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Original Article

Azacytidine induces cell cycle arrest and suppression of neuroendocrine markers in carcinoids

Vinita M. Alexander, Madhuchhanda Roy, Kristen A. Steffens, Muthusamy Kunnimalaiyaan, Herbert Chen

Endocrine Surgery Research Laboratory, Department of Surgery, University of Wisconsin, Madison, Wisconsin, USA.

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Abstract: Neuroendocrine tumors (NETs) hypersecrete neuropeptides that cause debilitating symptoms of carcinoid syndrome, including cardiac abnormalities. Surgical resection is the only potentially curative treatment for NETs; however, 90% of NE cancer patients are not candidates for surgery due to extensive hepatic sites involved with NETs. Recently, DNA methyltransferase inhibitors (DNMTI) such as azacytidine (AzaC) have shown efficacy in clinical treatments of hematological malignancies, but effects on NETs are not well-studied. We hypothesized that this novel class of drugs inhibits NET cell growth and decreases NE markers. Three carcinoid types—human midgut (CDNT2.5), pulmonary (H727), and gastrointestinal (BON)— were treated with AzaC (0-100uM) over 6 days. MTT Assays were used to measure cellular proliferation. Western blots were performed with antibodies against chromogranin A (CgA), Neuron-Specific Enolase (NSE), and Cyclin B1. Flow cytometric data was collected from AzaC-treated CNDT2.5 cells for DNA cell cycle analysis. Results showed that treatment of CDNT2.5, H727, and BON carcinoid cells with AzaC resulted in a dose-dependent reduction in tumor cell proliferation. Flow cytometric analysis showed that AzaC-treated cells accumulate in the G2 Phase of cell cycle. AzaC treatment led to: significant decreases in CgA and NSE, indicating that AzaC inhibits neuroendocrine markers; and significant increases in the levels of Cyclin B1, further supporting the flow cytometric data and conclusion that AzaC induces G2/M arrest. The data indicate that AzaC suppresses cell growth in three different carcinoid types, reduces neuroendocrine markers in CNDT2.5 cells, and inhibits cell proliferation by inducing G2/M phase arrest. The results suggest that DNMTIs may be a novel class of therapeutic agents that can effectively control tumor growth and the release of bioactive peptides in patients with NETs.

Keywords: Azacytidine, neuroendocrine tumors, Chromogranin A, Neuron Specific Enolase (NSE), G2/M arrest

Introduction

Neuroendocrine tumors (NETs) most commonly arise at sites along the gastroenteropancreatic axis. In fact, nearly 55% of NETs occur in the GI system while the rest (30%) are mainly found in the lungs [1]. Carcinoids are a special type of NET that are derived from the neuroendocrine (NE) cell system. In the GI tract, such tumors form deep in the mucosa and can grow to reach to the mucosal surface and underlying submucosa. Carcinoid tumors of the gastroenteropancreatic axis are traditionally classified based on their site of origin: the foregut (e.g. lung and pancreas carcinoids, as exemplified by the human cell lines H727 and BON, respectively); the midgut (e.g. small intestine carcinoids like the primary ileal carcinoid cell line, CNDT2.5); and the hindgut (rectal carcinoid) [2].

NETs are problematic because the tumor cells hypersecrete neuropeptides, amine hormones, and associated hormone products that can cause debilitating symptoms of the Carcinoid Syndrome, including severe diarrhea, abdominal cramps, and cardiac abnormalities. The acidic glycopeptide chromogranin A (CgA) is a secretory protein and a precursor of vasostatin that is found in the vesicles of neurons and endocrine cells that usually regulates the secretions of a neuroendocrine cell. CgA is commonly excessively co-secreted with hormones in NETs and is used as a clinical marker of the disease [3]. Neuron-specific enolase (NSE) is a glycolytic

enzyme, which is found in central and peripheral neurons and neuroendocrine cells. NSE is considered a neuroendocrine tumor marker and is found elevated in the plasma of patients with NETs, often in tandem with elevated CgA levels. Various published reports have suggested that NSE blood levels can be used in the diagnosis of neuroendocrine cancers [4,5].

