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Effects of novel somatostatin-dopamine chimeric drugs in 2D and 3D cell culture models of neuroendocrine tumors

Aura D Herrera-Martínez^{1,2,3}, Rosanna van den Dungen¹, Fadime Dogan-Oruc¹, Peter M van Koetsveld¹, Michael D Culler^{4,†}, Wouter W de Herder¹, Raúl M Luque^{2,3,5,6}, Richard A Feelders¹ and Leo J Hofland¹

¹Department of Internal Medicine, Division of Endocrinology, Erasmus Medical Center, Rotterdam, the Netherlands

²Maimonides Institute for Biomedical Research of Cordoba (IMIBIC), Córdoba, Spain

³Reina Sofia University Hospital, Córdoba, Spain

⁴Ipsen Bioscience, Cambridge, Massachusetts, USA

⁵Department of Cell Biology, Physiology, and Immunology, University of Córdoba, Córdoba, Spain

⁶CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Córdoba, Spain

Correspondence should be addressed to L J Hofland: l.hofland@erasmusmc.nl

[†](M D Culler is now at Culler Consulting, LLC, Cambridge, Massachusetts, USA)

Abstract

Control of symptoms related to hormonal hypersecretion by functioning neuroendocrine tumors (NETs) is challenging. New therapeutic options are required. Since novel *in vitro* tumor models seem to better mimic the tumor *in vivo* conditions, we aimed to study the effect of somatostatin and dopamine receptor agonists (octreotide and cabergoline, respectively) and novel somatostatin-dopamine chimeric multi-receptor drugs (BIM-065, BIM-23A760) using 2D (monolayer) and 3D (spheroids) cultures. Dose-response studies in 2D and 3D human pancreatic NET cell cultures (BON-1 and QGP-1) were performed under serum-containing and serum-deprived conditions. Cell proliferation, somatostatin and dopamine receptor expression (SSTs and D2R), apoptosis, lactate dehydrogenase, as well as serotonin and chromogranin A (CgA) release were assessed. The following results were obtained. 3D cultures of BON-1/QGP-1 allowed better cell survival than 2D cultures in serum-deprived conditions. SSTs and D2R mRNA levels were higher in the 3D model vs 2D model. Octreotide/cabergoline/BIM-065/BIM-23A760 treatment did not affect cell growth or spheroid size. In BON-1 2D-cultures, only BIM-23A760 significantly inhibited CgA release –this effect being more pronounced in 3D cultures. In BON-1 2D cultures, cabergoline/BIM-065/BIM-23A760 treatment decreased serotonin release (maximal effect up to 40%), being this effect again more potent in 3D cultures (up to 67% inhibition; with BIM-23A760 having the most potent effects). In QGP-1, cabergoline/BIM-065 treatment decreased serotonin release only in the 3D model. In conclusion, cultures of NET 3D spheroids represent a promising method for evaluating cell proliferation and secretion in NET cell-line models. Compared to 2D models, 3D models grow relatively serum independent. In 3D model, SST-D2R multi-receptor targeting drugs inhibit CgA and serotonin secretion, but not NET cell growth.

Key Words

- ▶ somatostatin-dopamine chimeric drugs
- ▶ neuroendocrine tumors
- ▶ 3D cell cultures
- ▶ hormone secretion

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Introduction

Neuroendocrine tumors (NETs) are rare, usually slow-growing, heterogeneous tumors that originate from the diffuse neuroendocrine system in the gastrointestinal and respiratory tract. Their incidence has increased in the last years, but better prognosis and encouraging increased survival has been reported due to advances in the diagnostic and therapeutic options (Dasari *et al.* 2017). Despite this, treatment of NETs remains challenging. Tumor heterogeneity and the fact that a high proportion of patients present with metastatic disease at diagnosis may contribute to it.

The treatment aim of NETs is twofold, that is symptom control by inhibition of hormonal overproduction in case of functional NETs and stabilization of tumor growth (Riihimäki *et al.* 2016, Herrera-Martínez *et al.* 2017, 2018). Somatostatin receptors (SSTs) are widely expressed in NETs, especially the SST₂ and SST₅ subtypes (Kubota *et al.* 1994, O'Toole *et al.* 2009, Nakayama *et al.* 2010), where they exert different functions (Herrera-Martínez *et al.* 2018). Somatostatin analogs (SSAs) represent the first therapeutic option in non-resectable well-differentiated NETs, especially in functioning tumors (Falconi *et al.* 2016), where they exert antitumor activity/tumor stabilization and symptoms control (Modlin *et al.* 2008, Veenstra *et al.* 2013, Pavel *et al.* 2017). Classical SSAs bind mainly to SST₂ receptors. However, it has been observed that SSAs that bind multiple SSTs subtypes have increased efficacy in inhibiting growth hormone overproduction, especially when used in combination with dopamine agonists (DAs) (Flogstad *et al.* 1994, Marzullo *et al.* 1999). As such, there is increasing interest in multiple receptor targeting by the use of dopastatins, which are chimeric somatostatin/dopamine receptor subtype-2 (D2R) ligands that have high affinity to SST₂, SST₅, and D2R (Couvelard *et al.* 2017). Since O'Toole *et al.* demonstrated the co-expression of SST₂ and D2R in GEP-NETs (O'Toole *et al.* 2006), the interest in multi-target directed therapies for NETs has increased. Nevertheless, the functional role of co-targeting SST₂ and D2R in NETs remains to be fully established.

