1. Introduction

1.1. Angiogenesis

The term Angiogenesis refers to the growth of new vessels from pre-existing ones and it is a physiological process in the human body involved in: wound healing, in the reproductive cycle in females, rebuilding of the uterus lining, maturing of the egg during ovulation and also in pregnancy in order to build the placenta to establish the circulation between mother and foetus – Figures 1 and 2 [Holland et al, 2003].

This process can be described in an orderly series of events as illustrated in Figure 3:

1. Affected tissues (by disease or another kind of injury) produce and release angiogenic growth factors (or induce surrounding cells to secrete them) that diffuse into nearby tissues
2. Angiogenic growth factors approach specific receptors of endothelial cells of former blood vessels
3. Binding of the growth factors to respective receptors leads to activation of endothelial cells, and signals are sent to the nucleus (production of new molecules, such as enzymes)
4. Digestion of the basement membrane of the blood vessels by the enzymes produced
5. Proliferation of the endothelial cells and migration through the digested spaces of the membrane towards the affected tissue (tumor)
6. Integrins (avb3, avb5) – adhesion molecules - work as hooks to help the growing new blood vessel developing towards the tissues
7. Production of matrix metalloproteinases (MMP) to digest the tissue ahead the growing vessel extremity in order to accommodate it. As the vessel extends, the tissue is restructured around the vessel
8. Growing endothelial cells organize into hollow tubes
9. New blood vessel tubes connect to each other in order to form blood vessel loops to circulate blood
10. Structural stabilization of the new blood vessels by pericytes (smooth blood cells) and beginning of the blood flow.
1.2. **Angiogenesis and Cancer**

In 1971, Dr. Judah Folkman proposed that tumor cells and vascular endothelial cells from a neoplasm may be the major components of a highly integrated ecosystem and that through the diffusion of chemical signal from tumor cells, endothelial cells may change from a resting stage to an exponential growth phase. Angiogenesis was already considered by Dr. Folkman to be a relevant target for cancer therapy [Folkman, 1971]. In his studies with Frederick Becker, he realized that in an isolated perfused organ, tumor growth was severely limited in the absence of neovascularization [Folkman et al, 1963]. In the prevascular phase, in which angiogenic activity is absent or insufficient, tumors remain small, with an area of a few cubic millimeters. Their growth is relatively slow; sometimes it takes years for doubling their size [Holland et al, 2003]. In experimental
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Tumors, the size limits in which angiogenesis is blocked or absent are between 0.2 mm to 2 mm in diameter, depending on the capacity of tumor cells to survive under different degrees of hypoxia (Figure 4). Above this size, diffusion is not enough to supply the cells with oxygen and nutrients and to remove waste products and tumor ceases its growth (dormant stage) [Homgren et al, 1995]. To study the behaviour of the cancer growth in the presence of angiogenesis, tumors were suspended in the aqueous fluid of the anterior chamber of the rabbit eye, remaining in a dormant state: viable, avascular, and limited in size (<1 mm³). Neovascularization of iris vessels was induced, but these vessels were too distant from the tumors floating in the aqueous fluid, blocking angiogenesis. When a spheroid tumor was apposed to the proliferating iris vessels, the tumor becomes neovascularized and could grow (Figure 5). Both locations in the eye had nutrients available, but only one could support angiogenesis and therefore could continue to grow [Gimbrone et al, 1972].

![Figure 4: Tumor growth without angiogenesis](Figures/tumor_growth_no_angiogenesis.png)

![Figure 5: Cancer growth in the presence of angiogenesis](Figures/tumor_growth_with_angiogenesis.png)

1.3. Regulation of angiogenesis in normal and tumoral tissues

Angiogenesis is the result of a complex balance between tightly regulated oncogenes and suppressor genes, stimulatory and inhibitory peptides, proteases and endogenous inhibitors, and microenvironmental factors such as the level of oxygen [Holland et al, 2003] or copper ion [Brem et al, 1998]. Angiogenesis is regulated by both activator and inhibitor molecules. If inhibitors predominate, vessel growth is blocked (normal situation) and if there is a need for new blood vessels, angiogenesis activators
increase in number and inhibitors decrease, leading to the angiogenesis process (Figure 6).

