Differential Cytotoxicity of Novel Somatostatin and dopamine Chimeric compounds on Bronchopulmonary and Small intestinal Neuroendocrine tumor cell lines

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Abstract

Introduction—Survival rates for gastrointestinal (GI) and bronchopulmonary (BP) neuroendocrine tumors (NETs) have not significantly altered (overall 67%, 5 yr survival) in thirty years (1973–2004) while the incidence has increased (~1,000%) in the same time-frame. No effective or specific antineoplastic agent(s) is available for treatment although somatostatin analogs inhibit tumor secretion. Given the coexistence of somatostatin and dopamine regulatory receptors on NET cells, we evaluated the antiproliferative efficacy as well as the signaling and transcriptional targets their ligands.

Methods—The cytotoxic effects of twelve somatostatin/dopamine compounds were evaluated in three NET cell lines and real-time PCR and ELISA studies performed to delineate antiproliferative signaling pathways.

Results—The atypical BP-NET, NCI-H720, was most sensitive to the sst_5 analog BIM23206 (IC_{50}:2.4pM) and showed similar sensitivity to lanreotide and the sst_2 analog BIM23120. The typical BP-NET, NCI-H727, was most sensitive to BIM23120 (0.7nM) and to the pan somatostatin receptor analog (BIM23A779). The GI-NET, KRJ-I, was most sensitive to $sst_{2,5}$ analogs lanreotide (1nM) and BIM23244 (7.4nM). Lanreotide activated ERK1/2 phosphorylation and $p21^{WAF1/CIP1}$ transcription but inhibited Ki67 transcription. NCI-H720 was most sensitive to the $sst_{2,5}$ and D_2-selective compound BIM23A761 (4.2nM) as was NCI-H727 (5.5nM). KRJ-I did not respond to any chimeric analog. BIM23A761 activated JNK signaling and caused inhibition of *Ki67* transcripts. $P21^{WAF1/CIP1}$ transcription was activated only in NCI-H727 cells.

Conclusion—The different responses of each individual cell line suggests that NETs from different locations arising from different NE cells may require cell-specific antiproliferative agents based on the unique receptor profile of individual lesions.

Introduction

Neuroendocrine tumors (NET) – previously considered as "carcinoids" – account for 0.66% of all malignancies; however, it is noteworthy that the incidence has increased 3–10% per year over the last 30 years [1, 2]. Although they predominantly occur in the gastrointestinal tract (67.5%), a substantial percentage is found in the bronchopulmonary system (25.3%) [3]. A paucity are evident in the ovaries (1.01%), testes (0.07%) or thymus (0.38%) but they can

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occur in any location that contains cells of the diffuse neuroendocrine system 1. In rare circumstances (<10%), such tumors may present as metastatic disease from an unknown primary site 4. Of note is the high proportion (22.4%) of associated non-carcinoid malignancies that occur concurrently with NETs, especially of the small bowel 1.

It is also noteworthy that there has been no improvement in survival in the last 3 decades (SEER data base 1973–2004) 3, 5 and there exists no effective, safe and targeted therapy for the disease. The overall five-year survival rate for gastrointestinal NETs is 67.2% 5 while for bronchopulmonary NETs it is 50–88% 3, with a worse prognosis for atypical lesions that are defined by a higher mitotic count (2–10 mitoses per square millimeter in 10 high-power fields) and/or the presence of necrosis 6. Given the rising incidence of these lesions and the failure to improve outcome, there is a substantial need to identify more effective treatment modalities 1, 5.

Although the only curative therapy for NETs is surgery, more than 85% of NETs are metastatic at diagnosis hence *ab initio,* less than 15% of patients have any likelihood of having curative intervention 7. Currently available therapy (biotherapy, radiotherapy, surgical resection, and debulking) focuses on disease stabilization and symptom control. Tumor regression is very rare and estimated at 2–5% 7. The majority of contemporary therapy for NET disease is based on empirical data derived from experience with adenocarcinomas; few adequately powered prospective studies exist and it is unclear at a cellular and molecular level how the individual NETs studied differ from each other 5.

The most thoroughly investigated group of agents are the long-acting somatostatin analogs, which include lanreotide and octreotide. These compounds, which have been assessed in phase III clinical studies 8, are effective in controlling most symptoms associated with the hypersecretory activity of NET cells. Lanreotide is effective in the treatment of active postoperative acromegaly 9, but the antiproliferative role of both it and octreotide in gastrointestinal and bronchopulmonary NETs is limited. Direct mechanisms of somatostatin analog action require the activation of five receptors (sst_{1-5}), belonging to the family of G-protein coupled receptors (GPCRs) and the induction of cell cycle arrest or apoptosis, mainly through the regulation of phosphotyrosine phosphatase and MAP kinase activities 10. The indirect mechanisms involve inhibition of tumor angiogenesis and secretion of factors which are required for tumor growth.

Dopamine regulates secretion and gene expression mediated by D_2 receptors coupled to $G_{\alpha i}$ proteins, with a resultant inhibition of adenylyl cyclase, a decreased cAMP production, and suppression of activated phosphokinase A 11. Recently, chimeric molecules that possess potent, selective agonist activity for both somatostatin and dopamine receptors have been synthesized. These compounds have been shown to be more effective in suppressing growth hormone and prolactin secretion from cultured human growth hormone-secreting pituitary adenomas than either octreotide or mono-receptor ligands alone 11−13.These agents have not been evaluated against human NETs of the gastrointestinal and bronchopulmonary system.

