A New Generation Somatostatin-Dopamine Analogue Exerts Potent Antitumoral Actions on Pituitary Neuroendocrine Tumor Cells

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Keywords

Pituitary neuroendocrine tumors · Chimeric compound · Somatostatin · Dopamine · Receptor

Abstract

Background: Pituitary neuroendocrine tumors (PitNETs) represent approximately 15% of all intracranial tumors and usually are associated with severe comorbidities. Unfortunately, a relevant number of patients do not respond to currently available pharmacological treatments, that is, somatostatin analogs (SSAs) or dopamine-agonists (DA). Thus, novel, chimeric somatostatin/dopamine compounds (dopastatins) that could improve medical treatment of PitNETs have been designed. **Objective:** This study aims to determine the direct therapeutic effects of a new-generation dopastatin, BIM-065, on primary cell cultures from different PitNETs subtypes. **Methods:** Thirty-one PitNET-derived cell cultures (9 corticotropinomas, 9 somatotropinomas, 11 non-

functioning pituitary adenomas [NFPAs], and 2 prolactinomas), were treated with BIM-065, and key functional endpoints were assessed (cell viability, apoptosis, hormone secretion, expression levels of key genes, free cytosolic $[Ca^{2+}]_i$ dynamics, etc.). AtT-20 cell line was used to evaluate signaling pathways in response to BIM-065. **Results:** This chimeric compound decreased cell viability in all corticotropinomas and somatotropinomas tested, but not in NFPAs. BIM-065 reduced ACTH, GH, chromogranin-A and PRL secretion, and increased apoptosis in corticotropinomas, somatotropinomas, and NFPAs. These effects were possibly mediated through modulation of pivotal signaling cascades like $[Ca^{2+}]_i$ kinetic and Akt- or ERK1/2-phosphorylation. **Conclusions:** Our results unveil a robust antitumoral effect in vitro of the novel chimeric compound BIM-065 on the main PitNET sub-

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types, inform on the mechanisms involved, and suggest that BIM-065 could be an efficacious therapeutic option to be considered in the treatment of PitNETs.

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Introduction

Pituitary neuroendocrine tumors (PitNETs) are mostly benign neuroendocrine neoplasms confined to the sella turcica that constitute approximately 15% of all intracranial tumors [1, 2]. These tumors are usually monoclonal, derived from the expansion of single precursor cells, and are associated with severe comorbidities related to hormonal hypersecretion and/or mass effects, such as hypopituitarism, visual defects, amenorrhea, galactorrhea, sexual dysfunctions, and growth abnormalities [3–5]. The expression of somatostatin and dopamine receptors (SST_{1–5} and D_{1–5}, respectively) has been largely studied in the different PitNETs subtypes, wherein high levels of certain SSTs/Ds, especially SST₂, SST₅, and D₂, have been described [6–10]. For this reason, somatostatin analogs (SSAs: i.e., octreotide, pasireotide, and lanreotide) and dopamine agonists (DA; i.e., cabergoline and bromocriptine) have been widely used as pharmacological treatments to manage this and other endocrine-related pathologies due to their multiple antiproliferative and anti-secretory actions [6, 11, 12]. Nevertheless, numerous reports have demonstrated that a high proportion of patients are unable to respond to these treatments, or that the responses show a high variability or decline over time [12–14]. Inasmuch as PitNETs often express, simultaneously, high levels of various SSTs and Ds, and both families of receptors have the capacity of heterodimerize resulting in altered pharmacological properties [15, 16], new chimeric somatostatin (SRIF)/DA compounds were developed in order to increase their efficacy and improve the control of the disease compared with individual SSA and/or DA [17].

In this context, BIM-23A760, a chimeric compound able to bind SST₂, SST₅, and D₂ with high affinity, was considered a promising therapeutic option to treat different PitNETs subtypes. In fact, the direct effects of this chimeric compound have been tested in different normal and tumoral pituitary cell models [8, 18–24]. Recently, our group has demonstrated that treatment with BIM-23A760 reduce hormone secretion and/or expression in a set of somatotropinomas (GHomas), prolactinomas (PRLomas), and corticotropinomas (ACTHomas) [8]. However, these results also showed the existence of two

PitNET cell populations that oppositely responded to the treatment of BIM-23A760, an inhibitory- and stimulatory population [8], suggesting that this compound might not be a successful therapeutic option in some patients with PAs. Moreover, BIM-23A760 has been finally withdrawn from clinical development after discovering a dopaminergic metabolite that accumulates and interferes with the activity of the parent compound in vivo [17].