Moreover, in the last decades, there is increasing interest in the development of better *in vitro* models for studying therapies in cancer models. Cell-based assays represent a simple, fast, and cost-effective tool for drugs discovery and evaluation (Edmondson *et al.* 2014). Most cell-based assays use traditional 2D monolayer cell cultures on flat, rigid substrates. 2D monolayer cultures have some limitations, and it has been recognized that

the effectiveness of drugs in 2D monolayer *in vitro* culture systems are often not representative for drug effectiveness *in vivo* (Edmondson *et al.* 2014). This discrepancy may be related to drug metabolism *in vivo* and/or the effects of the immune system. At the same time, 2D models lack the cell-cell contact, tumor-stromal interaction and tumor microenvironment (Edmondson *et al.* 2014, Costa *et al.* 2016). Therefore, during recent years, there is increasing interest in the development of *in vitro* models that better mimic the *in vivo* conditions in patients (Costa *et al.* 2016). Currently different methods have been developed to assemble 3D cell culture models. One of these, spheroid cultures, seem to appropriately reproduce tumor cells environment including, cell-cell signaling, growth kinetics, extracellular matrix deposition, nutrients-oxygen conditions, gene expression, drug resistance, cell heterogeneity and cell-cell physical interactions (Costa *et al.* 2016). 3D models with spheroids also allow the evaluation of long-term treatment and the generation of new treatment models (Harma *et al.* 2010, Kondo *et al.* 2011, Baker & Chen 2012, Kimlin *et al.* 2013). Moreover, such models seems to be a reliable method for evaluating treatment in NETs cell lines (Wong *et al.* 2012).

Based on the information described above, the aim of the current study was twofold: (1) to compare 2D and 3D NET cell culture models in terms of growth and hormone secretion, (2) to evaluate and compare the effects of two chimeric SSTs-D2R multi-receptor targeting drugs (BIM-065 and BIM-760) with the effects of the classical SST₂ and D2R targeting drugs octreotide (OCT) and cabergoline (CAB), respectively, on cell growth and serotonin and chromogranin A (CgA) secretion in both 2D and 3D pancreatic neuroendocrine tumor (PNET) culture models.

Materials and methods

Cell cultures

We used the human PNET cell lines BON-1 and QGP-1. The BON-1 cell line (kind gift from Dr Townsend, The University of Texas Medical Branch, Galveston, TX, USA) was derived from a lymph node metastasis of a human non-functional PNET. The QGP-1 cell line was established from a somatostatin-secreting pancreatic islet cell carcinoma and purchased from the Japanese Collection of Research Bioresources Cell Bank (JRCB, Osaka, Japan) (Kaku *et al.* 1980).

BON-1 cells were cultured in D-MEM/F12 (GIBCO Biocult Europe) containing 10% fetal calf serum (FCS),

L-glutamine, fungizone (1.25 mg/L) and penicillin (10⁵ U/L) (Bristol-Myers Squibb). QGP-1 cells were cultured in RPMI-1640 (GIBCO Biocult Europe) containing 10% FCS and penicillin (10⁵ U/L) (Bristol-Myers Squibb). Cell lines were cultured in 75 cm² flasks (Greiner bio-one, The Netherlands) at 37°C in a 5% CO₂ incubator. Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM) and resuspended in culture medium. Cell viability always exceeded 85%. Experiments were performed in optimal nutrient conditions (medium containing 10% FCS), as well as in serum-deprived conditions (medium containing 0.1% bovine serum albumin (BSA))

2D cultures (monolayer)

Cells were plated in 24-well plates with 1 mL medium at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment (50000 cells/well for BON-1 cells, 30000 cells/well for QGP-1 cells). Treatment was added after 24 h of incubation (day 0). For the serotonin and CgA assays, cells were cultured in medium containing 0.1% BSA. Cells were incubated for 24 h in medium with 10% FCS to allow cell attachment, after which the medium was refreshed with medium containing 0.1% BSA. In those experiments performed in 0.1% BSA, a higher number of cells was plated (85000 cells/well for BON-1 cells, 100000 cells/well for QGP-1 cells), again in order to obtain 65–70% cell confluence in the control groups at the end of the experiment.

3D cultures (spheroids)

Non-scaffold based 3D cell cultures were prepared. Cells were plated in cell-repellent-coated 24-wells plates, (Cellstar, Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) using a density of 750 cells/well and 1500 cells/well for BON-1 and QGP-1, respectively. After an initial 72 h of incubation in 1 mL medium with 10% FCS, spheroids were washed two times with medium containing 0.1% BSA, followed by incubation with the indicated drugs and the indicated incubation times, in medium containing 0.1% BSA. This day was defined as day 0 of incubation. Medium and cells were harvested after 3 and 7 days of incubation. In initial experiments, spheroids were also grown in complete medium containing 10% FCS for 3 and 7 days.

Drugs and reagents

CAB was purchased from Sigma-Aldrich (Cat.no. C-0246; Zwijndrecht, The Netherlands), OCT was purchased

from Novartis, and multi-chimeric drugs (BIM-065, BIM-23A760) were provided by Ipsen. The binding affinity of BIM-23A760 and BIM-065 is shown in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article). CAB was diluted in 100% EtOH until a stock solution of 10^{−4} M. BIM-065 and BIM-23A760 were diluted in 0.01 N acetic acid with 0.1% BSA, until a stock solution of 10^{−4} M. Further dilutions were made in culture medium until obtaining the final concentrations tested 10^{−7}–10^{−11} M.