We analyzed the effects of twelve novel somatostatin analogs and somatostatin/dopamine chimeric compounds (Table 1) on three human NET (carcinoid) cell lines (atypical bronchopulmonary NET: NCI-H720, a bronchial NET: NCI-H727, and a small intestinal NET: KRJ-I 14, 15) to investigate whether different NETs require cell-specific antiproliferative agents based upon the unique receptor profile of individual lesions. In addition, we examined the antiproliferative signaling mechanisms of somatostatin- and dopamine-mediated cell inhibition in each NET type.

Materials and Methods

Culture conditions

KRJ-I and NCI-H720 cells were cultured as floating aggregates at 37°C with 5% CO_2. KRJ-I cells were kept in Ham's F12 medium (Gibco™) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich), penicillin 100U/ml and streptomycin (100 μg/ml) [15−17]. For NCI-H720 a 1:1 solution of Ham's F12 and Dulbecco's minimal essential medium (DMES) supplemented with final concentrations of FBS (5%), insulin (0.005mg/ml), transferrin (0.01 mg/ml), sodium selenite (30nM), hydrocortisone (10nM), β-estradiol (10nM), HEPES medium (10mM) and L-glutamine (2mM) [14]. The adhesive growing NCI-H727 cells were kept at 37°C in RPMI 1640 medium containing final concentrations of FBS (10%), L-Glutamine (2mM), sodium pyruvate (1mM) and glucose (2.5g/l) [14]. For further processing, NCI-H727 cells were washed in PBS before Trypsin-EDTA was applied.

Proliferation studies

After being spun down, 5 minutes –1,500g, the pellets of the three cell lines were resuspended in each medium to a concentration of 5×10^5 cells/ml and seeded in 96 well plates at 100μl with 5×10^4 cells/well (2 plates/experimental condition). Lane 1 and 2 contained negative (medium only) and positive (pure cell suspension) controls [17]. The BIM compounds were provided by IPSEN (Milford, MA). Drugs were diluted in PBS and applied every 24 hrs in final concentrations from 10^{-10} to 10^{-6}.

Agent Profiles

The agent profiles and somatostatin and dopamine receptor subtype specificities (IC_{50}) of each of the tested compounds are provided in Table 1. All compounds are stable *in vitro* and degradation is not an issue (*MC – personal communication*).

MTT assay

After 72 hours of incubation (37°C; 5% CO_2) with each compound, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to a final concentration of 0.5mg/ml per well followed by additional incubation for 3 hours [17, 18]. The reaction was stopped and the formazan dye was solubilized by adding an equal volume (100μl) of acid-isopropanol (0.01N HCl in isopropanol). The optical density was read at 595 nm using a microplate reader (Bio-Rad 3500) [16, 17].

ELISA (CASE) Assay

The effects of somatostatin analogs, BIM23926 (sst_1), BIM23120 (sst_2), BIM23023, BIM23244 (both $sst_{2,5}$) and BIM23206 (sst_5) was measured on the ERK1/2 signaling pathway as described [19]. In separate studies, the effects of lanreotide on ERK1/2 signaling pathway and BIM23A761 on ERK/AKT/JNK and NFκB signaling pathways were evaluated [19]. Cells were stimulated with IC_{50} concentrations (from proliferation studies – Table 2) for 30 mins. To assess the specificity of ERK pathway activation, cells were pre-incubated with PD98059 (30 μM) (an inhibitor of ERK1/2 phosphorylation) for 10 min prior to addition of lanreotide and BIM23A761. ERK, AKT, JNK and NFκB phosphorylation were measured using an ELISA (SuperArray CASE™) as per the manufacturer's protocol. Briefly, stimulated cells were fixed (4% formaldehyde), and stained with either primary antibodies against the non-phosphorylated or phosphorylated forms of each protein (60 min, RT). After washing, and secondary antibody application (60 min, RT), cells were incubated with color developer (10 min, RT) and plates read at 450 nm. Thereafter, protein was assayed in each well (protein development – reading at 595nm). Results were calculated as antibody reading

(at 450nm)/protein concentration (measured at 595nm) and normalized to unstimulated cells. Phosphorylated signal was compared to total non-phosphorylated signal.

Real-Time PCR

RNA was extracted from 2×10^6 NCI-H70, NCI-H727 and KRJ-I, cells in log phase growth (*n*=3) or from normal brain (*n*=3) (TRIZOL®, Invitrogen, USA), and cleaned (Qiagen RNeasy kit and DNeasy Tissue kit, Qiagen Inc., USA) to minimize contaminating genomic DNA. RNA (2μg) was converted to cDNA (High Capacity cDNA Archive Kit, Applied Biosystems) [17, 20, 21]. Real time RT-PCR analysis was performed using Assays-on-Demand™ products and the ABI 7900 Sequence Detection System according to the manufacturer's suggestions [17, 20, 21]. Cycling was performed under standard conditions (TaqMan® Universal PCR Master Mix Protocol) and data normalized using geNorm [22] and expression of the novel house-keeping genes, *ALG9*, *TFCP2* and *ZNF410* [17, 21]

1. Receptor profile—Levels of sst_{1-5} and D_{1-5} were measured in NCI-H720, NCI-H727 and KRJ-I cell lines. Normal brain was used as a control. Receptor probes were used to quantify presence of somatostatin and dopamine receptors in each cell line. Transcript levels <0.1 normalized geNorm were considered absent.

