REVIEW

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The role of somatostatin and dopamine D_2 receptors in endocrine tumors

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Abstract

Somatostatin (SS) and dopamine (DA) receptors have been highlighted as two critical regulators in the negative control of hormonal secretion in a wide group of human endocrine tumors. Both families of receptors belong to the superfamily of G protein-coupled receptors and share a number of structural and functional characteristics. Because of the generally reported high expression of somatostatin receptors (SSTRs) in neuroendocrine tumors (NET), somatostatin analogs (SSA) have a pronounced role in the medical therapy for this class of tumors, especially pituitary adenomas and well-differentiated gastroenteropancreatic NET (GEP NET). Moreover, NET express not only SSTR but also frequently dopamine receptors (DRs), and DA agonists targeting the D_2 receptor (D_2) have been demonstrated to be effective in controlling hormone secretion and cell proliferation in *in vivo* and *in vitro* studies. The treatment with SSAs combined with DA agonists has already been demonstrated efficacious in a subgroup of patients with GH-secreting pituitary adenomas and few reported cases of carcinoids. The recent availability of new selective and universal SSA and DA agonists, as well as the chimeric SS/DA compounds, may shed new light on the potential role of SSTR and D_2 as combined targets for biotherapy in NET. This review provides an overview of the latest studies evaluating the expression of SSTR and DR in NET, focusing on their co-expression and the possible clinical implications of such co-expression. Moreover, the most recent insights in SSTR and D_2 pathophysiology and the future perspectives for treatment with SSA, DA agonists, and SS/DA chimeric compounds are discussed.

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Introduction

In recent years, somatostatin (SS) and dopamine (DA) receptors have been highlighted as two critical regulators involved in the negative control of hormonal secretion in a wide group of neuroendocrine tumors (NET), including pituitary adenomas (Ben-Jonathan & Hnasko 2001, Guillemin 2005, Ferone *et al*. 2009). Moreover, SS, DA, and their receptors represent two major systems that share a number of structural and functional characteristics (Rocheville *et al*. 2000*a*, Baragli *et al*. 2007, Srirajaskanthan *et al*. 2009). SS is a peptide present in mammals in two biologically active isoforms, consisting of 14 (SS-14) and 28 (SS-28) amino acids. Up to date, five human SS receptor subtypes (sst) have been cloned and characterized (Lamberts *et al*. 1996, Patel 1999, Moller *et al*. 2003). The transcript of the *sst*$_2$ gene can be present in two splice variants that differ only for the length of the cytoplasmic portion of the receptor (sst_{2A} and sst_{2B}). Somatostatin receptors (SSTRs) belong to the seven-transmembrane segment receptor superfamily and functionally couple to G proteins (Lamberts *et al*. 1996, Hofland & Lamberts 2003, Moller *et al*. 2003). SS binding to SSTR subtypes activates a series of second messenger systems, resulting in the inhibition of calcium channels and adenylate cyclase activity, ultimately leading to inhibition of hormone secretion (Reisine & Bell 1995, Patel 1997, 1999). Stimulation of other second messengers, such as phosphotyrosine phosphatases (PTPs), plays a role in SS- and somatostatin analogs (SSA)-mediated control of cell growth (Schally 1988, Lamberts *et al*. 1991, Hofland *et al*. 1995, Patel 1999, Hofland & Lamberts 2003, Florio 2008, Schonbrunn 2008). Among the SSTR, sst_3

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appears to be the subtype mostly related to the pro-apoptotic and antiproliferative effects of SS and SSA (Ferone *et al*. 2002, Hofland & Lamberts 2003).

DA is the predominant catecholamine neurotransmitter in the human central nervous system but also plays multiple roles in the periphery as a modulator of cardiovascular and renal function, gastrointestinal (GI) motility, as well as the endocrine system (Missale *et al*. 1998). DA exerts its functions via binding to dopamine receptors (DRs; Missale *et al*. 1998). DR are G protein-coupled receptors (GPCRs) acting predominantly via adenylate cyclase modulation. Up to now, five different DR, divided into two subfamilies, have been characterized. Activation of the D_1-like receptors (D_1 and D_5) results in a stimulation of adenylate cyclase activity, whereas on the contrary D_2-like subfamily (D_2, D_3, and D_4) stimulation leads to a Gi/Go protein-mediated decrease of intracellular cAMP (Missale *et al*. 1998). The DR subtypes have been demonstrated to exert heterogeneous roles in a number of different tissues and organs and show a tissue-specific distribution. Moreover, two different isoforms of the D_2 receptor have been isolated, the short (D_2 short) and long (D_2 long) form, hypothesized to activate distinct intracellular pathways and to mediate differential effects following ligand activation (Missale *et al*. 1998).

The SSTR and DR families share a 30% sequence homology and appear to be structurally related. Behavioral and clinical evidence in brain research indicated an interaction between the somatostatinergic and dopaminergic systems (Chneiweiss *et al*. 1985, Martin-Iverson *et al*. 1986, Izquierdo-Claros *et al*. 1997, Marzullo *et al*. 1999), recently confirmed by *in vitro* studies (Rocheville *et al*. 2000*a*, Baragli *et al*. 2007, Kidd *et al*. 2008). It is well known that SSTR and DR are widely expressed both in normal human neuroendocrine tissues and in tumors (Reubi *et al*. 1987, Pivonello *et al*. 2007*b*), such as pituitary adenomas (Stefaneanu *et al*. 2001, Moller *et al*. 2003, Saveanu *et al*. 2008, Ferone *et al*. 2009) and adrenal tumors (Pivonello *et al*. 2007*a*,*b*, de Bruin *et al*. 2009*a*). Since SSTR and D_2-like receptors mainly exert inhibitory functions, medical therapies targeting these receptors with selective agonists have been developed for the treatment of a number of neuroendocrine disorders. In the last decades, the knowledge on the pathophysiology of these two families of GPCRs in NET has progressively increased due to the new insights in receptor dimerization, internalization, and trafficking (Hofland & Lamberts 2003, Ferone *et al*. 2009, Lesche *et al*. 2009, Poll *et al*. 2010). Moreover, the recent availability and use of novel selective and universal SSAs and DA agonists, as well as the chimeric SS/DA compounds, has shed light on the potential role of SSTR and D_2 as combined targets for biotherapy in NET. Since SSTR and D_2 expression, co-expression, and their role as targets for medical treatment of patients with pituitary tumors have recently been extensively reviewed (Saveanu *et al*. 2006, Ferone *et al*. 2009), we will highlight in this review mainly the large and heterogeneous family of NET (previously known with the name of 'carcinoids'), which is emerging as a main field for SSTR and DR targeting therapy.