According to statistics released by the American Cancer Society in 2009, approximately 12,000 cases of neuroendocrine tumors and cancers are diagnosed annually in the U.S, and NETs like carcinoid and pancreatic islet cell cancers (in fact, in 60% of these cases) frequently metastasize to the liver. In fact, over 90% of patients with pancreatic carcinoid tumors and 50% of patients with pancreatic islet cell tumors develop hepatic metastases, and the most common cause of death from pancreatic NETs is hepatic failure [6]. Left untreated, patients with NE liver metastases have a five-year survival probability of less than 30%. Surgical resection is the only potentially curative treatment for NE cancers; however, 90% of NE cancer patients are not candidates for surgery due to the extent of hepatic sites involved with the NE tumors [7,8,9]. Furthermore, chemotherapeutic treatments have been applied to carcinoids with limited success. A recent review even concluded that chemotherapy is "almost ineffective" for patients with well-differentiated neuroendocrine tumors of the GI tract (carcinoids) [10].The unresponsiveness of well-differentiated NETs to current chemotherapy strategies and the limitations of surgical treatments highlight the need for novel treatment strategies for NETs.

The mechanism of carcinoid formation and growth is not known. However, DNA hypermethylation often leads to tumors. CpG islands are regions of DNA enriched with CpG and GpC dinucleotides that are rarely methylated in normal cells, except in association with imprinted genes [11]. Abnormal hypermethylation processes can silence important regulatory genes of cell growth and can cause tumorigenesis. The biological mechanism that leads to the selection of specific CpG for hypermethylation is unclear [12, 13].

Recently, DNA methyltransferase (DNMT) inhibitors such as Azacytidine (AzaC) and Zebularine have shown efficacy in the clinical treatments of lung cancer and hematological malignancies, since they can relieve such hypermethylation and thus possibly restore normal function to genes regulating cell division. In clinical trials in patients with myelodysplastic syndrome, AzaC has a cytotoxic effect on rapidly dividing cells but is not as potent in non-proliferating cells, so its toxicity seems to be limited to affected malignant tissues [14, 15]. Azacytidine is a pyrimidine nucleoside analog of cytidine, and rapidly dividing cells can incorporate AzaC into DNA or RNA during replication or transcriptional processes. The incorporated AzaC interferes with the actions of methyltransferases, because the enzyme becomes irreversibly bound to AzaC residues in DNA and jams the cells' proper methylation machinery. Still, the precise molecular underlying mechanism of action of AzaC is unclear [16], and the effects of DNMT inhibitors on NETs have not been studied.

In this study, we show that this novel class of drugs inhibits cell proliferation, promotes G2/M growth arrest, and reduces CgA and NSE expression in carcinoids. These results stand as the first description of DNMT Inhibitors applied to neuroendocrine carcinoids and suggest that AzaC may be a potential therapeutic treatment for patients with well-differentiated neuroendocrine tumors.

Materials and methods

Cell culture

BON GI carcinoid cells, a gift from Dr. B. Mark Evers (Galveston, TX), were originally obtained from a lymph node containing a metastasis from a human pancreatic carcinoid tumor and were cultured in DMEM/F-12 (+ 15 mM HEPES buffer) (Gibco, Carlsbad, CA, 11039) with 10% Fetal Bovine Serum (FBS) (Sigma, St. Louis, MO), 100 IU/mL penicillin and 100 µg/ml streptomycin (Gibco, Carlsbad, CA) [17].

CNDT2.5 midgut carcinoid cells, a gift from Dr. G Van Buren, were established from hepatic tissue containing a metastasis from a human ileal carcinoid tumor and were cultured in DMEM-F12 (+2.438 g/L Sodium Bicarbonate) (Gibco, Carlsbad, CA 11320) supplemented with 10% FBS (Sigma, St. Louis, MO), 100 IU/mL penicillin and 100 µg/ml streptomycin (Gibco, Carslbad, CA), 1X MEM Vitamin Solution (Gibco, Carslbad, CA), 1X mM Sodium Pyruvate solution (Gibco, Carlsbad, CA), 1X L-Glutamine solution,

and 1X Nonessential Amino Acids solution (Gibco, Carlsbad, CA) [18].