2. Cell cycle markers—Ki67 and $p21^{WAF1/CIP1}$ transcriptions were measured in response to lanreotide and BIM23A761. NCI-H720, NCI-H727, and KRJ-I cells were stimulated with IC_{50} concentrations of lanreotide and BIM23A761 respectively. Cells were cultured for 24 hrs prior to RNA extraction. RNA was isolated using the standard PCR protocol [17, 20, 21].

Statistical Evaluation

All statistical analyses were performed using Prism 4 (GraphPad Software, San Diego, CA). Sigmoidal dose responses and non-linear regression analyses were calculated to identify half-maximal concentrations (IC_{50}) for each drug. Alterations in signal transduction and transcriptional activation were assessed using 2-tailed paired t-tests.

Results

1. Receptor profile by PCR

Analysis of somatostatin and dopamine receptor levels using real-time PCR in each cell line demonstrated a different expression profile for each cell line.

NCI-H720—This cell line was derived from an "atypical" bronchopulmonary NET [14]. It is specific for somatostatin receptors 2 and 5 as well as the dopamine receptors 2 and 4 (Figure 1). The sst/da receptor profile of NCI-H720 is thus designated as $sst_{2,5}/D_{2,4}$.

NCI-H727 is cell line derived from a "typical" bronchial NET [14]. It is positive for $sst_{1-3,5}$ in addition to $D_{1,2,5}$. Very low levels of D_4 (<0.1 normalized geNorm) were also identified (Figure 1). The receptor profile of NCI-H727 is therefore $sst_{1,2,3,5}/D_{1,2,5}$.

KRJ-I is derived from a "typical" small intestinal NET [15]. It is positive for $sst_{1-3,5}$ as well as low levels of D_5 (=0.1 normalized geNorm) (Figure 1). The receptor profile of KRJ-I is $sst_{1,2,3,5}/D_5$.

2. Antiproliferative effects of somatostatin and somatostatin/dopamine chimeras

NCI-H720—Proliferation of this cell line is inhibited by the sst_5 agonist, BIM23206 (IC_{50} = 2.4pM) with a comparable sensitivity to Lanreotide ($sst_{2,5}$ agonist – 6.5pM) and the sst_2 agonist, BIM23120 (IC_{50} = 41pM) (Table 2). NCI-H720 cells were less responsive to the

$sst_{2,5}$ agonist, BIM23244 ($IC_{50} = 0.11nM$) and did not significantly respond to the sst_1 agonist, BIM23926. These results are consistent with the somatostatin receptor profile of the NCI-H720 cells (Figures 1A, B, D) and with ERK1/2 phosphorylation induced by these somatostatin analogs (Table 2). Of the two pan somatostatin receptor agonists tested, NCI-H720 cells were most sensitive to BIM23A719 (pan somatostatin receptor agonist 1, $IC_{50} = 6.2nM$).

Cell proliferation was also inhibited by the dopamine agonist, BIM53061 ($IC_{50} = 0.23nM$) and both BIM23A760 (sst/da agonist 2, $IC_{50} = 18.3nM$) and BIM23A761 (sst/da agonist 3, $IC_{50} = 4.2nM$).

NCI-H727—The typical BP-NET was most sensitive to the sst_2 agonist, BIM23120 ($IC_{50} = 0.7nM$) and to the pan somatostatin agonist 2, BIM23A779 ($IC_{50} = 1.9pM$) (Table 2). Neither sst_1, $sst_{2,5}$ nor any of the $sst_{2, 5}$ agonists caused inhibition of proliferation despite expression of transcripts for these receptors (Figures 1A, B, D). These results are consistent with the absence of ERK1/2 phosphorylation by these agents (Table 2).

Cell proliferation was also inhibited by the $sst_{2,5}$ and D_2 selective compound BIM23A761 ($IC_{50} = 5.5nM$).

KRJ-I—This was most sensitive to lanreotide ($IC_{50} = 1nM$), BIM23244 ($sst_{2,5}$ agonist, $IC_{50} = 7.4nM$) and BIM 23926 (sst_1 agonist, $IC_{50} = 8.5nM$) but was not responsive to either a pure sst_2 (BIM23120) or sst_5 (BIM23206) agonist (Table 2). The cell proliferation inhibitory effects were consistent with ERK1/2 phosphorylation by these compounds; neither BIM23120 nor BIM23206 induced phosphorylation (Table 2). KRJ-I cells were sensitive to both pan somatostatin receptor agonists but were most sensitive to the type 1 agent (BIM23A719).

KRJ-I cell proliferation could not be effectively inhibited by either the dopamine agonists ($IC_{50} = 0.7\mu M$) or the sst/da agonist chimeras which reflects the absence of significant transcripts for the inhibitory dopamine receptor (type 2) on this cell line (Figure 1F).