![Image: Control of endothelial cell proliferation by angiogenesis inhibitors and activators](National Cancer Institute, www.cancer.gov)

**Figure 6:** Control of endothelial cell proliferation by angiogenesis inhibitors and activators [National Cancer Institute, www.cancer.gov]

### 1.3.1. Angiogenesis Promoters

More than a dozen different proteins and small molecules have been identified as "angiogenic" - they are released by tumors, signalling for angiogenesis (Table 1). Among these molecules, two proteins appear to be the most important for sustaining tumor growth: vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). VEGF and bFGF are produced by many types of tumor cells (Figure 7) and also by certain types of normal cells [Holland et al, 2003].

<table>
<thead>
<tr>
<th>Known Angiogenic Growth Factors</th>
</tr>
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<tbody>
<tr>
<td>Angiogenin</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
</tr>
<tr>
<td>Del-1</td>
</tr>
<tr>
<td>Fibroblast growth factors: acidic (aFGF) and basic (bFGF)</td>
</tr>
<tr>
<td>Follistatin</td>
</tr>
<tr>
<td>Granulocyte colony-stimulating factor (G-CSF)</td>
</tr>
<tr>
<td>Hepatocyte growth factor (HGF) /scatter factor (SF)</td>
</tr>
<tr>
<td>Interleukin-8 (IL-8)</td>
</tr>
<tr>
<td>Leptin</td>
</tr>
<tr>
<td>Midkine</td>
</tr>
<tr>
<td>Placental growth factor</td>
</tr>
<tr>
<td>Platelet-derived endothelial cell growth factor (PD-ECGF)</td>
</tr>
<tr>
<td>Platelet-derived growth factor-BB (PDGF-BB)</td>
</tr>
<tr>
<td>Pleiotrophin (PTN)</td>
</tr>
<tr>
<td>Progranulin</td>
</tr>
<tr>
<td>Proliferin</td>
</tr>
<tr>
<td>Transforming growth factor-alpha (TGF-alpha)</td>
</tr>
<tr>
<td>Transforming growth factor-beta (TGF-beta)</td>
</tr>
<tr>
<td>Tumor necrosis factor-alpha (TNF-alpha)</td>
</tr>
<tr>
<td>Vascular endothelial growth factor (VEGF) /vascular permeability factor (VPF)</td>
</tr>
</tbody>
</table>

*Table 1: Known angiogenic growth factors [The Angiogenesis Foundation, www.angio.org]*
Basic fibroblast growth factor (bFGF) is a wide-spectrum mitogenic, angiogenic, and neurotrophic factor, expressed at low levels in many tissues and cell types, and reaches high concentrations in brain and pituitary. Basic FGF is involved in a large variety of physiologic and pathologic processes, including limb development, angiogenesis, wound healing, and tumor growth. Alavi and collaborators (2003) showed that bFGF and VEGF differentially activate Raf1 (pivotal regulator of endothelial cell survival during angiogenesis), resulting in protection from distinct pathways of apoptosis in human endothelial cells and chick embryo vasculature. Basic FGF activates Raf1 via p21-activated protein kinase-1 (PAK1) phosphorylation of serines 338 and 339, resulting in Raf1 mitochondrial translocation and endothelial cell protection from the intrinsic pathway of apoptosis, independent of the mitogen-activated protein kinase-1 (MEK1). In contrast, VEGF activates Raf1 via Src kinase (CSK), leading to phosphorylation of tyrosines 340 and 341 and MEK1-dependent protection from extrinsic-mediated apoptosis.