Human pulmonary carcinoid cancer cells (H727) were obtained from American Type Culture Collection (Manassas, Va) and maintained in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 IU/mL penicillin and 100 µg/ml streptomycin (Gibco, Carlsbad, CA), sodium pyruvate (Gibco, Carlsbad, CA), D-Glucose (Sigma, St. Louis, MO), and 10 mM HEPES (Mediatech, Manassas, VA). All cell lines were maintained at 37°C in 5% CO_2 and 95% air.

Cell proliferation assay

BON, CNDT2.5, and H727 cell proliferation was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay as previously described [19]. 2 x 10^4 BON cells, 2.5 x 10^3 CNDT2.5 cells, 3 x 10^4 H727 cells were seeded in quadruplicates into 24-well plates and were incubated overnight to allow cell adhesion. After incubation, cells were treated 0-100 µM Azacytidine (Sigma, St. Louis, MO). Treatment medium was replaced every 48 hours with a medium containing a fresh drug dilution. To perform the MTT assays over a six-day periods, cells were incubated in 250 µl of phenol red-free RPMI 1640 containing 0.5 mg/ml MTT solution for 3.5 hours. After incubation, 750 µl of dimethyl sulfoxide (Fischer Scientific, Pittsburg, PA) was added to each well and mixed thoroughly. Absorbance was measured at 540 nm using a spectrophotometer (µQuant; Bio-Tek Instruments, Winooski, VT) and the readings were plotted as an average ± standard error of the mean (SEM).

Immunoblotting

BON, CNDT2.5, and H727 cells were plated in 10cm plates and incubated overnight to allow cell adhesion. After incubation, cells were treated for 2 days with 0-100 µM of AzaC. Total protein was harvested as previously described and quantified using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions [19]. Denatured cellular extracts were resolved on a 10% SDS-PAGE gel, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and blocked in milk. Membranes were incubated overnight in primary antibodies with the following dilutions: 1:1000 for chromogranin A (CgA) (Zymed Laboratories, San Francisco, CA); 1:1000 for Neuron Specific Enolase (NSE) (Fitzgerald Industries International, Concord, MA); 1:1000 for Cyclin B1 (Cell Signaling Technology, Danvers, MA); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Trevigen, Gaithersburg, MD). Membranes were then incubated in appropriate amounts of horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibody (Cell Signaling Technology). Protein signals were detected using either Immun-star (Biorad) (CgA, NSE, and GAPDH) or Supersignal West Femto (Pierce Protein Research Products, Rockford, IL) (Cyclin B1) kits, according to the manufacturer's instructions.

Flow cytometry

CNDT2.5 cells were incubated in 10cm^2 cell culture plates overnight to allow cell adhesion. After incubation, cells were treated for 48 hours in duplicates with varying doses of AzaC. At the end of the 48 hour treatment period, cells were trypsinized, washed twice with PBS, centrifuged twice at 1000rpm for five minutes, and fixed in Ethanol as previously described [20]. Cells were then stained with 50µg/mL Propidium Iodide (PI) solution containing 100 µg/mL RNAse A and stored overnight in the dark at 4°C. On the second day, cells in solution were filtered through 40µm silk membranes and subjected to Flow Cytometric analysis. Samples were loaded into a BD FACSCalibur (BD Biosciences) multi-color two-dimensional flow cytometer and absorbance was measured with 488 nM and 633 nM laser beams to detect both fluorescence and propidum iodide. DNA cell cycle analysis of flow cytometric data was carried out using the software Modfit LT (Verity Software House, Topsham, ME, US).