3. Effects of Lanreotide and BIM23A761 on cell signaling pathways

NCI-H720—Lanreotide activated MAPK signaling ($182\pm35\%$, $p<0.01$ vs. control) through phosphorylation of ERK1/2 which could be inhibited (65 ± 17, $p<0.01$ vs. lanreotide) by the MEK inhibitor, PD98059 (Figure 2A). This is consistent with the inhibitory effects of Lanreotide on NCI-H720 cell proliferation. When cells were stimulated with the type 3 sst/da chimera, BIM23A761, ERK was not phosphorylated. In contrast, the JNK pathway was activated by this compound; pJNK levels were elevated ($61\pm4\%$, $p<0.05$ vs. control: Figure 3A). Neither NFκB nor AKT were significantly altered by BIM23A761.

NCI-H727—Lanreotide had no effect on ERK phosphorylation (Figure 2B) which is consistent with the observation that proliferation could not be inhibited (Table 2). The BIM23A761 sst/da chimera was associated, as in NCI-H720 cells, with significant activation of JNK phosphorylation ($475\pm27\%$, $p<0.01$ vs. control: Figure 3B). While ERK1/2 signaling was inhibited (17 ± 3, $p<0.05$ vs. control), neither the NFκB nor AKT signaling pathways were significantly activated.

KRJ-I—Lanreotide activated ERK1/2 signaling by $25\pm8\%$ ($p=0.05$ vs. control) which could be inhibited by the MEK inhibitor, PD98059 (Figure 2C). BIM23A761 did not significantly alter phosphorylation of any of the signaling pathways which was consistent with lack of any demonstrable effect of this sst/da chimera on KRJ-I cell proliferation (Table 2).

Effects of lanreotide and BIM23A761 on downstream transcriptional targets

NCI-H720—Real-time PCR analysis of cells incubated with lanreotide for 24 hr demonstrated that *Ki67* transcript levels were significantly decreased (45±7%) compared to unstimulated cells (*p*<0.01: Figure 4A). This is consistent with the observation that lanreotide inhibits NCI-H720 proliferation. The effect was specific since it could be inhibited by PD98059 (6±2%, *p*<0.05 vs. lanreotide alone). Lanreotide significantly increased *p21^{WAF1/CIP1}* transcript levels compared to unstimulated cells (140±27%, *p*<0.01 vs. control) and this increase was reversible by PD98059 (*p*<0.05 vs. lanreotide alone: Figure 4D). These results are consistent with the anti-proliferative effects of this compound on NCI-H720.

Real-Time PCR analysis of BIM23A761-stimulated cells demonstrated that *Ki67* transcript levels were decreased 99±17% (*p*<0.01 vs. controls: Figure 5A) confirming that this compound inhibited NCI-H720 cell proliferation. In addition, and in contrast to the effects of lanreotide in these cells, *p21^{WAF1/CIP1}* transcript levels were significantly decreased (74±27, p<0.05 vs. control).

NCI-H727—Real-Time PCR analysis of the effect of lanreotide on *Ki67* demonstrated that this was not significantly decreased compared to control cells (Figure 4A), while *p21^{WAF1/CIP1}* transcripts were not significantly up-regulated (Figure 4E); results consistent with the absence of an inhibitory effect of lanreotide on NCI-H727 proliferation.

PCR assessment of BIM23A761 demonstrated significant (*p*<0.01) down regulation of *Ki67* transcript levels (80±12% change to control: Figure 5B) while *p21^{WAF1/CIP1}* transcripts were elevated (1082±97%, *p*<0.01 vs. control).

KRJ-I—Real-Time PCR measurements of *Ki67* transcript levels demonstrated that lanreotide down regulated *Ki67* (73±12%; *p*<0.01 vs. control: Figure 4C) and this was inhibited by PD98059 (*p*<0.05 vs. lanreotide alone). Transcripts for *p21^{WAF1/CIP1}* were significantly elevated (175±21%, *p*<0.01 vs. control: Figure 4F), results consistent with the antiproliferative effects of this compound on KRJ-I.

In BIM23A761 treated KRJ-I cells, neither *Ki67* nor *p21^{WAF1/CIP1}* transcript levels were significantly decreased (Figure 5C, 5F), consistent with observations that BIM23A761 did not inhibit KRJ-I cell proliferation.

Discussion

This study reveals that individual lung and gastrointestinal NET cell lines exhibit substantially different receptor expressions for somatostatin and dopamine receptors which are differently coupled to the ERK and JNK signaling pathways. In addition, somatostatin analogs and somatostatin/dopamine chimeric compounds are potent inhibitors of NET cell proliferation, while cell lines from the same organ (lung: typical versus atypical NET) were differently sensitive to receptor targeting. These molecular observations confirm the need to individualize therapeutic strategies based upon analysis of the receptor profile of a specific lesion as opposed to a global strategy based purely upon a histopathological characterization or classification. The differences in ERK signaling activation to specific somatostatin analogs suggests that defining the receptor profile alone may not be sufficient to predict NET responsiveness to these analogs. This is highlighted by the results with lanreotide, BIM23244 and BIM23023 which all are predicted to inhibit cell function through activation of sst$_{2,5}$. In the current study, lanreotide was the most effective inhibitor in NCI-H720 and KRJ-I cells. In contrast, and despite expression of sst$_{2,5}$, none of these compounds inhibited NCI-H727 cell proliferation. The absence of an effect in this cell line may suggest either

abnormal receptor expression (cell membrane/protein level) as has been noted in acromegalic tumors 23 or receptor coupling resulting in non-functional and divergent signaling pathways as noted in rat somatotrophs 24. Because ERK1/2 phosphorylation studies demonstrated no responses to $sst_{2,5}$ ligands in NCI-H727 cells but did show an effect in NCI-H720 and KRJ-I cells, we conclude that effective receptor signaling may be a prerequisite for appropriate inhibition of NET proliferation.