SSTR expression in endocrine tumors

High levels of SSTR expression are generally detected in NET, including pituitary adenomas, GI and lung carcinoids, endocrine pancreatic tumors, small cell lung carcinomas, pheochromocytomas, paragangliomas, and medullary thyroid carcinomas (Reubi *et al*. 1992*a*,*b*, 1994*a*, 1996, Sestini *et al*. 1996, Papotti *et al*. 2000, 2001*a*,*b*, 2002, Ueberberg *et al*. 2005, Taboada *et al*. 2007). SSTR expression in all these tumors has been largely studied by means of both *in vivo* and *in vitro* techniques. The SSTR subtype expression has been demonstrated at the mRNA level using *in situ* hybridization, RNase protection assays, and real-time PCR (RT-PCR) and at the protein level using western blotting, autoradiography, and immunohistochemistry (IHC; Kubota *et al*. 1994, Reubi *et al*. 1994*b*, Vikic-Topic *et al*. 1995, Janson *et al*. 1998, Kimura *et al*. 1999, Papotti *et al*. 2000, 2001*a*, 2001*b*, Kulaksiz *et al*. 2002, Reubi 2004, Korner *et al*. 2005, Righi *et al*. 2010).

As for pituitary adenomas, well-differentiated gastroenteropancreatic (GEP) NET represent a major target for SSA treatment (Oberg 2002, Oberg *et al*. 2004*a*,*b*, Batista *et al*. 2006, Falconi *et al*. 2006, O'Toole *et al*. 2006*a*).

Both pancreatic NET (including gastrinomas, glucagonomas, and VIPomas) and GI NET express SSTRs in 80–100% of the cases. Insulinomas have a lower incidence of SSTR expression (50–70%). The receptor density can vary among tumors, but it can be considered high in the majority of cases. Undifferentiated NET express SSTR (mainly sst_2 subtype) less frequently (and in lower density) than well-differentiated ones (Reubi 2007). On the other hand, sst_5 mRNA expression was demonstrated to be positively correlated with histopathological features of tumor aggressiveness in primary insulinomas (de Sa *et al*. 2006). There is consensus, based on various methodologies, that among the different SSTR subtypes, sst_2 is usually the most prominent, followed by sst_1 and sst_5,

while sst_3 is less frequently expressed and sst_4 almost absent (Reubi 2003, 2004, Reubi & Waser 2003). This high and heterogeneous expression did not show any relevant correlation between the subtype(s) expressed and the primary tumor origin, or a specific hormone secretion (Reubi *et al*. 1998*b*, Papotti *et al*. 2002, Volante *et al*. 2008).

Moreover, also lung NET, both well- and poorly differentiated, have been shown to express SSTR *in vivo*, being subtypes 2, 3, and 5 the most represented (Berenger *et al*. 1996, Reubi *et al*. 1996, 1998*b*, 2000, Janson *et al*. 1998, Reisinger *et al*. 1998, Hofland *et al*. 1999, Papotti *et al*. 2000, 2001*a*). A recent study evaluating the tissue distribution of sst_{2A} and sst_3 in a large series (>200 cases) of pulmonary NET with clinically aggressive features (Righi *et al*. 2010) confirmed previous data from small series, showing a decrease in the expression of sst_2 and sst_3 from low-grade/intermediate-grade to high-grade tumors, and confirming a good correlation between immunohistochemical results and octreotide scintigraphy (Reisinger *et al*. 1998, Granberg *et al*. 2003, Reubi & Waser 2003).

The catecholamine-producing and -secreting tumor pheochromocytoma has been shown to express SS (Reubi *et al*. 1992*c*) and more than one SSTR receptor both at mRNA and protein level (Reubi *et al*. 1992*c*, Kubota *et al*. 1994, Mundschenk *et al*. 2003, Kolby *et al*. 2006), being the subtypes 1–3 the most represented (Hofland & Lamberts 2003, Unger *et al*. 2004). Similarly, *in vivo* and *in vitro* studies on medullary thyroid carcinomas detected the presence of all sst subtypes, except sst_4, and showed a clear positivity for SS, indicating that possible autocrine/paracrine circuits may be active in this tumor (Pacini *et al*. 1991, Kwekkeboom *et al*. 1993, Mato *et al*. 1998, Papotti *et al*. 2001*b*, Zatelli *et al*. 2006). As indicated above, SSTR expression is largely heterogeneous both within the same tumor type and even more between NET arising from different tissues. However, sst_2 is reported to be the mostly represented subtype, even if frequently other SSTR subtypes are co-expressed.

Furthermore, immune cells and human lymphatic tissues represent other important sites of SSTR expression in the human body. Monocyte-derived cells and mature T-lymphocytes have been clearly demonstrated to predominantly express sst_2 and sst_3 respectively (Ferone *et al*. 2002, Lichtenauer-Kaligis *et al*. 2004). Moreover, human lymphoid follicle centers (Reubi *et al*. 1998*a*), thymus, and spleen (Ferone *et al*. 2011) have been reported to express SSTR subtypes 2 and 3 as well. Sst_1, sst_2, and sst_3 have been demonstrated as the most represented SSTR in thymic tumors, both at the protein and at the mRNA level (Ferone *et al*. 2001). Moreover, a number of malignancies arising from immune cells, such as Hodgkin disease, T and B non-Hodgkin lymphomas (Reubi *et al*. 1992*d*, Lugtenburg *et al*. 2001*a*,*b*), myeloma, and plasmacytoma (Georgii-Hemming *et al*. 1999, Duet *et al*. 2005) heterogeneously express SSTR, although the role of SSTR targeting for diagnosis or treatment in these tumors is still debated. Finally, a clear positivity by SSTR scintigraphy has been observed in a number of chronic inflammatory diseases, such as sarcoidosis (van Hagen *et al*. 1994, Ameri *et al*. 2007) and rheumatoid arthritis (ten Bokum *et al*. 1999).