For all these reasons, a new generation of chimeric agonist for SST₂/SST₅/D₂ receptors (named BIM-065), with higher potency, efficacy, and safety, has been recently designed and developed by IPSEN that may be used for clinical purposes in the future [25, 26]. Therefore, the main aim of this study was to evaluate, for the first time, the direct effects of this new compound on relevant functional parameters (cell viability, apoptosis, hormonal secretion/expression, and [Ca²⁺]_i kinetics) in primary cell cultures from different PitNETs, including ACTHomas, GHomas, nonfunctioning pituitary adenomas (NFPAs), and PRLomas.

Materials and Methods

Reagents

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. BIM-065 and cabergoline were kindly provided by IPSEN Bioscience (Cambridge, MA, USA) and prepared following the instructions of the company. Octreotide was obtained from GP-Pharm (Barcelona, Spain), and pasireotide was generously provided by Novartis. All compounds were used at 100 nM based on previous studies [8, 27, 28].

Patients, Samples, and Primary Cell Cultures

Human PitNETs were obtained during transsphenoidal surgery from a total of 31 patients: 9 ACTHomas (mean age: 51 [29–79] years; 67% women), 9 GHomas (mean age: 48 [29–63] years; 44% women), 2 PRLomas (mean age: 25 [20–30] years; 50% women), and 11 NFPAs (mean age: 56 [41–75] years; 45% women). The appropriate classification of each pituitary sample collected was confirmed by 2 different methods: examination by expert anatomopathologists and by the molecular screening using quantitative real-time PCR (qPCR) as previously described [8, 28–30]. All techniques carried out in this study were conducted in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association and with the approval of the University of Cordoba/IMIBIC and Ethics Committees from all the Hospitals involved in the study. Informed consent from each patient was obtained.

All human samples were placed in sterile cold medium (S-MEM, Gibco, Madrid, Spain) supplemented with 0.1% BSA, 0.01% L-glutamine, 1% antibiotic-antimycotic solution, and 2.5% 2-(4[2-hydroxyethyl]-1-piperazine)-ethane sulfonic acid. The samples were rapidly moved to our laboratory and dispersed into

single cells by mechanical and enzymatic disruption and cultured onto different tissue culture plates following the methods and reagents previously described [28, 29].

Cell Line and Culturing

The mouse corticotroph pituitary derived cell line AtT-20/D16v-F2 (ATCC® CRL-1795) was cultured and maintained in Dulbecco's Modified Eagle's Medium complemented with 10% FBS, 100 U/mL penicillin/streptomycin, 0.024 M of 2-(4-[2-hydroxyethyl]-1-piperazine)-ethane sulfonic acid, and maintained at 37 °C and 5% CO₂, under sterile conditions, as previously reported [29]. The passage numbers used for the experiments were ranged between 20 and 25. Additionally, both cell lines were checked for mycoplasma contamination by PCR [31].

Measurement of Cell Viability

Cell viability of PitNET cell cultures (10,000 cells/well in 96-well plates) was evaluated every 24 h until 72 h in response to BIM-065, octreotide, pasireotide, or cabergoline using Alamar-blue reagent (Invitrogen, Madrid, Spain). In experiments using AtT-20 cell line, 6,000 cells/well were seeded in 96-well plates. The treatment was daily refreshed after each measure. Reduction of Alamar-blue was quantified using a FlexStation III system (Molecular Devices, Sunnyvale, CA, USA), as previously reported [8, 30, 32].

Measurement of Apoptotic Rate

Apoptotic rate was determined by measuring caspase 3/7 activity in response to BIM-065 using the Caspase-Glo 3/7 assay (Promega, Madrid, Spain) according to manufacturer's instructions. Briefly, 25,000 cells/well were plated in a 96-well white microplate and cultured for 24 h at 37 °C in an atmosphere containing 5% CO₂. Then, cells were incubated for another 24 h with BIM-065 and vehicle. After the incubation period, 100 µL of Caspase-Glo 3/7 reagent was added to each well and luminescence was measured at room temperature using FlexStation III system (Molecular Devices, Sunnyvale, CA, USA) for 3 h.

Measurement of Pituitary Hormone Release

To evaluate the effect of BIM-065 on pituitary hormone secretion, 150,000–200,000 cells/well were used. Media were recollected after 24 h of treatment and ACTH, GH, chromogranin-A (CgA), and PRL were evaluated using commercial ELISA kits (reference numbers: EIA-3647, EIA-3552, EIA-4937, and EIA-1291, respectively; DRG, Mountainside, NJ, USA) following the manufacturer's instructions. All the information regarding specificity, detectability, and reproducibility for each of the assays can be accessed at the website of the company.

Measurement of Dynamics of Free Cytosolic Calcium Concentration ([Ca²⁺]_i)

To assess the direct effect of BIM-065 on free cytosolic calcium mobilization, 50,000 cells/cover slip were plated, and changes in [Ca²⁺]_i in single cells of all types of PitNETs were measured using fura-2AM (Molecular Probes, Eugene, OR, USA), as previously described [8, 29, 30].