Results

Azacytidine inhibits GI & pulmonary carcinoid cell proliferation

Human carcinoid cells (BON, CNDT2.5, and H727) were treated with varying doses of AzaC over a six-day period, and MTT assays were performed every 48 hours. In BON cells, relative to the control treatment: 30 µM and 100 µM AzaC treatments over two days reduced growth

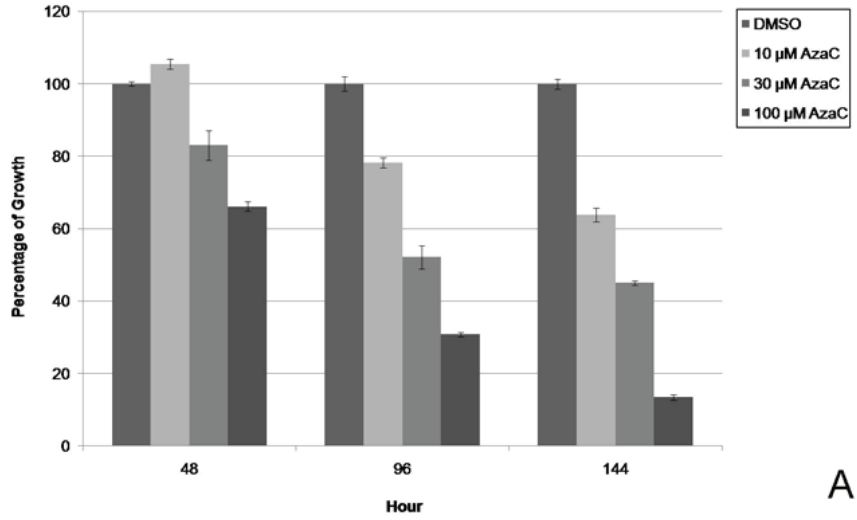
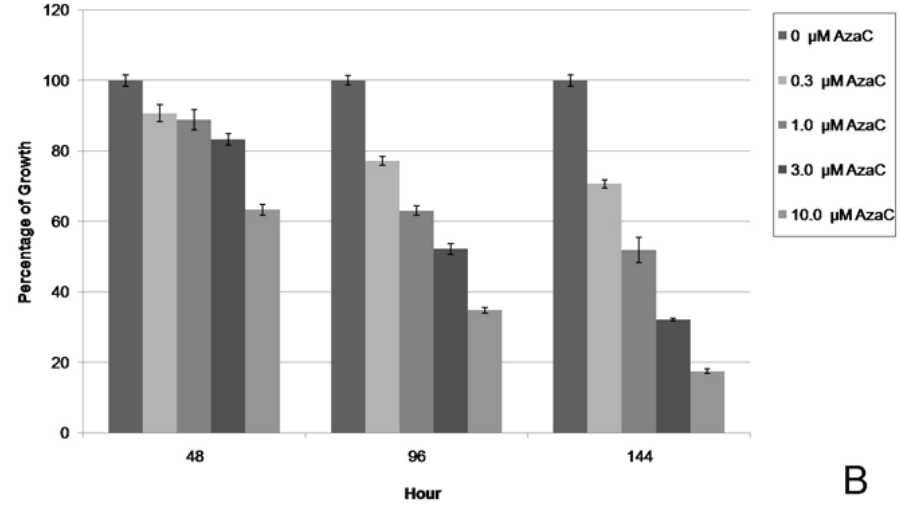
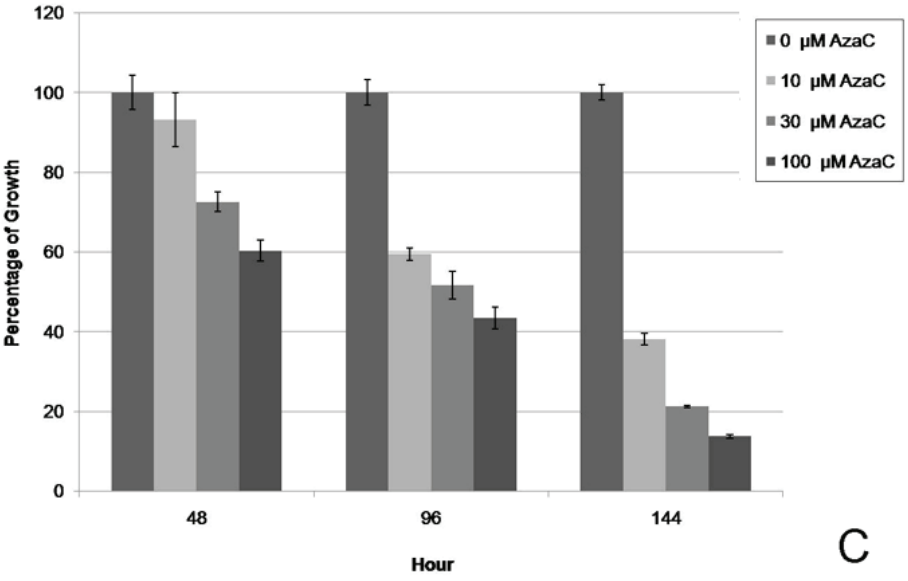