DR expression in endocrine tumors

It has been extensively demonstrated, with various techniques, that DR are expressed in the large majority of pituitary adenomas, including GH-secreting, prolactin (PRL)-secreting, ACTH-secreting, and clinically non-functioning tumors (Panetta & Patel 1995, Stefaneanu *et al*. 2001, Pivonello *et al*. 2004*b*, Zatelli *et al*. 2005, Ferone *et al*. 2008, Saveanu *et al*. 2008). Furthermore, DR expression has been well characterized in other NET, such as pheochromocytomas (Pupilli *et al*. 1994, Pivonello *et al*. 2004*a*, 2007*b*) and paragangliomas (Wu *et al*. 2001).

Beyond the above-mentioned NET, the presence of DR – mainly D_2 – has been demonstrated in a small group of well-differentiated endocrine tumors (including lung and thymic carcinoids), associated with ectopic ACTH secretion and Cushing's syndrome (Pivonello *et al*. 2007*a*).

The observation of the expression of D_2 in GEP NET cell lines (Lemmer *et al*. 2002) has been followed by recent studies evaluating the expression of D_2 in a series of patients with NET (mainly GEP NET).

O'Toole *et al*. evaluated the quantitative expression of D_2 mRNA by RT-PCR in a series of 35 GEP NET. The expression of D_2 was detected in all samples, although D_2 expression level was similar to those observed in somatotroph adenomas only in the 17% of cases (O'Toole *et al*. 2006*b*).

Recently, Grossrubatscher *et al*. demonstrated the presence of D_2 in well-differentiated NET of different sites and in normal islet cells by IHC. They found a high expression of D_2 receptors among the tumors examined (85%), being bronchial carcinoids, islet cell tumors, and the NET of the duodenum the tumors with the highest receptor protein expression (Grossrubatscher *et al*. 2008). Moreover, they observed that a worse clinical outcome was more frequent among patients with less D_2 immunoreactivity,

although they could not find a significant correlation between the presence of D_2 receptors and Ki-67 expression in the tumors.

It has to be emphasized that, as for SSTR, comparison between results obtained with different techniques is difficult, and in some cases, mRNA detection with PCR may overestimate quantities expressed.

SS and DA receptor co-expression in endocrine tumors

Co-expression of SSTR and D_2 in pituitary adenomas has been recently reviewed extensively (Jaquet *et al*. 2005*b*, Ferone *et al*. 2009, Saveanu & Jaquet 2009). Here, we would like to underline few important aspects: the D_2 receptor is the GPCR mostly represented in the pituitary tumors, overall it is associated with two or more SSTR subtypes (preferentially sst_2 and sst_5), and there is a high variability in SSTR and D_2 expression due to the heterogeneity of these tumors (Zatelli *et al*. 2005, Ferone *et al*. 2008, Saveanu *et al*. 2008, de Bruin *et al*. 2009*c*).

Up to date, only few studies in the literature demonstrated SSTR and D_2 co-expression in endocrine tumors beyond the above-mentioned pituitary adenomas (Table 1). The majority of these studies were carried out on immortalized cell lines from lung and GEP carcinoids, small cell lung cancer (SCLC), and medullary thyroid carcinoma (Ferone *et al*. 2005, Kidd *et al*. 2007*b*, 2008, de Bruin *et al*. 2009*b*, Arvigo *et al*. 2010). To our knowledge, only two studies have been published so far describing SSTR and D_2 co-expression in patients with NET (not including pituitary adenomas). O'Toole *et al*. (2006*b*) evaluated quantitative co-expression of sst_1, sst_2, sst_3, and sst_5 with D_2 mRNA by RT-PCR in a series of 35 GEP NET. They observed co-expression of sst_2 and D_2 in 100% of cases and sst_3 and sst_5 in 89%. Moreover, they correlated quantitative mRNA expression in tumors of this group of patients with that observed in GH-secreting adenomas and showed that sst_2 levels were similar between GEP and somatotroph tumors, whereas sst_5 and D_2 levels were higher in the latter.

Recently, Srirajaskanthan *et al*., using IHC, demonstrated co-expression of sst_2, sst_5, and D_2 in a wide variety of NET (56 cases). They observed an overall expression of D_2 in 81% of the cases and sst_2 and sst_5 were identified in 93% of cases. Both D_2 and SSTR expression was higher in low- and intermediate-grade tumors compared with high-grade tumors (Srirajaskanthan *et al*. 2009).

Preliminary data by Pivonello *et al*. (2008) described sst_2, sst_5, and D_2 co-expression in ten different cases of pancreatic endocrine tumors, evaluated by IHC.

Table 1 Most recent studies reporting co-expression of somatostatin and D_2 receptors in human tumors (excluding pituitary adenomas)

Author (years)	**Materials**	**Receptor evaluation**	**Results**
Ferone *et al*. (2005)	NSCLC cell line	mRNA/protein (W.B.)	sst_2, sst_3, sst_5, and D_2 co-expression
O'Toole *et al*. (2006*b*)	35 GEP NET patients	mRNA	100% sst_1, sst_2, and D_2 co-expression
Kidd *et al*. (2008)	Atypical BP NET cell line	mRNA	sst_2, sst_5, and D_2 co-expression
	Typical BP NET cell line		sst_1, sst_2, sst_3, sst_5, and D_2 co-expression
	GI NET cell line		D_2 absent
Pivonello *et al*. (2008)	Ten pancreatic NET patients	Protein (IHC)	100% sst_2 and D_2 co-expression
			80% sst_2, sst_5, and D_2 co-expression
De Bruin *et al* (2009*b*)	Pancreatic carcinoid cell line	mRNA	sst_2, sst_5, and D_2 co-expression
	MTC cell line		sst_2, sst_5, and D_2 co-expression
	SCLC cell line		sst_2, sst_5, and D_2 co-expression
Srirajaskanthan *et al*. (2009)	56 NET patients (48 GEP NET, seven unknown prymary NET, one ovarian NET)	Protein (IHC)	92.9% sst_2 and sst_5 co-expression
			80.3% sst_2, sst_5, and D_2 co-expression
Arvigo *et al*. (2010)	PCa cell line	mRNA/protein (W.B.)	sst_1, sst_2, sst_3, sst_5, and D_2 co-expression
Feelders *et al*. (2010)	18 ectopic ACTH-producing tumors patients (five BP NET, three SCLC, three thymic carcinoid, two pancreatic NET, two MTC, one gastrinoma, one NSCLC, one esthesioneuroblastoma)	Protein (IHC)	50% sst_2 and D_2 co-expression

NSCLC, non small cell lung cancer; W.B., western blot; GEP, gastroenteropancreatic; NET, neuroendocrine tumors; BP, bronchopulmonary; GI, gastrointestinal; MTC, medullary thyroid cancer; SCLC, small cell lung cancer; PCa, prostate cancer.