Measurement of MAPK and PI3K-Akt Signaling Pathways by Western Blotting

The mouse corticotroph PitNET-derived cell line AtT-20/D16v-F2 (ATCC® CRL-1795) was used to further explore the sig-

naling pathways modulated in response to BIM-065 due to the limited number of primary PitNET cells available for the culture experiments. About 500,000 cells were cultured in 6-well plates and incubated for 24 h with BIM-065 and vehicle-treated controls, as described previously [30]. Extracted protein samples were incubated with the primary antibodies (p-AKT [Ser47; Ref. CS9271S], Akt [Ref. CS9272], Bcl-2 [Ref. CS3498], p-ERK1/2 [Ref. CS4370], ERK1/2 [Ref. CS154], from Cell Signaling [Danvers, MA, USA]) and the appropriate secondary antibodies (secondary anti-rabbit antibody from Cell Signaling, Danvers, MA, USA), and developed using an enhanced chemiluminescence detection system (GE Healthcare, UK) with dyed molecular weight markers. A densitometric analysis of the bands was carried out with ImageJ software [33]. Relative phosphorylation of ERK1/2 and Akt was obtained from normalization of p-ERK1/2 or p-Akt against the total ERK1/2 or p-Akt, respectively. Bcl-2 was normalized by using total protein loading (ponceau staining).

RNA Isolation, Reverse Transcription, and Analysis of Gene Expression Levels by qPCR

Details of RNA extraction, quantification, reverse-transcription (RT), and qPCR using specific primers included in this study have been previously reported elsewhere by our group [8, 30, 34, 35]. It should be mentioned that since it is not possible to design a specific set of primers for qPCR that only amplified the short isoform of D₂, a set of primers that amplify both, the long and short, isoforms (D₂T) and a set of primers that only amplify the long isoform (D₂L) were used in this study, as previously reported [36]. In addition, new primer sequences were used in the present study to amplify CDKN1B (sense, ATAAGGAAGCGACCTGCAAC and antisense, TTGGGGAACCGTCTGAAA; accession number, NC_000012.12; product size, 88 pb) and CDK2 (sense, GCTCTCACTGGCATTCTCTT and antisense, GAGGTTTAAGTCTCGGTGGA; accession number, NC_000012.12; product size, 109 pb). It should be noted that, as previously reported and based on the stringent criteria to maximize specificity and efficiency, the qPCR technique, as applied, can be used to accurately quantify copy numbers for all human transcripts included in this study [36]. A no RT sample was used to ascertain that no detectable genomic DNA was present in the total RNA preparation. Moreover, to control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, the expression level (copy-number) of each transcript was adjusted using a normalization factor (NF) calculated from beta actin, hypoxanthine-guanine phosphoribosyltransferase 1, and glyceraldehyde 3-phosphate dehydrogenase expression levels.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical differences were assessed by paired parametric *t* test or one-way ANOVA followed by Dunnett's test for multiple comparison (according to normality evaluated by Kolmogorov-Smirnov test). As previously reported [29], to normalize values within each treatment and minimize intragroup variations in the different in vitro experiments (i.e., different age of the tissue donor and metabolic environment), the values obtained were compared with vehicle-treated controls (set at 100%). All experiments were performed in a minimum of 3 independent primary pituitary cultures from different patients (3–4 replicates/treatment per experiment), unless otherwise indicated. *p* values ≤ 0.05 were considered statistically significant and

a trend for significance was indicated when p values ranged between >0.05 and <0.1 . All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software; La Jolla, CA, USA).

Results

Expression Profile of SST₂, SST₅, and D₂ and Effects of BIM-065 on ACTHomas

SST₂, SST₅, and D₂, the target receptors for BIM-065, were highly expressed in human ACTHomas, with relative order of D₂T>D₂L>SST₅>>SST₂ (Fig. 1a), suggesting that corticotropinomas represent an appropriate model to test the effect of the new dopastatin compound. We also measured the expression of the two truncated SST₅ variants, SST₅TMD4 and SST₅TMD5, since the presence of these isoforms have been associated to a poor response to SSAs [7, 37]; however, the expression levels of these truncated SST₅ variants were negligible (<10 copies) in this cohort of samples.