Cell proliferation assay

Measurement of total DNA content

After the incubation period, monolayer cells (2D model) and spheroids (3D model) were harvested for DNA measurement, as a measure of cell number. The procedure for the total DNA measurement has been previously described in detail ([Downs & Wilfinger 1983](#), [Hofland *et al.* 1990](#)). Briefly, the cell pellet was treated with 150 µL of ammonia solution (1 mol/L) – Triton X 100 (0.2% v/v). 15 min later, sonification was performed (Soniprep 150; amplitude 1400 microns). Thereafter, for 2D model, 1 mL of assay buffer (100 mM NaCl, 100 mM EDTA, 10 mM Tris; pH 7.0) was added, and 20 µL of the solution was mixed with 200 µL of Hoechst dye H33258 solution (1 µg/mL). The fluorescence was measured with the excitation and emission wavelengths set at 350–455 nm and referenced to a standard curve of calf thymus DNA (type II, no D-3636; Sigma-Aldrich). For 3D model, the sensitive Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) was used following the protocol from the manufacturer. The fluorescence was measured at 485–535 nm respectively and referenced to the curve of the assay kit.

Spheroids growth measurement

The growth (spheroid size) tracking of the 3D model was performed by image analysis of spheroids using a Zeiss Axiovert 200/M-based phase-contrast microscope connected to a digital camera. Photographs of spheroids were measured at days 0, 3 and 7 of incubation. The growth was determined by measuring the cross-sectional area using the ImageJ software. Data (measurement in pixels) were compared to untreated control spheroids.

Quantitative RT-PCR

mRNA expression levels of SST subtypes (SST₁, SST₂, SST₃, SST₅) and D2R in BON-1 and QGP-1 monolayer and spheroids was evaluated by quantitative RT-PCR (qPCR).

We used a previously described method (Ferone *et al.* 2002). In short, poly(A⁺) mRNA was isolated using Dynabeads Oligo (Deoxythymidine)₂₅ (DynaL AS, Oslo, Norway). The poly(A⁺) mRNA was eluted in H₂O (65°C) twice for 2 min each and used for cDNA synthesis in a Tris buffer (50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM dithiothreitol, and 10 mM MgCl₂) with 10 U of ribonuclease inhibitor, 2 U of avian myeloblastosis virus Super Reverse Transcriptase and 1 mM of each deoxynucleotide triphosphate in a final volume of 40 µL. This mixture was incubated for 1 h at 42°C, and the resulting cDNA was diluted fivefold in 160 µL sterile H₂O. The total reaction volume (25 µL) consisted of 10 µL of cDNA and 15 µL of TaqMan Universal PCR Mastermix (Applied Biosystems). Primers were used at final concentration of 300 nM and probe at 200 nM. Real-time qPCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems, Roche) in the ABI Prism 7700 Sequence Detection System (PerkinElmer, Foster City, CA, USA). After two initial heating steps at 50°C (2 min) and 95°C (10 min), samples were subjected to 40 cycles of denaturation at 95°C (15 s) and annealing at 60°C (60 s). All samples were assayed in duplicate. Values were normalized against the expression of hypoxanthine-guanine phosphoribosyl-transferase (*HPRT*; used as housekeeping gene). Dilution curves were constructed to calculate PCR efficiencies (*E*) for every primer-probe set. To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template. The sequence of used primers and efficiencies are described in Supplementary Table 2. The relative expression of genes was calculated using the comparative threshold method, $2^{-\Delta C_T}$ (Schmittgen & Livak 2008), after efficiency correction of target and reference gene transcripts (*HPRT*) (Pfaffl 2001).

Chromogranin A and serotonin secretion assay

The medium of monolayer and spheroids cultures was collected from each well. Ascorbic acid (0.1%) was added into all the samples that were used for the serotonin assay. The commercially available human chromogranin A (CgA) ELISA kit (Epitope Diagnostics Inc, San Diego, CA, USA) and the serotonin high sensitive ELISA (IBL International, Hamburg, Germany) were used following the instructions of the manufacturer.

Apoptosis-lactate dehydrogenase (LDH) ELISA

Cells were plated in 24-well plates at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment. At the end of the experiment, medium and cells were collected. A commercially available ELISA (Cell Death Detection ELISA Plus, Roche) was used to determine apoptosis (DNA fragmentation) in cell lysates according to the protocol of the manufacturer. Similarly, a ELISA kit was used to measure the LDH (Pierce LDH Cytotoxicity Assay Kit, Thermo Scientific), to determine LDH release in the medium.

Statistical analysis

For the statistical analysis, the GraphPad Prism version 5 software was used (GraphPad Software). Comparisons between groups were analyzed by the Kruskal–Wallis test (non-parametric data). Differences were taken to be statistically significant at $P < 0.05$. Results are expressed as mean $\pm$ S.E.M. and as the percentage of the respective controls. Log transformation was used for calculating the IC₅₀.

Results

Pancreatic NET cell-line model in 2D (monolayer) and 3D (spheroids) cultures

In the BON-1 2D-model, the DNA content increased 5.8-fold after 3 days and 10.5-fold after 7 days of incubation in 10% FCS. In medium containing 0.1% BSA (serum-deprived conditions), the DNA content increased fourfold after 3 days and 4.8-fold after 7 days of incubation (Fig. 1A). In the BON-1 3D-model in medium with 10% FCS, the DNA content increased in a time-dependent manner after 3 (2.8-fold) and 7 days (4.6-fold); in serum-deprived conditions, a comparable increase in cell growth was observed after 3 (1.9-fold) and 7 days (4.1-fold; Fig. 1B).