Moreover, other preliminary data by Feelders *et al.* showed sst_2 and D_2 co-expression by IHC in 18 patients with ectopic ACTH-producing tumors from different sites. Sst_2- and D_2-positive staining was found in 10/18 and 13/18 tumors respectively. Co-expression of both receptors was observed in 9/18 tumors (Feelders *et al.* 2010).

Figure 1 shows a schematic representation of human tumors already demonstrated to co-express SS and DA D_2 receptors.

SS and DA analogs treatment in endocrine tumors

NET previously considered as 'carcinoids' are heterogeneous and ubiquitous neoplasias that account for about 1% of all malignancies.

Recent estimates have shown an increasing incidence (3–10% per year) over the last 30 years and a sharp increase in their prevalence, as well as survival (Gustafsson *et al.* 2008, Yao *et al.* 2008). Surgery is still the only curative therapy for NET, but unfortunately it can be really effective in a minority of patients (about 15%). This is due to the very high prevalence of metastatic disease already at the time of diagnosis (85% of diagnosed NET; Modlin *et al.* 2006). In this respect, the control of clinical symptoms and the stabilization of the disease are the primary goal of the currently available therapies (Modlin *et al.* 2006).

Most of the NET of any site have a more or less indolent clinical trend and can benefit from specific therapeutic approaches with biological agents with antisecretive and antiproliferative activity (Colao *et al.* 2010). The use of these agents requires knowledge of the biological mechanisms underlying neuroendocrine tumorigenesis, and in this respect up to now, medical therapy of NET is mainly based on biotherapy with SSAs (Arnold *et al.* 2009).

The 'classical' clinically available SSA octreotide and lanreotide, with a preferential binding affinity for sst_2, at standard dosages have been demonstrated to improve the symptoms related to functioning NET in 64% of patients, with a biochemical response in 66% of cases (Grozinsky-Glasberg *et al.* 2008). Only in about 10% of cases there is a significant reduction of tumor mass, while tumor stabilization occurs in 35–50% of patients (Grozinsky-Glasberg *et al.* 2008,

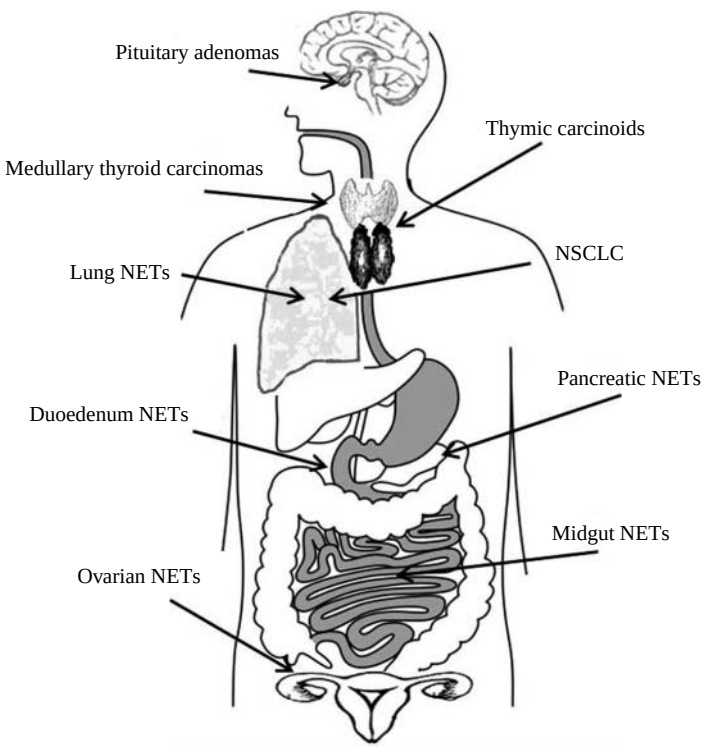

Figure 1 Schematic representation of human tumors already demonstrated to co-express somatostatin and D_2 receptors. The presence of both SSTR and D_2 receptors in pituitary adenomas is well known and has already been extensively reviewed. Recently, a significant number of studies have been published (listed in Table 1) focusing on SSTR and D_2 receptor co-expression in a number of neuroendocrine neoplasms, such as GEP and lung NET, and non-endocrine tumors. These new studies arise from the availability of new universal and subtype-specific SSAs and D_2-selective DA agonists and from the possibility to test these compounds as targeted combination therapy, with potentially synergistic effects. NETs, neuroendocrine tumors; NSCLC, non small cell lung cancer.

Colao *et al*. 2010). A first recent double-blind, placebo-controlled randomized phase IIIb study of octreotide LAR or placebo in patients with well-differentiated metastatic midgut NET demonstrated that octreotide more than doubled the time to tumor progression compared with placebo, in both functioning and non-functioning NET (Rinke *et al*. 2009). More in detail, patients with low-grade (Ki-67 $<2\%$) and low hepatic tumor load NET, even if non-functioning, are likely to benefit from octreotide treatment as first-line therapy (Rinke *et al*. 2009).

Different from GEP NET, and due to the limited number of clinical studies investigating the effect of 'cold' (Kosmidis 2004, De Dosso *et al*. 2007) or radio-labeled SSA in pulmonary NET (van Essen *et al*. 2007), there are no standardized guidelines yet for SSA treatment in these kind of tumors, especially for the most aggressive histotypes, where the role of biotherapies is still debated and not well clarified (Righi *et al*. 2010). Well-defined therapeutic approaches still reside on surgery (McMullan & Wood 2003), chemoradiotherapy, or liver embolization (Kosmidis 2004).

In addition, patients with GEP or lung NET receiving sst_2-preferring analogs frequently experience a loss of response (the 'escape from response' phenomenon or tachyphylaxis) to treatment, either in the short or in the long term, following initiation of treatment (Wynick & Bloom 1991, Ricci *et al*. 2000, Hofland & Lamberts 2003, Oberg 2010). In this context, although final evidences are lacking, receptor desensitization and degradation (leading to receptor downregulation) may be involved in an 'early' escape (weeks–months) to SSA treatment (Hofland & Lamberts 2003). On the other hand, cellular events such as the overgrowth of SSTR-negative clones, mutations in SSTR genes, and/or tumor dedifferentiation, frequently observed in cancer natural history, are more likely related to the 'late' escape to SSA (years) observed in NET (Hofland & Lamberts 2003, Tulipano & Schulz 2007).

