus inactivation, of GSK-3 β . Phosphorylation was demonstrated at concentrations as low as 1 μ M in H727 cells (Fig. 4A) and 0.5 μ M in BON cells (Fig. 4B). These results demonstrate that MG-132 is associated with phosphorylation of GSK-3 β in carcinoid cells.

DISCUSSION

Metastatic carcinoid disease is the second most common cause of isolated hepatic metastases and results in a less than 40% 5 year survival (2,3). Operative resection is the only curative therapeutic modality, but complete resection is often not possible on widespread disease. As other available treatment modalities have limited efficacy, there is a great need for the development of alternative treatment strategies as palliative and curative options to control widespread carcinoid disease.

Inhibition of GSK-3 β reduces tumor growth in several other kinds of cancers, including prostate, pancreas, and colorectal adenocarcinoma (19–21). There is also a growing body of literature suggesting that methods to inactivate GSK-3 β in NE cancer cells may be a novel treatment approach (11,14,22,23). We have previously reported that in medullary thyroid cancer, pheochromocytoma and carcinoid cancer cells, treatment with ZM-336372 and Lithium Chloride inhibits GSK-3 β signaling and is associated with inhibition of cellular growth and a change in the NE phenotype (14,23). These results suggest that inactivation of GSK-3 β may be a potential therapeutic target for carcinoid disease.

In the current study, we report that the proteasome inhibitor MG-132 inhibits GSK-3 β signaling in human GI and pulmonary carcinoid cells. At baseline, GSK-3 β signaling is active in these cells. However, with MG-132 treatment, dose dependent phosphorylation of GSK-3 β was noted. Likewise, treatment with MG-132 was associated with the suppression of the neuroendocrine tumor markers ASCL1, a basic helix-loop-helix transcription factor that could regulate the NE phenotype, and CgA.

We additionally report that MG-132 was shown to be associated with increasing levels of cleaved, active, caspase-3 as well as an increasing amount of cleaved PARP, indicating induction of apoptosis. This is a result similar to what has been observed in osteoma cells (9).

The mechanism by which MG-132 regulates cellular proliferation and apoptosis is complicated and controversial. It has been previously reported that inhibition of the UPS is sufficient to induce apoptosis in rapidly dividing cells (24). It is possible that the inhibition of such a central cellular mechanism is sufficient to trigger the initiation of cellular death. The inhibition of GSK-3 β as well as the alteration of the NE phenotype may be indirectly related to MG-132 treatment via the alteration of proteasomal degradation of regulatory proteins. Further studies into this mechanism will be needed to further elucidate the connection as both MG-132 and GSK-3 β constitute potential future targets for neuroendocrine malignancy growth and symptom control.

As mentioned, proteasome inhibitors have shown promise in a variety of tumors and this work extends the pre-clinical *in vitro* data to include human carcinoid cell lines. As has been noted, however, the *in vivo* effects of proteasome inhibitors is not always on par with the *in vitro* results. A number of novel proteasome inhibitors have been evaluated in *in vivo* cancer models though direct experience with MG-132 in murine xenograft cancer models has been limited (25). It has been shown in other *in vivo* models, however, that MG-132 can be given safely to rats and mice, though it is unclear how the dosages correspond to the *in vitro* concentrations used in this study (26,27).

In summary, our results demonstrate that the proteasome inhibitor MG-132 is associated with inactivation of the GSK-3 β signaling pathway and the decrease of ASCL1 and CgA, known NE tumor markers. Likewise, GSK-3 β inhibition decreases cellular proliferation via apoptosis. We submit that MG-132 and other proteasome inhibitors warrant further preclinical investigation in animal models as validation of its potential as a future therapy for patients with intractable carcinoid disease.

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A

B

Fig. 1. MG-132 significantly inhibits growth of carcinoid tumor cells in vitro. Pulmonary (NCI-H727) and GI (BON) carcinoid tumor cells were treated with MG-132 at the indicated concentrations (0 to 4 μM) for up to 6 days and cell viability was measured every 2 days with the MTT assay ($P < 0.001$). The results show that MG-132 leads to the suppression cellular proliferation in NCI-H727 (A) and BON (B) cells. (OD, optical density)

Fig. 2. MG-132 decreases the levels of ASCL1 and CgA in carcinoid cancer cells. Pulmonary (NCI-H727) (A) and GI (BON) (B) carcinoid cancer cells were treated with MG-132 (0 to 4 μ M) for 48 hours and cell lysates were analyzed by Western blotting for the expression of ASCL1 and CgA. Treatment with MG-132 results in a dose dependent inhibition of the two neuroendocrine tumor markers. Analysis for GAPDH confirmed equal loading.

Fig. 3.

MG-132 is associated with an induction of apoptosis. Pulmonary (NCI-H727) (A) and GI (BON) (B) carcinoid cancer cells were treated with MG-132 (0 to 4 μ M) for 48 hours and cell lysates were analyzed by Western blotting for the expression cleaved caspase-3 and cleaved PARP. Treatment with MG-132 results in a progressive increase in both cleaved caspase-3 and cleaved PARP. Analysis for GAPDH confirmed equal loading.

Fig. 4.

MG-132 is associated with phosphorylation GSK-3 β at Ser⁹. Pulmonary (NCI-H727) (A) and GI (BON)(B) carcinoid cancer cells were treated with MG-132 (0 to 4 μ M) for 48 hours and cell lysates were analyzed by Western blotting for pGSK-3 β and GSK-3 β . Treatment with MG-132 results in a progressive increase in phosphorylated GSK-3 β . Analysis for GAPDH confirmed equal loading.