In the QGP-1 2D-model in medium containing 10% FCS, a 4.2- and 6-fold increase in cell growth was observed after 3 and 7 days of incubation, respectively. In medium containing 0.1% BSA, DNA content increased only 2.2- and 2.4-fold after 3 and 7 days, respectively (Fig. 1C). In the QGP-1 3D model, the DNA content increased 2.3- and 4-fold after 3 and 7 days, respectively, in medium with 10% FCS and 1.9- and 3.8-fold after 3 and 7 days, respectively, in serum-deprived conditions (Fig. 1D).

Figure 1

BON-1 and QGP-1 cell growth in 2D- (monolayer) and 3D- (spheroids) cultures in different medium conditions. Cell growth (DNA content per well) was evaluated in untreated control cells at day zero (0) and after three (3) and seven (7) days of incubation. Panels A (2D) and B (3D) cultures of BON-1 cells; panels C (2D) and D (3D) represent cultures of QGP-1 cells. Dotted lines represent cell cultures in medium containing 10% FCS and the continuous black lines represent cell cultures in medium containing 0.1% BSA. Values represent the mean $\pm$ S.E.M. and are shown as a percentage of control at day 0. Asterisks: * P < 0.05; ** P < 0.01; *** P < 0.001.

In order to evaluate the attenuated cell proliferation in serum-deprived conditions in the 2D-model, we determined the apoptosis and LDH production in both culture models. In both BON-1 (Fig. 2A) and QGP-1 (Fig. 2C) 2D models, apoptotic rate at day 7 was 7-fold and 1.5-fold higher, respectively, in cells grown in medium with 0.1% BSA compared to cells cultured with 10% FCS medium. However, apoptotic rate was similar or even lower in cells grown in serum-deprived medium, compared to medium with 10% FCS in the BON-1 (Fig. 2B) and QGP-1 (Fig. 2D) 3D models.

In serum-deprived medium, but not in medium with 10% FCS, LDH release was detectable in QGP-1 2D-model after 3 and 7 days of incubation and in BON-1 2D-model after 7 days (Supplementary Table 3). On the other hand, LDH was not detectable, even after 7 days of treatment in the 3D model of both cell lines in serum-deprived and FCS-containing medium (data not shown).

In addition, in contrast to the 2D model, spheroid size and DNA content in BON-1 3D model strongly increased in a time-dependent manner both in FCS-containing and

Figure 2

DNA fragmentation (apoptosis) in 2D and 3D cultures in BON-1 and QGP-1 cell lines in different medium conditions. Panels A (2D) and B (3D) represent BON-1 cultures; panels C (2D) and D (3D) represent QGP-1 cultures. White bars represent cell cultures in medium containing 10% FCS and the gray bars represent cell cultures in medium containing 0.1% BSA. Apoptosis was higher in BON-1 spheroids after seven days (absolute values of 0.0041 ± 0.0009 – 0.003 ± 0.001 in optimal and serum-deprived conditions respectively) than in QGP-1 spheroids (absolute values of 0.001 ± 0 – 0.001 ± 0 in optimal and serum-deprived conditions respectively). Values represent the mean $\pm$ S.E.M. and are shown as a percentage of cells cultured in 10%FCS. Asterisks: * P < 0.05; *** P < 0.001.

in serum-deprived conditions (Fig. 3A and B respectively). Similar to what has been observed in BON-1, the size and the DNA content in QGP-1 spheroids increased in a time-dependent manner when cultured in optimal serum conditions (Fig. 3C). However, in serum-deprived conditions, the increase in DNA content and spheroid size were dissociated, for example, DNA content increased in a time-dependent manner, but spheroid size progressively decreased (Fig. 3D).

SSTs and D2R expression in monolayer and spheroids cultures

Both BON-1 and QGP-1 cells primarily expressed SST₁, SST₅ and D2R. Overall, the mRNA levels of SSTs subtypes and D2R were considerably higher in BON-1 cells, compared to QGP-1 cells (Supplementary Fig. 1). Since the 3D model may better mimic the gene expression in solid tumors, we evaluated the time-dependent mRNA levels of SSTs

Figure 3

Cell proliferation and spheroid size in BON-1 and QGP-1 cells grown as 3D spheroids in different medium conditions. Cells were followed by imaging using a Zeiss Axiovert 200/M-based phase-contrast microscope using a 5× objective. Representative images of BON-1 and QGP-1 spheroids after their formation (day 0) and after three and seven days culture are depicted in the right panels. Left panels: Increase in BON-1 spheroid cell growth and spheroid size in medium containing 10% FCS (A) and 0.1% BSA (B); Increase in QGP-1 spheroid cell growth and spheroid size in medium containing 10% FCS (C) and 0.1% BSA (D). Continuous black lines represent cell growth (DNA content), dotted lines represent spheroid size. Values represent mean $\pm$ s.e.m. and are shown as a percentage of control at day 0. Asterisks: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

subtypes and D2R in both systems. Overall, the mRNA expression of SSTs and D2R was significantly higher in 3D model with spheroids compared to 2D cultures, except for D2R in BON-1 cells and for SST₅ in QGP-1 cells after 7 days of culture (Supplementary Fig. 2).