VEGF is a homodimeric protein (40 to 45 kDa) with a signal sequence secreted by a wide variety of cells and the majority of tumor cells, and is upregulated by the ras oncogene [Carmeliet, 2005]. There are five different isoforms of 121, 145, 165, 189, and 206 amino acids, produced by a variety of normal and neoplastic cells. VEGF_{165} (VEGF-A) is the predominant molecular species and in situ hybridization studies demonstrated the VEGF-A mRNA expression in many human tumors [Dvorak, 2002]. Two receptors
for VEGF (VEGFR) are found mainly on vascular endothelial cells, the 180 kDa fms-like tyrosine kinase VEGFR-1 (Flt-1) and the 200 kDa human kinase insert domain containing VEGFR-2 (KDR). VEGF binds to both receptors, but VEGFR-2 transduces the signals for endothelial proliferation and chemotaxis. VEGFR-1 may sequester VEGF, preventing its interaction with VEGFR-2. VEGFR-1 has also significant roles in haematopoiesis, in monocytes and bone-marrow-derived cell recruitment. During embryogenesis, VEGF expression is essential for development of the embryonic vascular system as well for angiogenesis in the female reproductive tract, and in tumors [Ferrara, 2005]. In addition, it is involved in the induction of MMPs and in the paracrine release of growth factors by endothelial cells, and may be expressed by tumor cells mediating a chemotactic signal [Ferrara and Kerbel, 2005]. VEGF is an endothelial cell mitogen and motogen angiogenic in vivo. VEGF has a very important role in the pathogenesis of many human cancers characterized by neovascularization, which involve: up-regulation of VEGF that leads to the angiogenic switch, allowing the development of vasculature for transfer of oxygen and nutrients to the growing tumor mass. This up-regulation may be the consequence of oncogene activation, inhibition of tumor suppression molecules, release of growth factors, tumor hypoxia and necrosis [Carmeliet, 2005]. VEGF induces anti-apoptotic factors, protecting tumor vasculature from apoptosis, mediates secretion and activation of MMPs and facilitates tissue digestion and thus tumor angiogenesis. The disordered and irregular shape of the tumor vessels leads to insufficient perfusion of the tumor mass, stimulating VEGF release and creating a positive feedback loop for tumor angiogenesis [Carmeliet, 2005].

1.3.2. Inhibitors of angiogenesis

Dr. Judah Folkman hypothesized that a large primary tumor secretes not only stimulators of its own angiogenesis but also angiogenesis inhibitors (Table 2, Figure 8) that are released into the circulation and inhibit angiogenesis - and thus further growth - of any metastases of the primary tumor (Figure 9). This can explain why, some time after a successful therapy, large metastases appear in the patient’s body.

There are two classes of angiogenesis inhibitors: direct and indirect. Indirect inhibitors decrease or block expression of a tumor cell product, neutralize the tumor
product or block its receptor on endothelial cells. Direct inhibitors block directly vascular endothelial cells from proliferating, migrating or increasing the survival rate when exposed to pro-angiogenic proteins (VEGF, bFGF, etc) [Holand et al]. Once these type of inhibitors target genetically stable endothelial cells, rather than unstable mutating tumor cells, the probability of an individual to acquire drug resistance is small [Holand et al].

### Known Angiogenesis Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor Type</th>
<th>Protein/Compound Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Angioarrestin</td>
<td>- Interferon inducible protein (IP-10)</td>
</tr>
<tr>
<td>- Angiostatin (plasminogen fragment)</td>
<td>- Interleukin-12</td>
</tr>
<tr>
<td>- Antiangiogenic antithrombin III</td>
<td>- Kringel 5 (plasminogen fragment)</td>
</tr>
<tr>
<td>- Cartilage-derived inhibitor (CDI)</td>
<td>- 2-Methoxyestradiol</td>
</tr>
<tr>
<td>- CD59 complement fragment</td>
<td>- Placental ribonuclease inhibitor</td>
</tr>
<tr>
<td>- Endostatin (collagen XVIII fragment)</td>
<td>- Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>- Fibronectin fragment</td>
<td>- Platelet factor-4 (PF4)</td>
</tr>
<tr>
<td>- Gro-beta</td>
<td>- Prolactin 16kD fragment</td>
</tr>
<tr>
<td>- Heparinas</td>
<td>- Proliferin-related protein (PRP)</td>
</tr>
<tr>
<td>- Heparin hexasaccharide fragment</td>
<td>- Retinoids</td>
</tr>
<tr>
<td>- Human chorionic gonadotropin (hCG)</td>
<td>- Tetrahydrocortisol-5</td>
</tr>
<tr>
<td>- Interferon alpha/beta/gamma</td>
<td>- Thrombospondin-1 (TSP-1)</td>
</tr>
<tr>
<td>- Metalloproteinase inhibitors (TIMPs)</td>
<td>- Transforming growth factor-beta (TGF-b)</td>
</tr>
<tr>
<td></td>
<td>- Vasculostatin/Vasostatin (calrecticulin fragment)</td>
</tr>
</tbody>
</table>