Incubation of cultured ACTHoma cells with different doses of BIM-065 (10^{-9} to 10^{-6} M) revealed clear inhibitory effects on cell viability (Fig. 1b). Interestingly, the 4 doses employed reduced cell viability at similar levels after 48–72 h of incubation. It should be noted that, due to the limited number of cells available for the culture experiments, we could not test a wider range of doses; however, we had the opportunity to expand the doses employed in one ACTHoma cell preparation, in which a clear dose-response reduction on cell viability was observed at lower concentrations of BIM-065 (10^{-13} to 10^{-9} M; online suppl. Fig. 1A; for all online suppl. material, see www.karger.com/doi/10.1159/000500812). Specifically, BIM-065 does not seem to significantly alter cell viability at 10^{-13} or 10^{-11} M, whereas, as previously observed (Fig. 1b), it clearly reduced this parameter at 10^{-9} M after 72 h of incubation (online suppl. Fig. 1A). Based on these results and, in order to compare the effects of BIM-065 with previously selected, efficacious doses of SSAs (i.e., octreotide and pasireotide) and BIM-23A760 (the first-generation dopastatin compound) in PA cell cultures [8, 28], we selected the 10^{-7} M dose in further experiments.

Our results indicated that BIM-065 and octreotide produced a comparable significant reduction on cell viability in ACTHoma cell cultures (31.1 vs. 26.1%); however, pasireotide did not significantly alter cell viability (Fig. 1c). Interestingly, we could also compare the effects of BIM-065 and octreotide at a lower dose (10^{-9} M) in an ACTHoma cell preparation, and the results clearly indi-

cated that BIM-065 seems to be more effective than octreotide in reducing cell-viability after 72 h of incubation (41 vs. 10%, respectively; online suppl. Fig. 1B). In addition, we observed that incubation with BIM-065 (10^{-7} M) increased apoptosis after 24 h compared with vehicle-treated controls (Fig. 1d). Moreover, BIM-065 also appeared to decrease ACTH release in all ACTHomas analyzed, although this difference did not reach statistical significance probably due to the limited number of samples ($p = 0.07$; Fig. 1e); whereas, it did not alter mRNA expression levels of POMC (ACTH-precursor), somatostatin, and dopamine receptors (SST₂, SST₅, D₂T, D₂L; Fig. 1f) or tumor markers commonly associated with progression and aggressiveness of PitNETs such as PTTG1, CDKN1B, and CDK2 [38–40] (online suppl. Fig. 5A).

We also analyzed the direct effects of BIM-065 on $[Ca^{2+}]_i$ dynamics in single cells, since this is a key second messenger involved in the regulation of pituitary cell physiology and is closely related to hormone secretion through the modulation of secretory vesicle release [30, 41, 42]. This showed that BIM-065 treatment evoked a rapid inhibitory effect in the kinetics of $[Ca^{2+}]_i$ in all ACTHomas cases analyzed (3/3), exhibiting an averaged 26.4% of reduction in 44% of responsive corticotropinoma cells (Fig. 1g). Finally, to further test the capacity of BIM-065 to induce functional responses in pituitary tumor cells, we measured the levels of phosphorylation of several components of two important signaling pathways (MAPK and PI3K-Akt) in the PitNET-derived cell line AtT-20 (Fig. 2). As previously observed in primary ACTHoma cell cultures, treatment with BIM-065 (10^{-7} M) markedly reduced cell proliferation in cultured AtT-20 cells as compared to vehicle-treated controls (Fig. 2a). Interestingly, short-term incubation with BIM-065 (10 min) clearly increased p-Akt ($p = 0.004$), which was followed by a clear reduction of the antiapoptotic protein Bcl-2 (Fig. 2b). In contrast, long-term incubation with BIM-065 (24 h) upregulated p-ERK1/2 levels, although this latter effect did not reach statistical significance ($p = 0.1$; Fig. 2b).

Expression Profile of SST₂, SST₅, and D₂ and Effects of BIM-065 on GHomas

Similar to ACTHomas, SST₂, SST₅, and D₂ were highly expressed in human GHomas, with relative order of D₂T>D₂L>SST₅>>SST₂ (Fig. 3a). This expression profile is consistent with previous results evaluating and comparing the mRNA levels and protein content [43, 44], therefore supporting that somatotropinomas also represent an appropriate model to test the effect of BIM-065.

Fig. 1. ACTH-secreting adenomas. **a** Expression profile of SST₂, SST₅, D₂ (total and long isoforms), SST₅TMD4, and SST₅TMD5 ($n = 5$). **b** Dose-response experiment of cell viability in response to BIM-065 (10⁻⁹ to 10⁻⁶ M; $n = 3$). **c** Effect of BIM-065, octreotide, and pasireotide on cell viability (10⁻⁷ M; $n = 3$; 24–72 h treatment). **d** Effect of BIM-065 ($n = 3$) on apoptosis (24 h treatment). **e** Measurement of ACTH secretion (24 h treatment; $n = 3$). **f** mRNA expression levels of key genes in response to BIM-065 measured by qPCR and adjusted by NF ($n = 4$). **g** Summarized

table and representative profile of [Ca²⁺]_i kinetics assay in response to BIM-065 ($n = 3$). PRC, proportion of responsive cells showing changes in [Ca²⁺]_i in response to the treatment; PMR, percentage of maximum response; and time, time of response to treatment administration. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ statistically significant differences.