In the 2D model of BON-1, a time-dependent decrease in the mRNA expression of SST₂ and SST₃ was observed (Fig. 4A). In the QGP-1 2D model, a time-dependent increase in SST₅ expression and a non-significant increase in D2R mRNA levels was observed (Fig. 4B).

In the 3D models, the mRNA levels of SSTs subtypes and D2R was higher in both cell lines compared to the 2D models of both cell lines. Similar to the BON-1 2D model, mRNA levels of SST₂ decreased and the expression of D2R increased in a time-dependent manner in the spheroid 3D model (Fig. 4C). In QGP-1 spheroids, the mRNA levels of SST₁, SST₂ and SST₅ decreased in a time-dependent manner, whereas the expression of D2R significantly increased (Fig. 4D).

Proliferation assays

In BON-1 cells, no statistically significant effect was observed on cell growth (DNA content) after treatment with CAB, BIM-065, BIM-23760 or OCT in 2D models after 3 days of incubation in serum-deprived conditions (Fig. 5A). Similarly, no effect on cell growth of any of the drugs tested was observed after 3 and 7 days of incubation in medium containing 10% FCS (Supplementary Fig. 3A and B respectively). In the BON-1 3D model, also no statistical significant effect was observed on the spheroid growth after 3 or 7 days of treatment in serum-deprived conditions (Fig. 5B and C respectively). Similar results were observed on QGP-1 2D-model in optimal, FCS-containing conditions (Supplementary Fig. 3C and D), as well as in serum-deprived conditions (Fig. 5D) and in spheroid 3D-models (Fig. 5E and F).

In agreement with the absence of an effect of the drugs on cell growth (DNA content), no statistically significant effect was observed on spheroid size after three and seven days of incubation in BON-1 (Supplementary Fig. 4A and B respectively) or in QGP-1 spheroid 3D-models (Supplementary Fig. 4C and D respectively).

CgA and serotonin secretion

It was not possible to measure serotonin secretion in BON-1 and QGP-1 cells cultured in medium with FCS, due to a matrix effect in the serotonin assay (data not shown). In order to determine the effect of the drugs on

serotonin secretion, PNET cells were cultured in medium containing 0.1% BSA, as described earlier.

CgA and serotonin secretion was determined in 2D models only after 3 days of incubation because of the increased apoptosis and decreased cell viability observed after seven days in serum-deprived medium with 0.1% BSA. In the BON-1 2D-model, BIM-23A760 (10^{-8} – 10^{-10} M) slightly, but statistically significant, inhibited CgA release (11–20%) after three days of treatment (Fig. 6A). The other evaluated drugs did not inhibit significantly the secretion of CgA at concentrations between 10^{-7} and 10^{-10} M. CgA release was not measurable in QGP-1 cells.

In the same 2D culture system, serotonin release in BON-1 cells was decreased in a dose-dependent manner by CAB (10^{-7} – 10^{-10} M; 14–34%), BIM-065 (10^{-7} – 10^{-11} M; 16–24%) and most potently by BIM-23A760 (10^{-7} – 10^{-8} M; 37–43%; Fig. 6B). Octreotide 10^{-7} – 10^{-10} M did not decrease serotonin secretion in this model in a statistically significant manner. In the QGP-1 2D-model, no statistically significant effect was observed on serotonin secretion after 3 days of incubation with 10^{-7} – 10^{-10} M of CAB, BIM-065, BIM-23A760 or OCT (Fig. 6C). At clinically feasible concentrations of CAB and OCT (10^{-8} – 10^{-9} M), the most potent compound for inhibiting serotonin secretion in the 2D-model was BIM-23A760 (43% ($P<0.001$)–26% ($P<0.01$) respectively) in BON-1 cells.

CgA and serotonin release in 3D models were determined only after 7 days of incubation because hormone concentrations in the medium after 3 days of incubation were not high enough (spheroids still too small) to measure accurately. In the BON-1 3D-model, CAB (10^{-7} – 10^{-10} M) inhibited CgA release by 18–23% and BIM-065 (10^{-7} – 10^{-10} M) by 31.7–32.3% (Fig. 7A). BIM-23A760 and OCT (10^{-7} – 10^{-10} M) did not inhibit CgA secretion in a statistically significant manner (Fig. 7A). Comparable to the 2D model, CgA release was not measurable in QGP-1 3D-model. In BON-1 spheroids, serotonin secretion was decreased in a dose-dependent manner by CAB (10^{-7} – 10^{-11} M; 36–40%); BIM-065 (10^{-7} – 10^{-10} M; 9–42%) and BIM-23A760 (10^{-7} – 10^{-11} M; 38–62%; Fig. 7B). No statistically significant effect was observed on serotonin secretion when BON-1 cells were treated with OCT (Fig. 7B). In QGP-1 spheroids, CAB (10^{-7} – 10^{-11} M) and BIM-065 (10^{-7} – 10^{-11} M) decreased serotonin production by 29–38% and 25–47%, respectively (Fig. 7C). BIM23A70 and OCT did not significantly inhibit serotonin secretion in this model (Fig. 7C). At clinically feasible concentrations of CAB and OCT (10^{-8} – 10^{-9} M), the most potent compound for inhibiting serotonin secretion in the 3D-model was BIM-23A760 (51% ($P<0.01$)–62%

Figure 4

mRNA expression profile of somatostatin receptor subtypes and dopamine type 2 receptor in BON-1 and QGP-1 cell lines using 2D and 3D culture systems. Relative mRNA relative expression (normalized to *HPRT*) in 2D (monolayer) cultures of BON-1 (A) and QGP-1 (B) cell lines and relative mRNA relative expression in 3D (spheroid) BON-1 (C) and QGP-1 (D) spheroids. Receptor expression after 3 and 7 days was compared to day 0. ND: not detectable. Asterisks: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to monolayer (2D) or spheroid (3D) at day 0.