An examination of pan somatostatin receptor agonists demonstrated that the type 1 (BIM23A719) was most effective in NCI-H720 and KRJ-I cells (IC_{50} in NCI-H720 = 6.2 nM and IC_{50} in KRJ-I = 0.53 nM) while the type 2 agonist (BIM23A779) was most effective in NCI-H727 cells (IC_{50} NCI H727 = 1.9 pM). Of note is the fact that the pan somatostatin receptor agents were not as effective as individualized somatostatin agonists in the atypical BP NET (NCI-H720) as in the "typical" BP NET. The sensitivity of the latter to a pan somatostatin compound conforms to a previous study demonstrating that SOM230 was more effective than octreotide in inhibiting NCI-H727 proliferation25. This also confirms our observation that NCI-H727 cells are not effectively inhibited by a $sst_{2,5}$ compound despite expressing these receptors25, 26.

Targeting the D2 receptor is an effective mechanism for suppressing NET secretion 11⁻13 but its antiproliferative effects are not well characterized, particularly on lung and gastrointestinal NETs. Targeting D_2, which is coupled to G_{ai} and through coupling to phosphodiesterase activity reduces intracellular cAMP 27, is associated with inhibition of MAPK signaling 28. Activation of inhibitory D_2 receptors would therefore be predicted to inhibit cell proliferation. In the current studies, NCI-H720 cells which express the inhibitory dopamine receptors ($D_{2,4}R$) exhibit a decrease in cell proliferation when exposed to BIM53061 (IC_{50} = 21nM). This compound also inhibits KRJ-I (μM concentrations) but not NCI-H727 cell proliferation. These differences could reflect expression of the stimulatory $D_{1,5}$ receptors in these cell lines and suggests that a D_2 agonist would probably be ineffective in neoplastic NE cells expressing multiple dopamine receptor subtypes.

Somatostatin/dopamine chimeras target multiple receptor complexes to effect growth inhibition 13. Proliferation of NCI-H720 was inhibited by both sst/da agonists 2 and 3 (IC_{50}: 4.2–18 nM). These agents, however, were less effective (<100-fold) than targeting specific somatostatin receptors alone e.g. sst_5 or the pan somatostatin receptor agonist 1 on NCI-H720 cells. Targeting the dopamine in addition to somatostatin receptors is not therefore additionally inhibitory in this cell line. In NCI-H727 cells, sst/da agonist 3 was an effective inhibitor of proliferation. The KRJ-I cell line was not effectively inhibited by any of the sst/da compounds.

Having determined that NET cells lines were differentially sensitive to somatostatin analogs and sst/da chimeras, we next evaluated the mechanisms (signaling pathways, downstream transcriptional targets) by which two agents, lanreotide ($sst_{2,5}$) and BIM23761 (sst/da agonist 3), inhibited cell proliferation. These agents were chosen because they were both potent (lanreotide: 6.5pM NCI-H720 and 1nM KRJ-I; BIM23761: 4.2nM NCI-H720 and 5.5nM NCI-H727) and they significantly inhibited only two (lanreotide: H720 and KRJ-I; BIM23761: NCI-H720 and NCI-H727) of the three cell lines; one cell line therefore could be used as a negative control.

Lanreotide stimulated ERK1/2 activity in both NCI-H720 and KRJ-I cells but not in NCI-H727 cells. This resulted in a decrease in transcripts for *Ki67* and an elevation of the cell cycle inhibitor, $p21^{WAF1/CIP1}$. Preincubation with the MEK inhibitor, PD98059, decreased these lanreotide-mediated alterations in $p21^{WAF1/CIP1}$ expression. In studies in Chinese Hamster epithelial ovary CHO-K1cells, lanreotide-stimulated ERK activity led to

accumulation of phosphorylated P27^{Kip1}, inhibition of cyclinE-cdk2 kinase activity, accumulation of hypo-phosphorylated p105Rb and cell cycle arrest 29, 30. Over expression of P21$^{WAF1/CIP1}$, a cyclin-dependent kinase (cdk) inhibitor, leads to G1 and G2 or S-phase arrest 31. Our findings indicate that the inhibitory effects of lanreotide on H720 and KRJ-I are associated with cell cycle arrest at least via *p21$^{WAF1/CIP1}$* transcriptional upregulation.