Figure 1. AzaC Inhibits GI and Pulmonary Carcinoid Cell Proliferation. BON GI, CNDT2.5 midgut, and H727 pulmonary carcinoid cells were treated with increasing doses of AzaC over a 6-day period, and cell viability was quantified via the MTT assay. A dose-dependent decrease in cell proliferation was observed in all three carcinoids (BON, CND2.5, and H727 carcinoids, shown in **A, B, and C,** respectively).



by 16.9% (±4.1%) and 33.8% (±1.4%). Relative to the control treatment: 10 μM, 30 μM, and 100 μM AzaC treatments over four days reduced growth by 21.7% (±1.5%), 47.9% (±3.3%), and 69.2% (±0.6%), respectively; and by 36.2% (±2.0%), 55% (±0.6%), and 86.5% (±0.8%) over six days (**Figure 1A**).



In CNDT2.5 cells, relative to the control treatment: 0.3 μM, 1 μM, 3 μM, and 10 μM AzaC treatments over two days suppressed cell proliferation by: 9.3% (± 2.4%), 11.1% (± 2.8%), 16.7% (±1.6%); 36.7% (±1.5%); by 22.8% (±1.3%), 36.9% (±1.3%), 47.8% (±1.5), and 65.2% (±.8%) over four days of treatment; and by 29.4%(±1.2%), 48.1% (±3.6%), 67.9% (±0.3%), and 82.5% (± 0.6%) over six days of treatment (**Figure 1B**).