Higher doses of the drug may reverse, even if temporarily, the 'early' escape but, going forward with the treatment, most patients with lung and GEP NET eventually become un-responders (Lamberts *et al*. 1996, Hofland & Lamberts 2003).

Targeting D_2 has been shown as an effective mechanism for suppressing secretion of hormones by NET, and especially in pituitary adenomas (Ren *et al*. 2003*a*, Pivonello *et al*. 2004*b*, Jaquet *et al*. 2005*a*, Ferone *et al*. 2007*a*). In particular, in patients with prolactin-secreting pituitary adenomas, the role of D_2 agonist treatment is well established (i.e. inhibition of circulating prolactin levels and induction of tumor shrinkage). However, despite the multiple evidences for antiproliferative and antisecretive effects by DA agonists from *in vitro* studies, the *in vivo* efficacy of these compounds has not well been established yet in NET, particularly in lung and GEP NET (Kidd *et al*. 2008).

Many years ago, for the first time, Child *et al*. (1978) treated two cases of hypergastrinemia due to a gastrinoma with bromocriptine, unfortunately without any results. Ishibashi *et al*. (1994) showed the ability of bromocriptine, a dopaminergic agonist, in inhibiting the growth of human SCLC cells, implanted as tumor xenograft in athymic nude mice, in a dose-dependent manner. In the human neuroendocrine pancreatic cell line BON some authors have demonstrated the expression of D_2 (de Bruin *et al*. 2009*a*) and the effect of the D_2 receptor agonist drug quinpirole in decreasing intracellular cAMP levels (Lemmer *et al*. 2002). Recent studies also demonstrated a significant inhibitory effect of cabergoline, a D_2 preferential compound, in inhibiting cell proliferation both in lung carcinoma and in prostate cancer cell lines, endogenously expressing the D_2 (Ferone *et al*. 2005, Arvigo *et al*. 2010, Ruscica *et al*. 2010).

Recently, bromocriptine and cabergoline have been shown to be effective in suppressing ACTH secretion in a small group of patients with lung carcinoid (Reith *et al*. 1987, Pivonello *et al*. 2007*a*), as previously observed *in vitro* by Farrell *et al*. (1992). Moreover, preliminary data by Pivonello *et al*. (2008) demonstrated an *in vitro* inhibitory effect of cabergoline on cell growth in ten different cases of pancreatic endocrine tumors (seven cases defined as well-differentiated tumors and three defined as well-differentiated carcinomas). In this context, a recent report showed a sevenfold decrease in the pancreatic polypeptide levels and the reduction of liver metastases in a patient with pancreatic polypeptide secreting islet cell tumor during therapy with DA agonists (Pathak *et al*. 2004).

Despite the fact that combined treatment with SSAs, DAs have already been demonstrated efficacious in a subgroup of patients with GH-secreting pituitary adenomas (Colao *et al*. 2007) and in one case of an ACTH-secreting lung carcinoid (Pivonello *et al*. 2005), up-to-date clinical studies reporting the effect of combined SSA/DA in NET are lacking.

New insights in SS and DA receptor signaling, trafficking, and interaction

In recent years, a number of studies have investigated the pathophysiological role of SSTR and D_2, leading to novel insights with possible important clues for clinical management of NET (Hofland & Lamberts 2003, Tulipano & Schulz 2007).

It is well known that the five SSTR subtypes share common signaling pathways, although particular SSTR subtypes can activate distinct signaling pathways as well (Ben-Shlomo & Melmed 2010, Hofland *et al*. 2010).

Recently, Duran-Prado *et al*. (2009) reported the first evidence for the existence of two novel sst_5-truncated variants (termed sst_5TMD4 and sst_5TMD5) in pituitary adenomas, which are absent in the normal pituitary gland. The sst_5TMD4 variant was reported as particularly abundant in octreotide-resistant somatotropinomas and the authors speculated about its possible role in the attenuated response to SSA observed in some pituitary tumors (Duran-Prado *et al*. 2010).

The intracellular pathways activated by SSTR activation appear different in different types of tumor cells and depend on the specific SSTR distribution pattern, signaling elements, as well as to receptor desensitization, internalization, and cross talk (Lahlou *et al*. 2004, Schonbrunn 2008). Moreover, it has been suggested that different SSA, in the same cell type, may elicit differential effects, due to the activation of different subsets of intracellular mediators. This phenomenon, also named biased agonism, seems to depend on the typical agonist–receptor interactions (Schonbrunn 2008, Ben-Shlomo *et al*. 2009, Cescato *et al*. 2010). In two recent *in vitro* studies octreotide and pasireotide (SOM 230) have been demonstrated to modulate sst_{2A} receptor phosphorylation and trafficking in a clearly distinct manner, despite their approximately similar binding affinity to this SSTR subtype (Poll *et al*. 2010). Pasireotide appeared to be more potent than octreotide in inducing internalization and signaling of human sst_3 and sst_5 receptors (Lesche *et al*. 2009). The observed behavior of SOM 230 as only a partial agonist of sst_2 sheds light on the importance of the agonist-induced receptor conformation in affecting receptor signaling and regulation, more than binding affinity alone.

It has to be reminded that like many other GPCRs, SSTR undergo agonist-induced endocytosis following the agonist binding to the receptors. The activated receptor is then phosphorylated by the G protein-coupled receptor kinases (GRKs) and subsequently