($P < 0.001$) respectively) in BON-1 cells and BIM-065 in QGP-1 cells (43% ($P < 0.01$)–39% ($P < 0.001$) respectively).

Discussion

In the present study, we have systematically compared 2D (monolayer) and 3D (spheroid) culture systems of PNET cells in serum-containing and serum-deprived conditions.

Since both model systems may provide complementary information, the effects of SSTs-D2R multi-receptor targeting drugs, SSAs and DAs were evaluated. To the best of our knowledge, this is the first characterization of these PNET cell lines using both culture systems in different nutrient conditions.

In solid tumors, monolayer cell cultures cannot mimic the structure and drug resistance conferred by

Figure 5

Effect of cabergoline, BIM065, BIM23A760 and octreotide on growth of BON-1 and QGP-1 cells cultured in 2D (monolayer) or 3D (spheroids) in medium with 0.1% BSA. 2D cultures of BON-1 cells after three days of incubation (A); 3D cultures of BON-1 cells after three (B) or seven (7) days of incubation (C). 2D cultures of QGP-1 cells after 3 days of incubation (D); 3D cultures of QGP-1 cells after 3 (E) or 7 (F) days of incubation with the indicated drugs. Cell growth (DNA content) is expressed as the percentage of untreated control (mean $\pm$ SEM).

elements of the tumor microenvironment (Breslin & O'Driscoll 2013). This situation may explain the eventual lack of correspondence between some *in vitro* findings and their application in *in vivo* conditions (Costa *et al.* 2016). In this context, 3D models seem to be a promising method to evaluate anticancer therapies, since they better mimic the 3D structure of the tumor and the surrounding microenvironment (Breslin & O'Driscoll 2013). These characteristics may explain, for example, the lower apoptotic rate and increased drug resistance found in breast cancer spheroids when compared to monolayer cultures (Au leong *et al.* 2017). In our study, a significant lower apoptotic rate was also observed in spheroids when compared to monolayer cultures.

Spheroids also seem to appropriately mimic nutrients availability and hypoxia (Mehta *et al.* 2012). Cells located

in the peripheral spheroid-area have higher proliferation rates compared to those senescent or necrotic cells located in the center of the spheroid (Mehta *et al.* 2012). A difference in the proliferation rate is explained by their easier access to oxygen and nutrients (Tredan *et al.* 2007) and may be graphically represented with the difference in color intensity inside the BON-1 spheroids, which progressively increased after three/seven days of incubation. According to our results, spheroid cultures of BON-1/QGP-1 cells allowed better cell survival in serum-deprived conditions, admitting longer incubation periods with optimal (time-dependent) increase in the DNA content, even in serum-deprived conditions. This may be useful for evaluating long-term drug effects. The complete lack of correspondence between the DNA content and the spheroid size in QGP-1 3D-model in serum-deprived

Figure 6

Effect of cabergoline, BIM065, BIM23A760 and octreotide on chromogranin A and serotonin secretion in 2D (monolayer) cultures of BON-1 and QGP-1 cells. Cells were incubated with the indicated drugs during three days in medium with 0.1% BSA. Chromogranin A (CgA) release in BON-1 cells (A); serotonin release in BON-1 cells (B); serotonin release in QGP-1 cells (C). Values represent mean $\pm$ S.E.M. and are shown as a percentage of untreated control. Asterisks: * P < 0.05; ** P < 0.01; *** P < 0.001, compared to untreated controls (serotonin absolute values in controls of BON-1: 521.5 $\pm$ 57.8; QGP-1: 192.2 $\pm$ 16.3 pg/mL; CgA absolute values of BON-1 controls: 721.6 $\pm$ 38.8 ng/mL).

Figure 7

Effect of cabergoline, BIM065, BIM23A760 and octreotide on chromogranin A and serotonin secretion on 3D (spheroid) cultures of BON-1 and QGP-1 cells. 3D spheroids were incubated during seven days with the indicated drugs in medium with 0.1% BSA. Chromogranin A (CgA) release in BON-1 cells (A); serotonin release in BON-1 cells (B); serotonin release in QGP-1 cells (C). Values represent mean $\pm$ s.e.m. and are shown as a percentage of untreated control. Asterisks: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to untreated controls (serotonin absolute values in BON-1 controls: $238.21.5 \pm 21.6$; QGP-1: 13.67 ± 1.8 pg/mL; CgA absolute values of BON-1 controls: 297.6 ± 14.89 ng/mL).

conditions, suggests that nutrient conditions may influence cell compactness and, as a consequence, the spheroid size. These data emphasize that spheroid size is not always a reliable measure of spheroid cell growth and we strongly advocate that spheroid size in 3D culture models should be correlated with other tumor growth markers before drawing conclusions, especially about drug effectiveness.