Fig. 2. AtT-20 corticotrope cell line. **a** Effect of BIM-065 (10^{-7} M; $n = 5$) of cell proliferation (24–72 h treatment), measured by Alamar-blue reduction. **b** Representative Western Blots and quantification of levels of p-Akt/total Akt, Bcl-2/ponceau, and p-ERK1/2/ total ERK1/2 in response to BIM-065 (10^{-7} M; $n = 3$). Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ statistically significant differences.

It should be mentioned that the expression levels of the truncated SST₅ variants were also negligible in this set of samples.

Specifically, treatment with BIM-065 (10^{-7} M) significantly decreased cell viability in GHoma cell cultures after 72 h of incubation. Our data also revealed that the efficacy of BIM-065, octreotide, and pasireotide to reduce cell viability was comparable in GHoma cell cultures (35.6, 24.7, and 41.8%, respectively; Fig. 3b) with respect to vehicle-treated control. BIM-065 also increased apoptosis (Fig. 3c) and strongly reduced GH secretion after 24 h of incubation (Fig. 3d). In line with this, we also had the opportunity to compare the effects of BIM-065 versus octreotide/pasireotide (10^{-7} M) in two independent GHoma cell cultures and found that all the compounds inhibited GH release (online suppl. Fig. 2).

Treatment with BIM-065 did not alter the mRNA expression levels of GH or other genes of interest mentioned above (SST₂, SST₅, D₂T, D₂L [Fig. 3e]; PTTG1, CDKN1B [online suppl. Fig. 5B]), except for CDK2,

whose expression increased in response to BIM-065 compared to vehicle-treated control (online suppl. Fig. 5B). Finally, BIM-065 treatment clearly reduced $[Ca^{2+}]_i$ levels (32.74% of reduction) in 2 out of the 3 GHomas analyzed, affecting the 30% of cells (Fig. 3f).

Expression Profile of SST₂, SST₅, and D₂ and Effects of BIM-065 on NFPA

NFPAs expressed high levels of SST₂, SST₅, and D₂ and negligible levels of truncated SST₅TMD4 and SST₅TMD5, with relative order of D₂T>D₂L>>SST₂>SST₅ (Fig. 4a). Remarkably, in contrast to that found with ACTHomas and GHomas, none of the NFPAs analyzed in this study responded to BIM-065 in terms of cell viability (Fig. 4b; online suppl. Fig. 3). Conversely, similar to ACTHomas and GHomas, NFPA cells were clearly responsive to BIM-065 in terms of increase in apoptosis (Fig. 4c) and reduce of CgA-secretion (Fig. 4d) after 24 h of incubation. Moreover, as we observed with ACTHomas and GHomas, treatment with BIM-065 did not produce any significant

Fig. 3. GH-secreting adenomas. **a** Expression profile of SST₂, SST₅, D₂ (total and long isoforms), SST₅TMD4, and SST₅TMD5 ($n = 4$). **b** Effect of BIM-065, octreotide, and pasireotide on cell viability (10^{-7} M; $n = 4$; 24–72 h treatment). **c** Effect of BIM-065 ($n = 3$) on apoptosis (24 h treatment). **d** Measurement of GH secretion (24 h treatment; $n = 4$). **e** mRNA expression levels of key genes in response to BIM-065 were measured by qPCR and adjusted by NF ($n = 3$). **f** Summarized table and representative profile of $[Ca^{2+}]_i$

kinetics assay in response to BIM-065 ($n = 3$). PRC, proportion of responsive cells showing changes in $[Ca^{2+}]_i$ in response to the treatment; PMR, percentage of maximum response; and time, time of response to treatment administration. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ statistically significant differences.

alteration in the mRNA expression levels of gonadotropin pituitary hormones (FSHB, LHB, or CGA), SST₂, SST₅, D₂T, D₂L (Fig. 4e) or of the tumor markers PTTG1 or CDK2, while CDKN1B expression slightly decreased in response to BIM-065 compared to vehicle-treated control (online suppl. Fig. 5C). Finally, BIM-065 treatment also reduced $[Ca^{2+}]_i$ levels (30.18% of reduction) in 2/3 NFPA's analyzed but only in a limited percentage of NFPA cells (27%) as compared to ACTHoma and GHoma cells (43.50 or 30.43%, respectively; Fig. 4f).