Finally, in H727 cells,

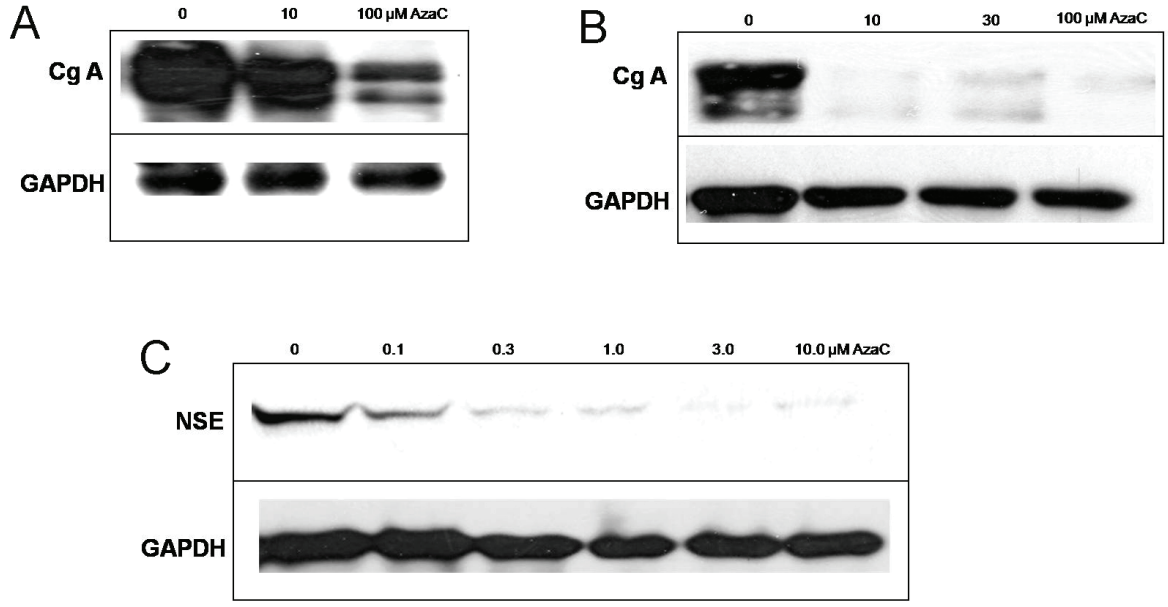


Figure 2. AzaC Suppresses Markers of Neuroendocrine Tumors. **(A)** AzaC inhibits chromogranin A (CgA) protein expression in BON GI carcinoid cells. BON cells were treated with AzaC for 2 days. Cells were lysed, and changes in neuroendocrine tumor markers were analysed by Western Blot. Treatment with increasing doses of AzaC for 2 days suppressed the protein expression of CgA, a well-known marker of NETs. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is presented as a loading control. **(B)** In H727 pulmonary carcinoid cells, AzaC inhibits protein expression of CgA, a well known marker of NETs. GAPDH is presented as a loading control. **(C)** In CNDT2.5 midgut carcinoid cells, AzaC inhibits protein expression of Neuron Specific Enolase (NSE), a well known marker of NETs. GAPDH is presented as a loading control.

compared to the control treatment: 30 μM and 100 μM AzaC treatments over two days reduced growth by 27.4% (± 2.4%) and 39.7% (±2.6%), respectively. Furthermore, relative to the control treatment, 10 μM, 30 μM, and 100 μM AzaC treatments over four days reduced H727 cell proliferation by 40.6% (±1.5%), 49.3% (±3.5%), and 56.6% (±2.7%), respectively; and by 61.9% (±1.5%), 78.7% (±0.3%), and 86.2% (±0.4%) after six days of treatment (**Figure 1C**).

AzaC suppresses markers of neuroendocrine tumors

To determine the effect of AzaC on NETs at the molecular level, we investigated changes in NE markers, either CgA (BON and H727) or NSE (CNDT2.5), via Western blot. Published reports suggest CNDT2.5 cells biologically have barely detectable levels of CgA, so levels of NSE were checked [18]. Western blot analysis showed AzaC treatment results in a dose-dependent reduction in CgA after 48 hours of treatment of BON GI carcinoid and H727 pulmonary carcinoid cells (**Figure 2A & 2B**); and also a dose-dependent reduction in NSE in CNDT2.5 midgut carcinoid cells (**Figure 2C**). These findings are significant because both CgA and NSE are considered blood biomarkers for neuroendocrine tumors, and their reduced levels in Carcinoid cells treated with AzaC indicates that AzaC suppresses hormone secretion and can alter the GI, midgut, and lung neuroendocrine malignant phenotype.

Azacytidine induces G2/M cell cycle arrest

To investigate the mechanism through which AzaC exerts its tumor-suppressive effects, flow cytometric analysis was performed on the CNDT2.5 cell lines. DNA cell cycle analysis was performed on flow cytometric data obtained from EtOH-fixed, AzaC-treated CNDT2.5 cells. In the control treatment, only 6.82% of the loaded CNDT2.5 cells were in the G2 phase. However, after 48 hours of treatments with AzaC of 1 μM, 3 μM, and 10 μM, cell cycle analyses showed that the proportion of CNDT2.5 cells in the G2