Similar to our results, previous studies have suggested that spherical (round) spheroids had decreased viability when compared to non-spherical (asymetric) spheroids, probably due to an increased distance between each tumor cell and the culture medium interface (Au *et al.* 2017). Despite the fact that the apoptosis was higher in BON-1 than in QGP-1 spheroids, apoptosis rate was significantly lower in the spheroid culture system than in the monolayer cultures after seven days of incubation, especially in serum-deprived conditions. Due to the increased apoptotic rate in monolayer cells cultured in serum-deprived conditions after 7 days, hormone secretion was only evaluated after 3 days of incubation. Serotonin secretion in 3D spheroids was lower than in monolayer cultures, due to lower cell numbers. After 3 days serotonin concentrations were below the detection limit of the assay. For this reason, serotonin production in the spheroid model was evaluated only after 7 days.

Previous studies have reported changes in the gene expression of spheroids when compared to monolayer cultures (Francia *et al.* 2004, Schmidt *et al.* 2016). In this sense, the up or downregulation of several genes may explain the differences in drug response in both culture models and may be associated to tumor treatment-resistance (Francia *et al.* 2004). Interestingly, in our study, mRNA levels of SSTs (BON-1 and QGP-1) and D2R (only in QGP-1) were higher in spheroids than in monolayer cultures, which could explain the increased effect of the evaluated drugs in the 3D culture system.

Both culture systems exhibited comparable changes in mRNA levels during culturing, for example a time-dependent decreased expression of SST₂ and increased expression of D2R. To the best of our knowledge, time-dependent changes in expression of SSTs and D2R in NET cells has not been reported before, but should be considered when interpreting data from *in vitro* studies. Importantly, both monolayer and spheroid cultures showed the same trend of changes, suggesting this is likely not an explanation for differences between the effects of the SSTs and D2R targeting drugs between the two culture models. Additionally, the increased expression of D2R in spheroids in both cell lines may also explain the potent effect of D2R targeting compounds in this culture system. As described earlier, NETs express both SSTs and D2R. If our results can be extrapolated to PNETs *in situ*, our data suggest that SSTs and D2R expression (and possibly response to SSA and D2R agonists) may change during disease progression. It would be valuable to confirm these findings using an *in vivo* model, since it suggests that the detection of expression level of SSTs and D2R in NET may only be a momentum.

Despite there are some differences between 2D and 3D culture systems, their usefulness may be complementary. As previously described, 2D monolayer cultures allow cell proliferation in stable conditions of nutrients, space and oxygen for all cells and the culture system is easy and not expensive. On the other hand, limitations include the lack of cell-cell and cell-extracellular matrix contact/signaling, cellular organization in layers and growth kinetics that are observed in solid tumors (Costa *et al.* 2016). These limitations are not present in 3D cultures with spheroids. 3D models also allow the study the interaction between cancer and stromal cells, reproducing the cellular distribution found in tumors *in vivo*. In addition, cells in 3D models are organized in three main layers (proliferative, senescent and necrotic layers),

growth kinetics and mRNA expression patterns mimic those ones observed in solid tumors (Costa *et al.* 2016). However, 3D cultures with spheroids also have disadvantages including the variability in size and shape of the spheroid, increased costs and time for performing experiments and specialized equipment for agitation-based phases is also required (Breslin & O'Driscoll 2013). In general, we consider 2D monolayer cultures as useful methods for evaluating the effects of drugs on cell proliferation, secretion and the underlying mechanisms, in particular after short incubation periods. In contrast, 3D cultures with spheroids can represent a valuable tool for evaluating the effects of a drug that requires serum-deprived conditions or longer incubation periods, as well as for evaluating the interaction between different cell types.

Cell cultures using spheroids have been used to assemble models of different cancer types including breast, cervical, colon, prostate, lung, pancreas and melanoma (Dufau *et al.* 2012, Ludwig *et al.* 2013, Vorsmann *et al.* 2013, Amann *et al.* 2014, Costa *et al.* 2014). The spheroid culture system was initially described in BON-1 and QGP-1 cell lines in 2012, and at that moment, the histone-deacetylase inhibitor trichostatin A was chosen as the model drug for evaluating 3D cultures in NETs (Wong *et al.* 2012). Recently, the effects of inhibitors of poly(ADP-ribose) polymerase-1 have been also evaluated in a NET 3D culture model (Samuel Adant *et al.* 2017). Despite the advantages of 3D cultures for the evaluation of drug effects in cancer, no other treatments have been evaluated in NETs.

NETs may cause specific clinical syndromes due to overproduction of hormones and bioactive peptides (Mougey & Alder 2007, Niederle *et al.* 2016). Consequently, symptoms control is a main goal in the treatment of these patients (Hofland *et al.* 2018). Despite SSAs are considered to be the preferred first-line treatment option in functionally active NETs, some patients may not respond or develop resistance (Falconi *et al.* 2016, Pavel *et al.* 2016), for this reason, novel therapeutic options are still required. In our study, serotonin secretion could only be measured in serum-deprived conditions, because of a serum-matrix effect in the assay. In contrast to 2D cultures, the use of 3D cultures allowed to study the more prolonged effects of drugs on serotonin secretion. Additionally, some transcription factors, which are present in serum may modify cell proliferation *in vitro*, thus serum-deprived conditions may also be required in some cases (Liang *et al.* 2013). Measurements in these conditions allow reliable results for the evaluation of drug responsiveness and

the underlying mechanisms. Furthermore, angiogenic properties of some cells *in vitro* may be modified when exposed to hormones and fatty acids, consequently, *in vitro* experiments should be designed in serum-deprived conditions as well (Vanetti *et al.* 2016, 2017).