Expression Profile of SST₂, SST₅, and D₂ and Effects of BIM-065 on PRLomas

We also had the opportunity to analyze the expression levels of SST₂, SST₅, D₂S, and truncated SST₅ variants in two available PRLoma samples and to test the response to BIM-065. Specifically, PRLoma cells expressed high levels of D₂ compared with SST₂ and SST₅ (and negligible levels of truncated SST₅ variants), with relative order of D₂T>D₂L>>>>SST₅>SST₂ in PRLoma-1 and D₂T>D₂L>>>>SST₂>SST₅ in PRLoma-2 (online suppl.

Fig. 4. Nonfunctioning pituitary adenomas. **a** Expression profile of SST₂, SST₅, D₂ (total and long isoforms), SST₅TMD4, and SST₅TMD5 ($n = 3$). **b** Effect of BIM-065 on cell viability (10⁻⁷ M; $n = 8$; 24–72 h treatment), measured by Alamar-blue reduction. **c** Effect of BIM-065 ($n = 3$) on apoptosis (24 h treatment), measured by Caspase-Glo 3/7 assay. **d** Measurement of CgA secretion (24 h treatment; $n = 3$), determined by commercial ELISA kit. **e** mRNA expression levels of key genes in response to BIM-065

were measured by qPCR and adjusted by NF ($n = 3$). **f** Summarized table and representative profile of [Ca²⁺]_i kinetics assay in response to BIM-065 ($n = 3$). PRC, proportion of responsive cells showing changes in [Ca²⁺]_i in response to the treatment; PMR, percentage of maximum response; and time, time of response to treatment administration. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. * $p < 0.05$ statistically significant differences.

Fig. 4A). BIM-065 decreased cell viability at all the doses tested after 72 h of incubation (online suppl. Fig. 4B) and this reduction was seemingly more pronounced than the effect of cabergoline (34.92 vs. 15.4%, respectively; online suppl. Fig. 4C). A decrease of PRL secretion was also observed in response to BIM-065 compared with vehicle-treated control after 24 h of incubation (online suppl.

Fig. 4D). Similar to than previously observed in other cultured PitNET cell types, the expression levels of PRL, SST₂, SST₅, D₂T, and D₂L were not significantly altered in response to BIM-065 (online suppl. Fig. 4F). Moreover, BIM-065 treatment did not alter [Ca²⁺]_i kinetics in the PRLoma analyzed (online suppl. Fig. 4E).

Discussion/Conclusion

In the present study, we have tested, for the first time and in a representative set of distinct PitNETs subtypes, the actions of a new peptidic chimeric compound, called BIM-065. BIM-065 has been designed with the same affinity to bind SST₂, higher affinity to bind SST₅, and slightly smaller affinity to bind D₂ compared to the previous chimeric compound known as BIM-23A760 (binding affinity of BIM-065 in nM: 0.03 for SST₂; 0.5 for SST₅; and 27.2 for D₂ [17, 25, 26]). Moreover, this second-generation dopastatin molecule has been designed with an improved chemical structure to avoid the generation of metabolites with potent dopaminergic activity as the one found to accumulate in patients enrolled in Phase IIB study in response to BIM-23A760 [17] and also to avoid the paradoxical stimulatory responses found in a small but relevant proportion of pituitary tumor cell populations after treatment with this latter compound [8].

Of particular novelty are the results showing that BIM-065 decreases cell viability, increases apoptosis, and inhibits ACTH secretion in corticotropinomas, without significantly altering mRNA expression levels of the genes of interest at the times evaluated. To our knowledge, this is the first time that the effect of a chimeric compound is evaluated in detail in ACTH-secreting PitNETs. Although BIM-065 induced a comparable reduction of cell viability than octreotide at 10⁻⁷ M, the results achieved at 10⁻⁹ M revealed a higher reduction of cell viability in response to BIM-065. Nevertheless, this observation should be taken with caution, as this comparison could only be tested in one ACTHoma. In contrast, pasireotide did not exert an inhibitory effect on cell proliferation despite the high expression levels of SST₅ and also SST₂, which might suggest that additional mechanisms distinct from the mere presence of a given receptor are required to exert an anti-proliferative effect. Indeed, several reports have described that the expression levels of other proteins such as filamin A, β -arrestins, or E-cadherin might be important for SSAs to exert their antitumor actions in pituitary tumors [45–49]. Interestingly, results of a previous study from our group demonstrated a decrease on ACTH secretion in response to BIM-23A760 in one ACTHoma analyzed [8], which is in accordance with the results of this report. In the same line, we observed a reduction on [Ca²⁺]_i levels in all ACTHomas tested in response to BIM-065, which is also in accordance with the [Ca²⁺]_i response observed in the previous study. Indeed, the reduction of [Ca²⁺]_i levels was similar in both studies (26.4% of decrease in response to BIM-065 versus 27% of decrease ob-