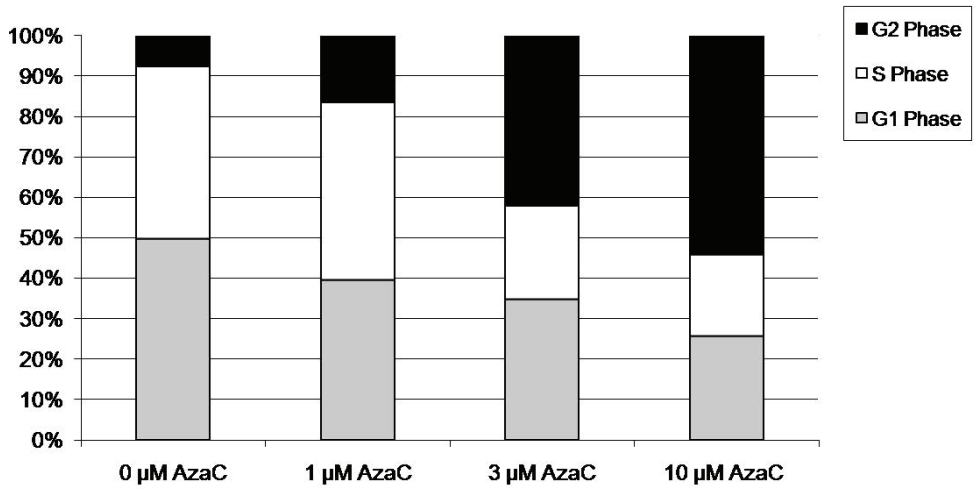


Figure 3. AzaC treatment induces G2/M arrest in CNDT2.5 cells. Flow cytometric data was obtained from AzaC-treated CNDT2.5 cells. In the control treatment, 6.82% of the CNDT2.5 cells were in the G2 phase. After 48 hours of treatments with AzaC with 1 μM, 3 μM, and 10 μM concentrations, cell cycle analyses detected an increased number of CNDT2.5 cells in the G2 phase (16.51%, 42.01%, and 54.19%, respectively).

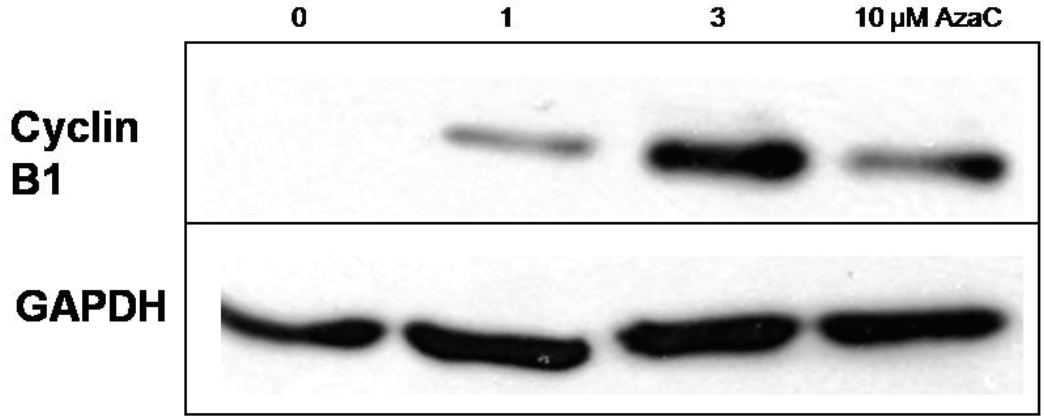


Figure 4. AzaC treatment induces markers of G2/M arrest in CNDT2.5 cells. Western blot analysis of AzaC-treated cell lysates was carried out. Increased expression of Cyclin B1, a marker for G2/M arrest, was observed with AzaC treatment in CNDT2.5 midgut carcinoid cells. GAPDH is presented as a loading control.