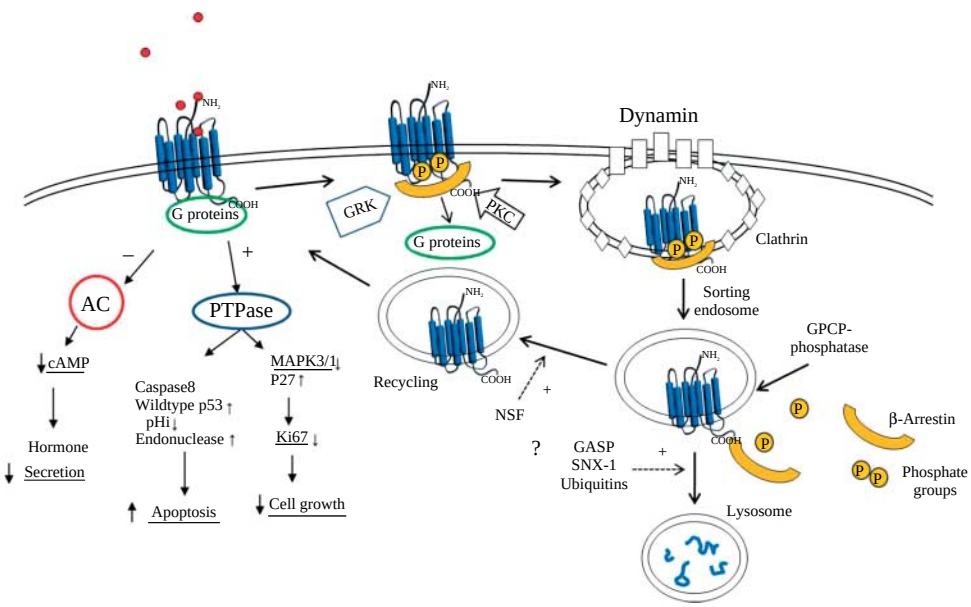

Figure 2 Simplified representation of SSTR signaling and trafficking. SS (or SSA) binding to SSTR activates G proteins and inhibits adenylyl cyclase (AC) activity, activates K^+ channels, and inhibits Ca^{++} channels. An antiproliferative effect may be mediated via the stimulation of PTP and modulation of mitogen-activated protein kinases (MAPK). An increase in apoptosis via p53 has been shown as well (Ferone *et al*. 2009). After agonist activation, SSTR are phosphorylated (mainly involving G protein coupled receptor kinase (GRK)) and recruited by cytoplasmic proteins termed arrestins that interrupt coupling between the receptor and G proteins (desensitization process). β-Arrestins also function as the link between the receptor and the components of the endocytic machinery, such as dynamin and clathrin. The internalized receptor is then directed to early endosomes in which it is dephosphorylated and dissociated from β-arrestins. The receptor is then directed to different intracellular compartments, leading to either recycling or degradation. Finally, the recycled receptor is back to the plasma membrane as functional (resensitized) receptor (Tulipano & Schulz 2007). The rate of recycled or degraded receptors seems to be influenced mainly by β-arrestin interaction and other regulatory intracellular proteins, such as ubiquitins (Gray & Roth 2002). NSF, *N*-ethylmaleimide sensitive factor; GASP, GPCR-associated sorting protein; SNX-1, sortin nexin-1; pHi, intracellular pH. Adapted, with permission, from Tulipano & Schultz (2007), Ferone *et al.* (2009).

recruited by cytoplasmic proteins, named arrestins, determining uncoupling between the receptor and its related G proteins (Oakley *et al*. 2000, 2001, Gurevich & Gurevich 2006). The receptor/arrestin complex is then internalized by dynamin-dependent endocytosis.

In this context, β-arrestins seems to play a pivotal role in the desensitization–internalization process of GPCRs, including SSTR (Bohm *et al*. 1997, Koenig & Edwardson 1997, Bloch *et al*. 1999, Tulipano & Schulz 2007). Different SSTR subtypes display a differential interaction with β-arrestins. sst_5 and sst_3 bind β-arrestin 2 with higher affinity than β-arrestin 1, resulting in a less stable receptor/arrestin complex and a faster recycling on cell membrane. On the contrary, sst_2 displays the same affinity for both β-arrestin 1 and 2 and is internalized into endosomes forming a tight SSTR/β-arrestin complex.

Moreover, while the recycling seems to be the most common process following the internalization of sst_2 and sst_5, degradation seems to be the most common for sst_3 (Jacobs & Schulz 2008, Reubi *et al*. 2010). In this respect, other regulatory factors of this already very complex system are intracellular proteins such as ubiquitins, SNX-1, GASP, and NSF that lead the early endosome to either cell membrane or the lysosomal pathway (Yu *et al*. 1993, Pippig *et al*. 1995, Gagnon *et al*. 1998, Tsao & von Zastrow 2000, Gray & Roth 2002).

The co-expression of different SSTR subtypes or the presence of different intracellular components involved in this trafficking could form the basis for differences in the SSTR trafficking in different kinds of tumors (Hofland & Lamberts 2003, Tulipano & Schulz 2007, Grant *et al*. 2008, Jacobs & Schulz 2008; Fig. 2).

These above-mentioned new insights are extremely important for the development of new therapeutic strategies targeting SSTR with SSA, especially for lung and GEP NET, that after an initial response frequently show escape from the effect of SSA. The mechanism behind such an escape from treatment has not been elucidated yet (see above) but could include receptor downregulation as result of SSA-activated receptor trafficking (Lamberts *et al*. 1996, Hofland & Lamberts 2003, Hofland *et al*. 2005*b*).

The regulation of D_2 seems even more complicated, with the receptor activation variably resulting in functional desensitization, sensitization, and up or downregulation (Pivonello *et al*. 2007*b*). Namkung and colleagues demonstrated that D_2 homologous and