served in response to BIM-23A760) but the percentage of responsive cells was higher with BIM-065 than with BIM-23A760 (43.50 vs. 24.0%, respectively) [8]. Of note, in contrast with the previous study, we did not observe any stimulatory effect in response to BIM-065 in the ACTHomas tested. In addition, we also demonstrate that treatment with BIM-065 increased p-Akt and p-ERK1/2 levels and decreased Bcl-2 in AtT-20 cells. These 2 pathways are tightly linked to cell growth, proliferation, and survival in tumor pathologies, including PitNETs [50, 51], albeit they displayed a distinct temporal course: p-Akt increased acutely, at 10 min, while p-ERK1/2 levels raised after long-term, 24-h incubation. A similar stimulatory response in ERK1/2 levels has been previously reported in PitNET cells after treatment with BIM-23A760 and the dopaminergic agonist BIM-53097, which was mainly associated to an activation of the dopaminergic signaling through D₂ that contributed to the antiproliferative effects of the 2 compounds in NFPA cell cultures [22]. Therefore, our results might suggest that the effects of BIM-065 on p-ERK1/2 and p-Akt proteins might be associated to a preferential activation of the D₂/dopaminergic-signaling. Moreover, the decrease in Bcl-2 levels supports the increase of apoptosis observed in response to BIM-065 in PitNETs. Therefore, the data presented provide novel evidence regarding how this new compound regulates some key intracellular signaling pathways in PitNETs cells. It should be noted that although a side-by-side study comparing BIM-065 and BIM-23A760 might have been interesting to make direct comparisons between both dopastatin compounds, the limitation of PitNET samples and the fact that BIM-23A760 was withdrawn from clinical development, prompted us to disregard this option, and to implement the direct, side-by-side comparison of BIM-065 with treatments currently used in medical practice (octreotide and pasireotide).

Results on GH-secreting PitNETs revealed that BIM-065 also induced profound direct actions on this tumor type, where we observed a higher reduction of cell viability and a comparable increase of apoptosis compared with our previous results with BIM-23A760 in somatotropinomas [8]. In the case of GH secretion, we observed a 53.2% of reduction of GH release, which is more striking than the decrease produced in response to BIM-23A760 or other chimeric compounds (SST₂-SST₅ or SST₂-D₂) in somatotropinomas or GH3 pituitary cell line [8, 18, 24, 52]. These results are in agreement with a very recent report showing that BIM-065 can reduce GH and IGF1 levels in healthy male volunteers with a safety and tolerability profile only limited by orthostatic hypotension [53]. On the

other hand, treatment with BIM-065 was not accompanied by decreased GH mRNA levels after long-term exposition (24 h) in human GH-secreting PitNETs, which is in contrast to our previous results with BIM-23A760 [8], but compares well with the results reported by Gruska et al. [24] in response to BIM-23A760 and BIM-23A761. Comparison of GH reduction between BIM-065 and SSAs did not show any relevant difference in the cases analyzed. Nevertheless, further experiments testing different BIM-065 doses and in additional tumor preparations will be necessary to unequivocally establish whether BIM-065 is more potent and efficacious than SSAs in somatotropinomas. An initial analysis of the possible mechanisms involved in the functional response observed in these tumors revealed that the new dopastatin evoked a clear reduction on $[Ca^{2+}]_i$ levels, which also agrees with the predominant inhibitory responses observed by our group in the face of BIM-23A760 [8]; yet, in clear discrepancy with that observed previously for BIM-23A760 in somatotropinomas [8], but in line with our present results in ACTHomas, we did not detect here in any stimulatory response from somatotropinoma cells.

It is currently rare to have access to PRLoma samples for in vitro testing, given, fortunately, to the frequent success of DAs treatment regimens to control or even cure this type of tumors. However, in the course of this study, we had the opportunity to test this compound on 2 PRL-secreting PitNETs. Interestingly, we observed clear differences in the response to BIM-065 and cabergoline in terms of cell viability between the 2 PRLomas analyzed, which could be due to their differential receptor expression pattern. Thus, the tumor that was highly responsive to BIM-065 expressed higher levels of SST₅ compared to SST₂ and higher levels (and similar between them) of both isoforms of D₂, which is a comparable expression pattern to that found in the rest of functioning PitNETs analyzed. Furthermore, these data are in line with previous results reporting that dopaminergic contribution is more important for the response to chimeric compounds, such as BIM-23A760, than the expression of SST₂ [54]. Consistent with previous results observed with chimeric compounds [8, 24], a profound decrease on PRL release was detected in response to BIM-065; whereas, in contrast with the data reported so far in human PRLomas, rat pituitary cell cultures and MMQ cell line [8, 24, 52], no changes were found on PRL mRNA levels. Nonetheless, our results showing that BIM-065 is able to decrease PRL release compare favorably with a very recent report showing that this compound inhibited PRL levels in healthy male volunteers [53]. Obviously, additional experiments

are necessary to confirm/complement the results obtained herein, and to explore the signaling pathways involved, particularly given the unexpected observation that the effects observed in PRLomas did not seem to be accompanied by parallel changes in $[Ca^{2+}]_i$ dynamics.