As previously mentioned, it is well known that SSA and DAs are important therapeutic options in the management of several endocrine tumors, especially in those with hormone overproduction (Flogstad *et al.* 1994). SSTs and D2R share 30% of their gene sequence (Alonso-Gordoa *et al.* 2015). Both family of receptors have been described in NETs (van Adrichem *et al.* 2016) and a potential functional role of somatostatin-dopamine receptor dimerization has been also reported (Culler 2011, Gatto & Hofland 2011). The antiproliferative effect of octreotide and cabergoline has been reported *in vitro* in several types of cancer (Liu *et al.* 2004, Zou *et al.* 2009, Ayiomamitis *et al.* 2014, Lin *et al.* 2017) but, similarly to other *in vitro* publications (Exner *et al.* 2018) decreased proliferation rate was not observed in our pancreas NET model. The ideal chimeric molecule would have potent SST₂ and D2R affinity, with moderate activity at SST₅ (Culler 2011). The antiproliferative effect of somatostatin-dopamine chimeras on cell proliferation seems to be more efficient than SSAs or DAs in enterochromaffin-like cells according to some studies (Kidd *et al.* 2007), but other authors have described a lower effect on cell proliferation compared to specific SSA in atypical lung carcinoids (Kidd *et al.* 2008). It has been suggested that BIM-065 may control tumor cell growth in pituitary adenomas (Alexandraki 2017), but its effect on cell proliferation in NETs has not been described yet. According to our results, this novel SSTs-D2R chimera does not significantly affect cell proliferation in pancreas NET cell lines. Kidd *et al.* described a cytotoxic effect of some other somatostatin-dopamine chimeras, including BIM-23A760, in neuroendocrine tumor cells (small intestine, typical and atypical lung carcinoid cell lines; Kidd *et al.* 2008). In contrast, we did not observe an antiproliferative effect of BIM-23A760, which may be explained by the different somatostatin and dopamine expression profile of these cell lines, when compared to BON-1 and QGP-1 cells. These findings suggest the need to individualize therapeutic strategies based upon the analysis of the receptor profile of the tumor.

In addition, it has been suggested that targeting the D2R receptor is an effective mechanism for suppressing hormone secretion by NET (Kidd *et al.* 2008). CAB represents a useful therapeutic tool in the treatment of other hormone-related syndromes (Cozzi *et al.* 1998,

Koch *et al.* 2006, Godbout *et al.* 2010), including pancreatic polypeptide and ACTH-producing NETs (Pathak *et al.* 2004, Pivonello *et al.* 2007). Additionally, somatostatin-dopamine chimeric molecules have shown short-term effectiveness on hormone secretion according to preclinical and clinical trials in endocrine tumors (Culler 2011), whereas octreotide is recognized as a therapeutic option for carcinoid syndrome (Davis *et al.* 1980, Baldelli *et al.* 2014). In our study, BIM-23A760 was the most effective drug for inhibiting serotonin release in BON-1 monolayer and BON-1/QGP-1 spheroids, followed by CAB. The effect of BIM-065 was higher in QGP-1 than in BON-1 spheroids and comparable to CAB and BIM-23A760. This is the first report on the effects of this compound on serotonin secretion in NETs. Interestingly, while octreotide is widely used for reducing 5-hydroxyindolacetic acid in patients with NETs (O'Toole *et al.* 2000), in our PNET model, octreotide did not significantly decrease serotonin release in BON-1 monolayer and BON-1/QGP-1 spheroid cultures. Previous *in vitro* reports have described the inhibition of serotonin release by octreotide in human midgut carcinoid tumor cells (Wangberg *et al.* 1991); the tumor cell origin, the SSTs/D2R expression profile and the incubation period may explain the differences with our results. In addition, the effect of both chimeric compounds seems to be comparable to the dopamine agonist, suggesting that their effect in this model may be predominantly mediated by the D2R. Importantly, the study of the molecular expression of SSTs and D2R in NETs may help to identify those patients suitable for treatment (Kanakakis *et al.* 2015). Also remarkable is the strong increase in the mRNA expression of D2R in the 3D-culture model of QGP-1 cells, which could have enhanced the effect of CAB and the somatostatin-dopamine chimeric drugs. Unfortunately, the incubation period in 2D- and 3D-culture systems was different and a direct comparison could not be performed.

In contrast, CgA secretion was only slightly inhibited by BIM-23A760 in monolayer cultures and by CAB and BIM-065 in spheroids, which may be related to the absence of effect on cell proliferation, since CgA is considered as a marker related to tumor load (Nobels *et al.* 1997). Clinical studies have described a dose-dependent reduction of serum CgA with octreotide (Al-Efraij *et al.* 2015), which was not observed in our pancreas model.

In summary, this study provides a comprehensive comparison of 2D monolayer and 3D spheroid culture systems in a pancreas model for NETs and suggests that spheroids represent a valuable tool for evaluating long-term drug effectiveness, especially in serum-deprived conditions. Compared to 2D models, 3D models

grow relatively serum-independent. In the 3D model, SSTs-D2R multi-receptor targeting drugs inhibit CgA and serotonin secretion, but not NET cell growth. These results further highlight the importance of determining the somatostatin/dopamine receptor profile in NETs in order to identify those patients that may benefit from these compounds.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0086>.

Declaration of interest

M C is former employee of Ipsen Bioscience. Wouter de Herder is an Associate Editor of Endocrine-Related Cancer. Wouter de Herder was not involved in the review or editorial process for this paper, on which he is listed as an author. The other authors have nothing to disclose.

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