In NCI-H720, BIM23A761 did not phosphorylate ERK1/2, AKT or NFκB but activated JNK phosphorylation. JNK was also phosphorylated in BIM23A761-treated NCI-H727 cells but phosphorylation of ERK1/2 was inhibited. Although BIM23A761 caused a decrease in transcripts for *Ki67* in both cell lines, an elevation of the cell cycle inhibitor, *p21$^{WAF1/CIP1}$* was only detected in NCI-H727 cells. In contrast, expression of this cdk inhibitor was significantly decreased in NCI-H720 cells. This seemingly discrepant observation raises the issue of the precise role of JNK signaling in apoptosis since JNK has been reported to function as both a pro- and anti-apoptotic agent 32. JNK induces phosphorylation of c-Jun at Ser63, a site important for c-Jun-dependent transcriptional activity 33 and the combination of c-Jun activation and *p21$^{WAF1/CIP1}$* suppression stimulates P53-dependant apoptosis 31. Our finding of suppressed levels of *P21$^{WAF1/CIP1}$* in NCI-H720 cells is consistent with a role for BIM23A761 in inhibiting cell proliferation via a P53-dependant apoptosis. In contrast, BIM23A761-mediated growth inhibition in NCI-H727 cells, like lanreotide in NCI-H720 and KRJ-I cells, is associated with P21$^{WAF1/CIP1}$-mediated cell cycle inhibition through the retinoblastoma pathway 29, 30. It is thus likely that individual NETs will not share completely similar transduction mechanisms for proliferative regulation and that specific assessments will be necessary to identify and predict the most efficacious therapeutic agent.

Synthetic derivatives of somatostatin, such as octreotide, are in common clinical use as therapy for acromegaly and gastroenteropancreatic tumors 34. While effective in the inhibition of the secretion of bioactive products and the amelioration of symptomatology 35, they have been disappointing as antiproliferative agents *in vivo* 7. The reasons for this are not known but indicate that secretory regulation of NET cells is an intrinsic property, whereas the proliferative drive may comprise many agents (angiogenic factors, growth factors) and has a substantial extrinsic basis, likely from the tumor micro-milieu.

We have used an *in vitro* model to study the effects of selective somatostatin and dopamine compounds on NET cells. With the caveat that our studies cannot measure the effects of these compounds on cells, e.g. fibroblasts, endothelial cells, in the NET microenvironment that regulate NET proliferation and secretion5, 36, 37, we demonstrate that targeting specific receptor subtypes on tumor cells, with selective somatostatin agonists may be more advantageous in "atypical" BP-NETs than a single generic compound. In contrast, BP-NETs and GI-NETs with an extensive somatostatin receptor expression profile may be more responsive to a pan somatostatin analog 25. Since some NETs express somatostatin and D$_2$ receptors, it is has been suggested that sst/da chimeric compounds may prove to be valuable in the management of NETs that exhibit both receptor profiles 38. Our results support this observation as somatostatin/dopamine chimeric compounds are as effective as pan somatostatin analogs in inhibiting NET cell proliferation. They do, however, appear to be less effective than single receptor targeted somatostatin agonist therapy for "atypical" BP NETs.

In conclusion, individual (site and cell specific) NETs respond differently to specific somatostatin analogs and sst/da chimeric compounds depending on the somatostatin and dopamine receptor profile coupling to signaling pathways. Antiproliferative effects are due to ERK1/2 phosphorylation and JNK signaling and activation of cdk inhibitors with a decrease in *Ki67* (Figure 6). If a therapeutic strategy that embraces receptor targeting is

utilized for NETs, it will require prior delineation of the specific receptor profile of the tumor and the selection of "receptor-tailored" pharmacotherapy to optimize effective treatment.

Acknowledgments

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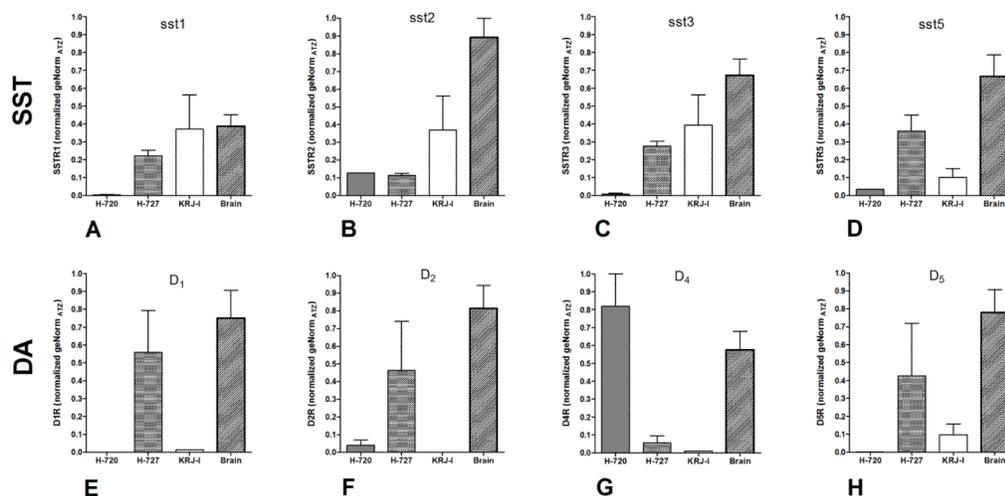
Figure 1. Somatostatin and dopamine receptor profiles in NCI-H720 (atypical), NCI-H727 (typical), and KRJ-I assessed by real-time PCR