phase increased to 16.51%, 42.01%, and 54.19%, respectively (**Figure 3**). To verify the induction of G2/M phase arrest by AzaC, which was suggested by the flow cytometric data, immunoblots were performed. AzaC-treated BON and CNDT2.5 protein lysates were probed for expression levels of Cyclin B1, which accumulates as a part of an inactive p34 cdc2 protein complex and plays a critical role during the G2/M phase transition of the cell cycle. Therefore, relative increases in the levels of Cyclin B1 indicate induction of G2/M cell cycle arrest [21]. Immunoblots on AzaC-treated CNDT2.5 protein lysates reveals that increasing treatment concentrations of AzaC resulted in a dose-dependent increase in the levels of Cyclin B1 (**Figure 4**).

Discussion

Patients with NETs can suffer from crippling symptoms, including cardiac abnormalities and dysphagia, and other hallmarks of the Carcinoid Syndrome as a result of the tumors' excessive hormone secretions. Well-differentiated neuroendocrine tumors are refractory to the chemotherapy treatments currently available [10]. Furthermore, although it is the only curative treatment available, surgery is often not an option for patients with NETs due to the extent of tumor metastases to liver sites [7,8]. These limitations point to a need for novel therapeutic strategies for NETs.

DNA Methyl Transferase (DNMT) Inhibitors are in clinical use in treatments of hematopoetic disorders, and their hypomethylating action has recently shown promise as an anti-cancer treatment strategy that might act by restoring normal function to abnormally hypermethylated genes. In particular, in a 2009 Phase III international drug trial, Fenaux et al showed that AzaC significantly extends the overall survival of patients with MDS (myelodysplastic syndrome, a bone marrow stem cell disorder) and delays progression of MDS to AML (acute myeloid leukemia). Patients receiving AzaC treatments were more likely to undergo complete or partial remission compared to those that received conventional care [24]. Both MDS Syndromes and NETs have been shown to be responsive to treatments of Imatinib, a tyrosine kinase inhibitor, and MGD0103, a histone deacetylase (HDAC) inhibitor, suggesting that MDS and NETs may respond to drugs with similar therapeutic mechanisms of action [25]. Given these findings, AzaC treatment may also have potential survival benefits for patients with neuroendocrine tumors.

For the first time, here we examine the effects of the DNMT Inhibitor Azacytidine on three neuroendocrine carcinoid types. We demonstrate here that AzaC can inhibit the *in vitro* proliferation of BON GI carcinoid, CNDT2.5 midgut carcinoid, and H727 pulmonary carcinoid cells. We also provide evidence for the accumulation of G2/M cell cycle markers with AzaC treatment, suggesting that AzaC inhibits cell proliferation by inducing G2/M cell growth arrest. This is in concordance with previously published reports that AzaC treatments induces G2/M growth arrest in fission yeast (*Schizosaccharomyces pombe*) and primary human cell types (fibroblasts, primary mammary epithelial cells, and human ovarian surface epithelial cells) [22, 23]. Immunoblot analysis of AzaC-treated carcinoids indicates a reduction in either CgA or NSE, markers of hormone secretion, in three well-differentiated neuroendocrine carcinoids— in the BON, H727, and CNDT2.5 carcinoid types. This immunoblot data suggest that Azacytidine suppresses neuroendocrine markers and therefore can reduce the excessive secretion of bioactive hormones caused by NETs. AzaC also has been shown to strongly affect only rapidly dividing cancerous cells and not non-proliferating normal cells, and it has also already been shown to be safe in clinical trials [14].

Thus, in this study, we show the anti-NET potential of AzaC on three well-differentiated carcinoid NETs, and we also provide the first description of AzaC treatments and the probable mechanism of action on the primary ileal carcinoid NET. Given its effectiveness, Azacytidine warrants additional pre-clinical investigation.

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Please address correspondence to: Herbert Chen MD, H4/722 Clinical Science Center, 600 Highland Avenue, Madison, Wisconsin 53792-7375, USA. Tel: (608) 263-1387, Fax: (608) 263-7652, E-mail: chen@surgery.wisc.edu

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