In contrast to previous results from our group and others with other dopastatins [8, 21], NFPA primary cell cultures did not show relevant alterations in terms of cell viability in response to BIM-065. Yet, surprisingly, the treatment with BIM-065 did induce a clear increase on apoptotic rate and a significant decrease in CgA in all cases tested. Although the vast majority of observations suggest that the cell proliferation and programmed cell death are effectively coupled [55, 56], there are several studies showing an imbalance between cell proliferation/survival index and apoptosis in pathological conditions such as breast, lung, or colorectal cancer [57–59]. Nevertheless, this observation is both, clinically relevant for this difficult-to-treat type of tumor, and biologically intriguing, and therefore further studies are warranted to explore the reasons why cell proliferation and apoptosis are apparently uncoupled in response to BIM-065 in NFPA primary cell cultures.

In summary, our data provide compelling evidence demonstrating that BIM-065 can directly and profoundly alter cell function and behavior in the most relevant subtypes of PitNETs. While not universal for all tumor types, BIM-065 acted reducing cell viability, likely by increasing apoptosis, and inhibiting hormone secretion, mostly by reducing $[Ca^{2+}]_i$ levels. Importantly, in contrast to previous dopastatin, treatment with BIM-065 did not evoke any stimulatory action in the tumor cells analyzed, while the proportion of responsive tumors/cells was higher with this compound than with BIM-23A760, suggesting that this novel dopastatin is a more efficacious and specific chimeric compound that could become a useful tool in the future treatment of PitNETs. Indeed, this compound could be used to treat patients harboring NFPAAs without a clear indication of surgical resection, patients with a tumor residue or with hormone hypersecretion persistence after transsphenoidal surgery or to control hormone hypersecretion and reduce tumor growth before surgery. In any case, further studies should be implemented to confirm and expand the original results provided herein, and a randomized clinical trial comparing BIM-065 with the pharmacological options currently available should be performed. Thus, this new generation chimeric compound may hopefully help to enhance the currently scarce pharmacological arsenal for the treatment of patients harboring PitNETs.

Acknowledgments

We thank Ipsen Bioscience (Cambridge, MA, USA) for kindly providing us the compounds BIM-065 and cabergoline. We also thank Novartis for generously providing us the pasireotide compound.

Statement of Ethics

All techniques carried out in this study were conducted in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association and with the approval of the University of Cordoba/IMIBIC and Ethics Committees from all the Hospitals involved in the study. Informed consent from each patient was obtained.

Disclosure Statement

E.V.-M., M.A.G.-M., A.S.-M., C.B.-A., J.P.C., and R.M.L. have received lecture fees and/or research grant support from Ipsen and Novartis. M.D.C. and T.L. are former employees of Ipsen. The rest of the authors have nothing to disclose.

Funding Sources

This work was supported by the following grants: Junta de Andalucía (CTS-1406, BIO-0139); Instituto de Salud Carlos III-FIS (co-funded by European Union [ERDF/ESF, “Investing in your future”; PI16/00264, and “Miguel Servet” Program CP15/00156]), Ministerio de Ciencia, Innovación y Universidades, (BFU2016-80360-R) and CIBERobn. Ciber is an initiative of Instituto de Salud Carlos III, Ministerio de Ciencia, Innovación y Universidades, Spain.

Author Contributions

Conception and design of research: M.C.V.-B., T.L., M.D.C., J.P.C., and R.M.L.; Performed experiments: M.C.V.-B., F.L.-L., A.C.F.-F., and A.D.H.-M.; Analyzed data: M.C.V.-B., A.C.F.-F., J.P.C., and R.M.L.; Interpreted results: M.C.V.-B., A.C.F.-F., M.D.G., M.D.C., J.P.C., and R.M.L.; Prepared figures: M.C.V.-B., A.C.F.-F., and R.M.L.; Acquisition of clinical/pathological data and samples: M.A.-G., E.V.-M., A.D.H.-M., C.B.-A., J.S., and A.S.; Wrote the manuscript: M.C.V.-B., M.D.G., J.P.C., and R.M.L.; Critically revised the manuscript and approved final version: M.C.V.-B., F.L.-L., M.A.-G., A.C.F.-F., E.V.-M., A.D.H.-M., C.B.-A., J.S., T.L., M.D.G., A.S., M.D.C., J.P.C., and R.M.L.

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