Sst_1, sst_3, sst_5 receptors were found exclusively in NCI-H727 and KRJ-I cell lines (**1A, 1C, 1D**), sst_2 were present in all cell lines (**1B**). D_1, D_2, D_4 and D_5 receptors were present in NCI-H727 cells (**1E, 1F**) while inhibitory D_2 and D_4 receptors were identified in NCI-H720 (**1G**). D_5 receptors were observed in KRJ-I cells (**1H**). All sst and D receptors were identified in brain tissue (positive control). Mean±SEM; *n*=3.
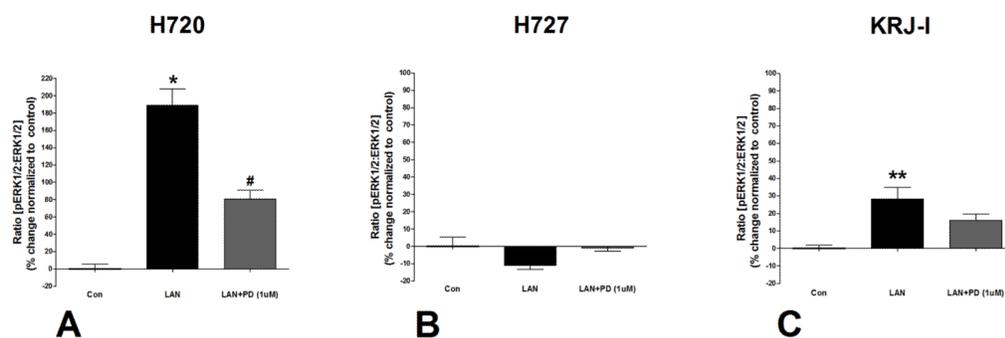
Figure 2. Effects of Lanreotide on ERK1/2 phosphorylation
Lanreotide phosphorylated ERK1/2 in NCI-H720 and KRJ-I cell lines (**4A, 4C**). Lanreotide had no effect on ERK1/2 phosphorylation in NCI-H727 (**4B**). The effects of lanreotide were inhibited by the MEK inhibitor, PD98059 (1μM) (**4A–C**). *$p<0.01$ vs. control; **$p<0.05$ vs. control; #$p<0.05$ vs. lanreotide. Mean±SEM; $n=4$.
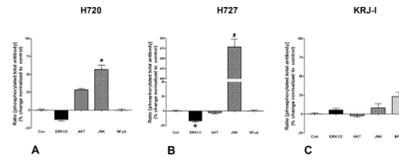
Figure 3. Effects of BIM23A761 on cell signaling pathways
BIM23A761 inhibited ERK1/2 phosphorylation and activated JNK signaling in NCI-H720 and NCI-H727 cells (**5A–B**). BIM23A761 has no effect on signaling pathways in KRJ-I cells (**5C**). *$p<0.05$ vs. control; #$p<0.05$ vs. control. Mean±SEM; $n=4$.
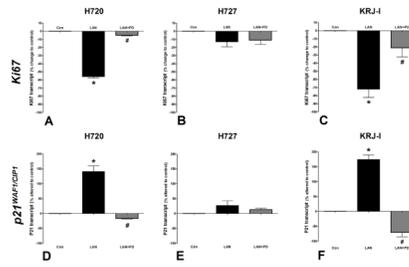
Figure 4. Real-time PCR analysis of the effect of Lanreotide on *Ki67* and *p21^WAF1/CIP1* transcripts

Lanreotide significantly inhibited *Ki67* transcription in all cell lines (**2A-C**). *P21^WAF1/CIP1* transcript was stimulated in all cell lines (**2D-F**). These effects were inhibited by PD98059 (1μM). *p<0.01 vs. control; #p<0.05 vs. lanreotide; **p<0.01 vs. lanreotide. Mean±SEM; *n*=4.
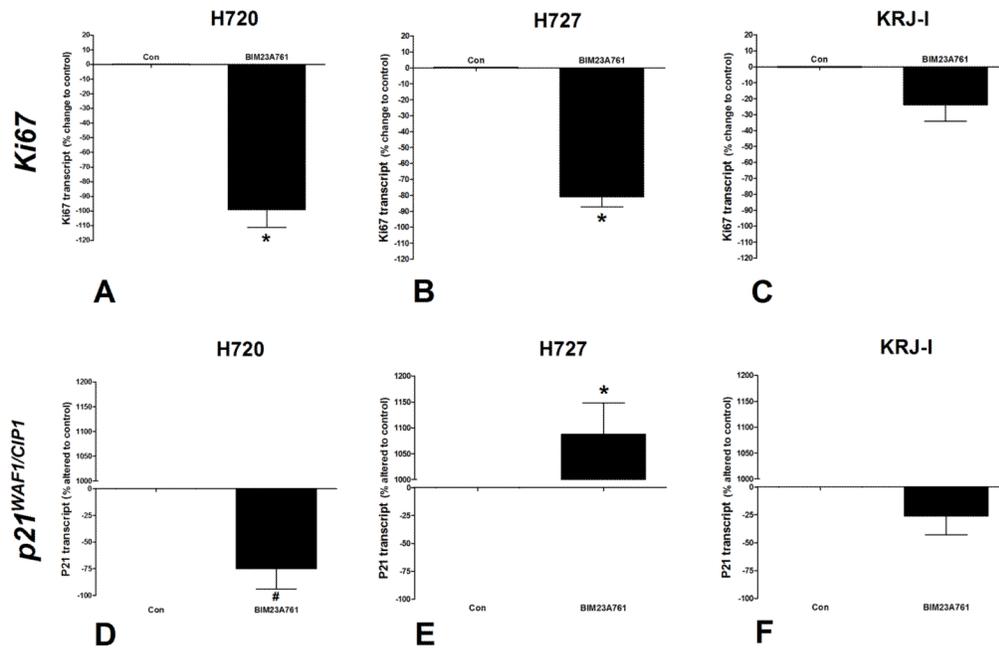
Figure 5. Real-time PCR analysis of the effects of BIM23A761 on *Ki67* and *p21^WAF1/CIP1^* transcripts