*Table 2: Known angiogenesis inhibitors (The Angiogenesis Foundation; www.angio.org)*
An example of an indirect inhibitor is Angiostatin, which is a polypeptide of approximately 200 amino acids, produced by cleavage of plasminogen. It binds to subunits of ATP synthase embedded at the surface of the cell in the plasma membrane. It induces cell arrest and apoptosis of endothelial cells, inhibits endothelial migration, angiogenesis in vitro and in the quail chorioalantoic membrane [O’Reilly et al, 1996; Sim et al 1997].

Several drugs have been developed against angiogenesis, such as Combretastatin A4 prodrug. It is a derivative of combretastatin (organic molecule extracted from the bush willow tree Combretum caffrum), and is activated by a phosphatase selectively amplified in proliferating endothelial cells. It induces apoptosis in human endothelial cells [Iyer et al, 1998]. In tumor-bearing mice, combretastatin A-4 significantly enhanced the antitumor effects of radiation therapy [Li et al, 1998]. Other drugs, which interact with a...
molecule called integrin, can also promote the destruction of proliferating endothelial cells as Vitaxin®, a monoclonal antibody directed against the alpha-v/beta-3 vascular integrin [Cheresh, 1998]. In Phase II clinical trials in humans, Vitaxin has shown some promise in shrinking solid tumors without harmful side effects [Cheresh, 1999]. Another drug, Bevacizumab (Avastin®), a humanized monoclonal antibody binds to VEGF, keeping it from binding to its receptors. It was approved by the US FDA in February 2004 for metastasized colorectal cancer therapy. This drug interferes with the VEGF signalling cascade, delaying tumor growth and extending the survival time [Ferrara, 2005].

The anticancer ribozyme Angiozyme™ (Ribozyme Pharmaceuticals, Inc, Boulder, Colo) inactivates mRNA for two VEGF receptors (Flt-1 and KDR), which causes disruption of the VEGF signalling pathway, inhibiting angiogenesis, and suppressing tumor growth in preclinical models (Lewis lung metastases and colon carcinoma). There are also angiogenesis inhibitors directed against the metalloproteinases that catalyze the digestion of the extracellular matrix.

### 1.4. Somatostatin and Somatostatin Receptors

Somatostatin is a natural small cyclic peptide widely expressed hormone. There are two forms of somatostatin synthesized in the organism, named as SS-14 and SS-28, according to their amino acid chain length (Figure 10). They result from a proteolytic cleavage of pro-somatostatin, which is derived from pre-pro-somatostatin. SS-14 has two cysteine residues that allow forming an internal disulfide bond [Lamberts et al., 1996].

The relative amounts of these peptides differ among tissues: SS-14 is the predominant form produced in the nervous system and the only one found in pancreatic secretion; SS-28 is secreted in large amounts by the intestine. SS-28 is a ten-fold more potent inhibitor of growth hormone secretion than SS-14 but less potent than the latter in inhibiting glucagon’s release [Holand et al., 2003]. Somatostatin acts by endocrine and paracrine pathways. The effects of somatostatin in the organism are described succinctly in Table 3.
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Figure 10: Somatostatin biosynthesis [www.vivo.colostate.edu]