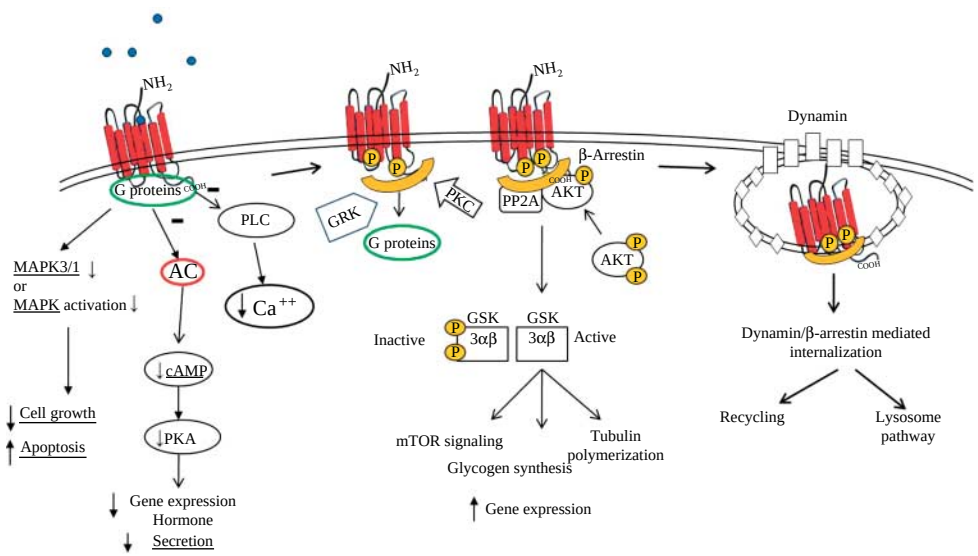

Figure 3 Simplified representation of D_2 signaling and trafficking. DA (or DA agonists) binding to D_2, via interaction with G proteins, inhibits adenylyl cyclase (AC) activity and phosphatidylinositol metabolism, activates voltage-activated potassium channels and decreases voltage-calcium currents, and modulates the activity of phospholipase C (PLC) and the mitogen-activated protein kinases (MAPKs). These processes result in lowering of gene expression, as well as to antisecretive effects in endocrine cells, and, via modulation of MAPK, in increased cell apoptosis and inhibition of cell growth (Pivonello *et al*. 2007*b*, Ferone *et al*. 2009). Like SSTR, D_2 phosphorylation after agonist activation is mediated by GRKs and heterologous receptor phosphorylation may occur via protein kinase C activation (PKC; Namkung & Sibley 2004). Moreover, subsequent recruitment of arrestins by the activated receptor may result not only in signal desensitization but β-arrestins can also act as a second messenger leading to glycogen synthase kinase 3αβ (GSK3αβ) activation and increased gene expression (Hofmann *et al*. 2009). It has to be underlined that recent studies suggested that GRK and β-arrestins may exert their functions in the absence of receptor phosphorylation, and phosphorylation-independent association with β-arrestin seems to play a major role in agonist-induced D_2 desensitization (Cho *et al*. 2010). Adapted, with permission, from Ferone *et al*. (2009).

heterologous phosphorylation (mediated by GRK and protein kinase C activation respectively) differently affect receptor internalization and recycling (Namkung & Sibley 2004, Namkung *et al*. 2009). Moreover, in a recent study, Cho *et al*. (2010) observed that D_2 desensitization and resensitization, different from the classical GPCR model, are mainly mediated by β-arrestin in a phosphorylation-independent manner (Fig. 3). Since SSTR and D_2 are often co-expressed in endocrine tumors, it is likely to hypothesize some interaction between the receptor/β-arrestin complex of the two receptor families, affecting signaling and trafficking of the activated receptors.

In addition, it is well known that both SSTR and DR may act not only as monomers but also as homo- and heterodimers as well. Such receptor oligomerization results in modified functional and pharmacological properties of the receptor complex (Rocheville *et al*. 2000*b*, Pfeiffer *et al*. 2001, Patel *et al*. 2002, Lee *et al*. 2003, Grant *et al*. 2004, Duran-Prado *et al*. 2008, Grant *et al*. 2008). SSTR and D_2 have been reported to physically interact by forming heterodimers with enhanced functional activity (Rocheville *et al*. 2000*a*) involving cellular events such as modified SSTR internalization, trafficking (Baragli *et al*. 2007, Grant *et al*. 2008), and signal transduction (Kidd *et al*. 2008; Fig. 4).

In the light of these insights, new SSA, which bind more than one SSTR subtype (Weckbecker *et al*. 2003), as well as chimeric compounds binding both SSTR and D_2, have been developed for treatment of SSTR and D_2 co-expressing tumors (Ferone *et al*. 2007*b*).

However, almost all studies carried out on cell lines showed receptor dimerization in transfected models highly expressing at least two receptor subtypes (Rocheville *et al*. 2000*b*, Pfeiffer *et al*. 2001, Ren *et al*. 2003*b*) and most of the *in vitro* studies evaluating the effect of these new chimeric compounds did not evaluate receptor interactions (Ferone *et al*. 2005, Saveanu *et al*. 2006, Florio *et al*. 2008, Kidd *et al*. 2008). In a very recent study, Arvigo *et al*. investigated the effect of this new generation of chimeric compounds in two non-neuroendocrine cell lines endogenously expressing SSTR and D_2 (Ferone *et al*. 2005, Ruscica *et al*. 2010) observing for the first time a direct and significant positive correlation between the amount of ligand induced sst_5/D_2 dimers and the observed antiproliferative effect (Arvigo *et al*. 2010).

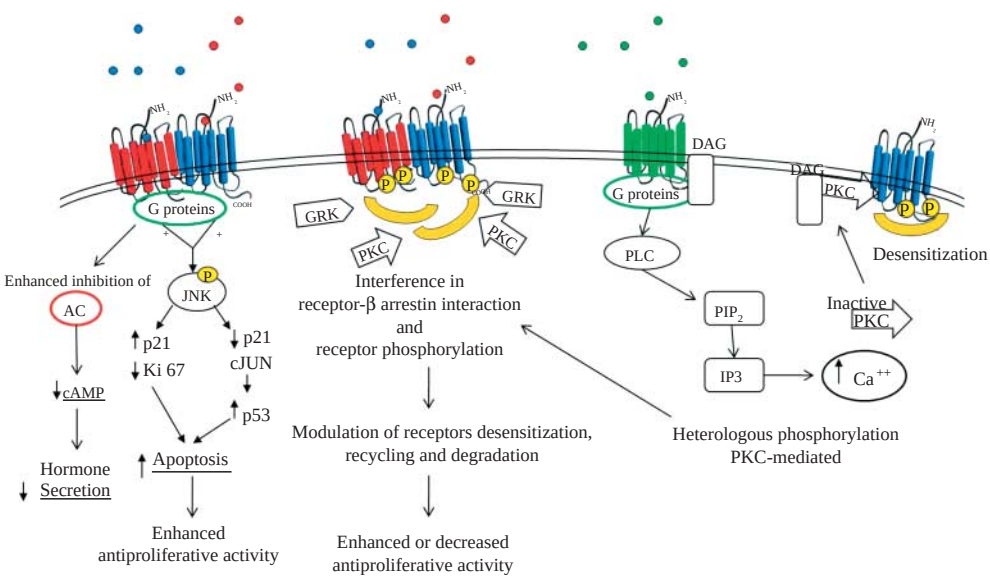

Figure 4 Schematic representation of possible interactions between SSTR and D_2 receptor. Like the majority of GPCRs, SSTR and D_2 can also interact at the cell membrane, when co-expressed. It is well known that these receptors can act as heterodimers after agonist binding (Rocheville *et al*. 2000*a*, Kidd *et al*. 2008). Kidd *et al*. suggested a possible explanation for the intracellular pathway following the activation of the heterodimer represented by an upregulation of p21WAF1/CIP1, via c-Jun N-terminal phosphorylation and a concomitant inhibition of Ki-67 transcription. However, they observed that a different dimer complex composition can lead to a decrease in P21 transcription and to an increase in p53 (Kidd *et al*. 2008). It could also be hypothesized that receptor dimerization can influence the single receptor phosphorylation and β-arrestin interaction, resulting in the modulation of receptor desensitization, recycling, and degradation. Moreover, recent evidences suggested a possible heterologous phosphorylation of both sst_2 and D_2 by co-expressed phospholipase C (PLC)-coupled receptors (e.g. cholecystokinin (CCK) or bombesin (BBS) receptors) that may result in the modulation of receptor desensitization and internalization (Elberg *et al*. 2002, Namkung & Sibley 2004). IP3, inositol phosphate 3. Adapted, with permission, from Ferone *et al*. (2009).