BIM23A761 significantly inhibited *Ki67* transcription in NCI-H720 and NCI-H727 cell lines (**3A, 3B**). Transcripts for *p21^WAF1/CIP1^* were inhibited by BIM23A761 in NCI-H720 (**3D**) and stimulated in NCI-H727 cells (**3E**). No significant effects were identified in KRJ-I cells (**3C, 3F**). $*p<0.01$ vs. control; $^{\#}p<0.05$ vs. control. Mean±SEM; $n=4$.
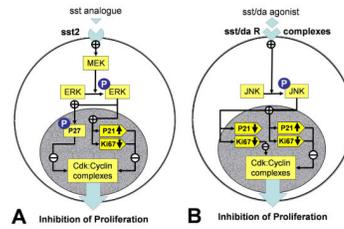
Figure 6. Somatostatin and sst/da chimera receptor signaling pathways in NET cell lines
Somatostatin receptor activation (largely via sst_2) results in MEK activation and downstream phosphorylation of ERK (**6A**). Activated ERK phosphorylates and activates the cdk inhibitor P27^{KIP1} and up-regulates P21$^{WAF1/CIP1}$ while inhibiting transcription of the cyclin, Ki67. The combination of cdk inhibitor upregulation and a decrease in Ki67 results in growth arrest. Sst/da receptor activation (largely $sst_{2/5}/D_2$) results in phosphorylation of JNK (**6B**). Activated JNK up-regulates P21$^{WAF1/CIP1}$ while inhibiting transcription of the cyclin, Ki67. This combination results in growth arrest. Depending on the sst/da receptor complex, *p21*$^{WAF1/CIP1}$ transcription can also be decreased.

Table 1

Human somatostatin receptor subtype specificity (IC$_{50}$) 39

Compound	sst1	sst2	sst3	sst4	sst5	D2
Somatostatin-14	1.95	0.25	1.2	1.77	1.41	-
Somatostatin-28	1.86	0.31	1.3	5.4	0.4	-
BIM23023	>1000	0.42	86.88	>1000	4.18	-
BIM23926	3.6	>1000	1000	833	788	-
BIM23120	1000	0.34	412	1000	213.5	-
BIM23206	>1000	166	1000	>1000	2.4	-
BIM23244	>1000	0.29	133	>1000	0.67	-
Lanreotide	>1000	0.75	98	>1000	12.7	-
BIM23A719	40	1	5.6	1000	0.37	-
BIM23A779	2.46	0.32	0.57	20.5	0.56	-
BIM53061	-	-	-	-	-	114.7
BIM23A758	486	0.1	324	>1000	27	8
BIM23A760	662	0.03	160	>1000	42	15
BIM23A761	602	0.128	196	>1000	8.5	25

- not done

Table 2

Summary of effects of somatostatin analogs and somatostatin/dopamine chimeric compounds on NET cell line proliferation and ERK1/2 phosphorylation

Compound	Action	H720	ERKp	H727	ERKp	KRJ-I	ERKp
BIM23023	sst$_{2/5}$ agonist	0.23 Nm	Y	No effect	N	0.28 μM	Y
BIM23926	sst$_1$ agonist	No effect	N	No effect	N	8.5 nM	Y
BIM23120	sst$_2$ agonist	41 pM	Y	0.7nM	Y	No effect	N
BIM23206	sst$_5$ agonist	2.4 pM	Y	No effect	N	No effect	N
BIM23244	sst$_{2/5}$ agonist	0.11 nM	Y	No effect	N	7.4 nM	Y
Lanreotide	sst$_{2/5}$ agonist	6.5 pM	Y	No effect	N	1 nM	Y
BIM23A719	pan sst agonist 1	6.2 nM		No effect		0.53 nM	
BIM23A779	pan sst agonist 2	No effect		1.9pM		0.35 μM	
BIM53061	Dopamine Agonist	21 nM		No effect		0.71 μM	
BIM23A758	sst/da agonist 1	No effect		No effect		No effect	
BIM23A760	sst/da agonist 2	18.3 nM		No effect		No effect	
BIM23A761	sst/da agonist 3	4.2 nM	N	5.5 nM	N	No effect	N