<table>
<thead>
<tr>
<th>Effects of Somatostatin in the organism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effects on the pituitary gland</strong></td>
</tr>
<tr>
<td>Somatostatin inhibits secretion of growth hormone</td>
</tr>
<tr>
<td><strong>Effects on the pancreas</strong></td>
</tr>
<tr>
<td>Somatostatin is secreted within pancreatic islets which secrete insulin and glucagon. It acts primarily in a paracrine pathway inhibiting the secretion of these peptides. It suppresses pancreatic exocrine secretions by inhibiting cholecystokinin-stimulated enzyme secretion and secretin-stimulated bicarbonate secretion.</td>
</tr>
<tr>
<td><strong>Effects on gastrointestinal Tract</strong></td>
</tr>
<tr>
<td>Somatostatin is secreted by scattered cells in the GI epithelium and by the enteric nervous system and inhibits secretion of many other GI hormones, including gastrin, cholecystokinin, secretin and vasoactive intestinal peptide. Somatostatin supresses secretion of gastric acid and pepsin, lowers the rate of gastric emptying, and reduces smooth muscle contractions and blood flow within the intestine. The overall effect is the decreasing of the nutrient absorption rate.</td>
</tr>
<tr>
<td><strong>Effects on the nervous system</strong></td>
</tr>
<tr>
<td>Somatostatin has a neuromodulatory activity in the CNS and a variety of complex effects on neural transmission</td>
</tr>
</tbody>
</table>

Table 3: Effects of somatostatin in the organism [www.vivo.colostate.edu]

Somatostatin interacts with specific receptors that belong to the G protein-coupled seven-transmembrane domain receptor (GPCR) superfamily, that bind to four core conserved residues of somatostatin and its analogs (Figure 11). Five subtypes of somatostatin receptors have been cloned (SSTR 1–5) (Table 4) [Lamberts et al, 1996; Grimberg, 2004] and the genes encoding these subtypes are located on different chromosomes. Alternative splicing can generate two forms of the SSTR 2 receptor (SSTR 2a and SSTR 2b). These five subtypes mediate a variety of signal transduction pathways: inhibition of adenylate cyclase and guanylate cyclase, modulation of ionic conductance channels and protein phosphorylation, activation of mitogenactivating protein kinase (MAPK) and phospholipase C. The inhibitory effects of somatostatin on adenylate cyclase activity and on the influx of calcium are linked to inhibition of secretion processes. The response is cell-type specific, determined by the distribution of ligand,
SSTR subtypes, SSTR signalling pathways, SSTR dimerization, cross-talk between G protein coupled membrane receptor families, SSTR internalization and desensitization [Grimberg, 2004].

Taniyama and collaborators (2005) examined the systemic localization of somatostatin receptors subtypes. They found out that various SSTR subtypes are present not only in parenchymal cells but also in various stromal cells (lymphocytes, fibroblasts, endothelial cells). They reported for the first time the presence of SSTR in the parotid gland (SSTR 2 and SSTR 5), bronchial gland (SSTR 1, 2, 3, 4, 5), parathyroid gland (SSTR 1, 3, 4) and duodenum (all subtypes). In pancreatic islet cells, only SSTR 2 was positive in all cases but the others SSTR subtypes are associated with intraislet heterogeneity distribution. In stomach, all subtypes of SSTR were detected in the mucosa, but none in enterochromaffin-like (ECL) cells of fundic gland.

Adams and collaborators (2005) reported the expression of SSTR in human umbilical vein endothelial cells (HUVECs) in vitro, during proliferation and quiescence. SSTR 2 and SSTR 5 were mostly expressed in proliferating HUVECs, presenting high variability in their expression. HUVEC were negative for SSTR 4 and the expression of SSTR 1 and 3 and cell cycle progression were unrelated. The authors suggest that dynamic changes in the expression of SSTR 2 and 5 during the cell cycle and the inhibition of proliferation with specific analogs are indicative that these receptors may have a role in angiogenesis. The authors considered these facts particularly important for the clinical application of somatostatin analogs, once they have effective antiproliferative action on different epithelial and neuroendocrine tumors.