Keeping all these data together, this suggests that not only the single receptor signaling and trafficking but also the cell types, as well as the receptor dimerization, are important components determining the final effect of a given ligand.

Future perspectives of SSA and DA agonist treatment

On the basis of the reported data on NET treatment using the classical SSA and DA, the newly developed compounds and the combined targeting of SSTR and D_2 all aim as primary goal to increase the tumor response rate (especially in terms of antiproliferative effect) and to reduce the impact of the escape to treatment.

In order to perform a really tailored target therapy, several SSA with different SSTR binding affinity have been developed and used for *in vitro* and *in vivo* studies (Weckbecker *et al*. 2003). Among these, an interesting compound is the BIM-23244, a SSA with a comparable high binding affinity for sst_2 and sst_5. *In vitro* studies using this compound have demonstrated that a co-activation of sst_2 and sst_5 suppresses GH production in octreotide-resistant GH-secreting adenomas, suggesting that this generation of compounds could improve the clinical utility of SSA (Saveanu *et al*. 2001).

Pasireotide (SOM 230), a novel multi-receptor ligand analog with high binding affinity for four of the five SSTR subtypes ($sst_{1–3}$ and sst_5), exhibits an affinity binding profile for human SSTR more similar to native SS than to either octreotide or lanreotide (Schmid & Schoeffter 2004, Schmid 2008, Ben-Shlomo *et al*. 2009). A number of preclinical studies suggest that SOM 230 is a promising candidate for clinical applications in situations where octreotide and lanreotide were shown to be weakly active or even ineffective, such as ACTH-secreting pituitary adenomas and octreotide-resistant GH-secreting adenomas (van der Hoek *et al*. 2005, Hofland *et al*. 2005*a*, Batista *et al*. 2006, Ben-Shlomo & Melmed 2007). Moreover, pasireotide was described to significantly reduce cell proliferation in a lung NET cell line (Ono *et al*. 2007) and to inhibit cell growth and catecholamine secretion in cell cultures of pheochromocytoma (Pasquali *et al*. 2008). Therefore, this new SSA is currently under clinical investigation to treat patients with acromegaly, Cushing's disease, and NET.

The SSA/DA chimeric molecules have been largely tested in *in vitro* studies.

Saveanu *et al*. (2006) demonstrated that the chimeric compounds BIM-23A387 and BIM-23A760 were more effective in controlling hormonal hypersecretion *in vitro* in a subgroup of GH-secreting adenomas that were partially responders to octreotide, compared to both sst_2 and sst_5 monospecific analogs, as well as to octreotide in combination with cabergoline. The same molecules tested in a non-small lung cancer cell line (Calu-6) and in a prostate cancer cell line (LNCaP) showed a greater antiproliferative effect than subtype-specific SS and DA agonists, alone or in combination (Ferone *et al*. 2005, Arvigo *et al*. 2010). In addition, a differential cytotoxicity of chimeric compounds was recently observed in bronchopulmonary and small intestinal NET cell lines (Kidd *et al*. 2008). Conversely, in a gastric enterochromaffin-like tumor cell line, the dopastatin BIM-27A760 did not display any additive effect in the inhibition of cell proliferation and hormone secretion (Kidd *et al*. 2007*a*). All these data suggest that specific compounds based on the individual tumor lesion receptor profile might be needed to achieve a significant antiproliferative effect in the different NET cell types (Kidd *et al*. 2008). Moreover, a possible explanation for the lack of efficacy of agonist drugs in the presence of a 'suitable' SSTR/D_2 profile may reside in the dynamic behavior of GPCRs, leading to receptor cross talk both at cell membranes and/or intracellular level (Ferone *et al*. 2007*b*, Saveanu *et al*. 2008).

The *in vitro* studies reported above are now to be supported by clinical evidences. There are several ongoing pre-clinical and clinical trials, involving pasireotide, dopastatin, and combined treatments with SSA and everolimus (a mammalian target for rapamycin inhibitor), aimed at demonstrating the *in vivo* safety and efficacy of these newly developed compounds (Ferone *et al*. 2009, Colao *et al*. 2010). It should be mentioned, however, that a recent phase II trial with BIM-23A760 in acromegaly was stopped due to a lack of efficacy. Therefore, further studies investigating the clinical efficacy of dopastatins are required.

Conclusions

SSTR and D_2 are widely co-expressed in pituitary adenomas. Recent studies demonstrated their co-expression in a large variety of well-differentiated NET, originating from different tissues such as GI tract, pancreas, lung, adrenal, and thymus as well.

These findings, together with the availability of new universal and subtype-specific SSAs and D_2-selective DA agonists, initiated researchers and clinicians to test targeted combination therapy and treatment with the so-called dopastatins, compounds with affinity for both SSTR and D_2.

However, the controversial results coming out from a number of *in vitro* studies highlight the importance for a better understanding of the pathophysiological basis, which regulates the complex system of GPCRs. The most recent insights of SSTR and D_2 pathophysiology show that not only cell and tissue specificity, receptor pattern, and single receptor binding affinity of drugs might affect receptor signaling and trafficking after ligand exposure.

Many other variables, such as the presence of truncated receptor forms with different biological effects than the wild-type receptor, receptor cross talk, receptor homo and heterodimerization, and different expression of intracellular regulatory molecules (e.g. β-arrestin and ubiquitins), can determine the success of a given receptor targeted therapy. In this context, the increasing need for a better understanding of the molecular mechanisms regulating SSTR and D_2 signaling, trafficking, and cross talk is the ultimate step for developing more specific, selected, and effective medical treatment for patients with NET.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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