Some studies permitted to specify functions to each SSTR. All five present an antiproliferative effect, by inhibition of mitogenesis or stimulation of apoptosis. SSTR 1, 2, 4 and 5 induce cell cycle arrest in G1; receptor 3 is proapoptotic, via induction of p53 and BAX; receptors 2, 3 and 5 are also antiangiogenic [Stafford et al, 2004].
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Figure 11: Model of the effector systems coupled to somatostatin receptors. A. General model of a receptor coupled to G protein. α, β, γ; protein subunits. B. Coupling of the somatostatin receptor (SSTR) to adenylyl cyclase (ACL). Activation of the receptor may result in a reduction (minus sign) in intracellular cyclic AMP (cAMP) concentrations through the inhibition (minus sign) of adenylyl cyclase. SST- somatostatin, G(i)- inhibitory G protein. Adapted from Lamberts et al, 1996

<table>
<thead>
<tr>
<th>PROPERTY</th>
<th>SUBTYPE 1</th>
<th>SUBTYPE 2</th>
<th>SUBTYPE 3</th>
<th>SUBTYPE 4</th>
<th>SUBTYPE 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal location</td>
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<td>17</td>
<td>22</td>
<td>20</td>
<td>16</td>
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<tr>
<td>G protein coupling</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Effector system</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenylyl cyclase activity</td>
<td>Reduced</td>
<td>Increased</td>
<td>Reduced</td>
<td>Not investigated</td>
<td>Reduced</td>
</tr>
<tr>
<td>Tyrosine phosphatase activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC50 (nM)</td>
<td>Somatostatin-14</td>
<td>2.2</td>
<td>4.1</td>
<td>6.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Somatostatin-28</td>
<td>&gt;1000</td>
<td>2.1</td>
<td>4.4–35</td>
<td>&gt;1000</td>
<td>3.6</td>
</tr>
<tr>
<td>Octreotide</td>
<td>&gt;1000</td>
<td>5.4</td>
<td>10.9</td>
<td>45.0</td>
<td>66.0</td>
</tr>
<tr>
<td>Lanreotide (BIM-23014)</td>
<td>&gt;1000</td>
<td>1.8</td>
<td>43.0</td>
<td>66.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Distribution in normal human tissue</td>
<td>Brain, lung, stomach, pancreas, kidneys, liver, and pancreas</td>
<td>Brain and kidneys</td>
<td>Brain and pancreas</td>
<td>Brain and lungs</td>
<td>Brain, heart, adrenal glands, placenta, pituitary, small intestine, and skeletal muscle</td>
</tr>
</tbody>
</table>

*Data on the five subtypes were obtained from the following reports: subtype 1 and 2, Yamada et al.; subtype 3, Yamada et al.; subtype 4, Braun et al.; and subtype 5, O’Carroll et al.*

Table 4: Properties of the Five Cloned Subtypes of Human Somatostatin Receptors [Lamberts et al, 1996]

1.4.1. Somatostatin Receptors’ expression in tumors

Tumors from somatostatin-target tissues frequently express a high density of multiple SSTRs, for example, pituitary adenomas, pancreatic endocrine tumors, carcinoids, paragangliomas, pheochromocytomas, small cell lung cancers, medullary thyroid carcinomas, breast cancers and malignant lymphomas [Stafford et al, 2004].

Somatostatin receptor expression is highly variable among individuals and different tumor types [Reubi, 2003]. Some tumors have a high density of receptors (meningiomas or medullblastosomas); others, for example lymphomas, present a much
lower density [Casini Raggi et al, 2002]. Some tumors, as most neuroendocrine tumors (e.g. gastroenteropancreatic tumors), have a homogenous somatostatin receptor distribution. There are tumors such as breast cancer with a high heterogenous somatostatin receptor distribution, alternating regions of high density with others without receptors - tumoral polyclonality [Casini Raggi et al, 2002]. Receptor homogeneity is considered of high importance in the use of somatostatin receptors as therapeutic or diagnosis targets. Another example of heterogeneity distribution is the situation of most of colorectal cancers that express only a few octreotide (somatostatin analog) binding sites comparing with the high density of such receptors in the peritumoral vessels [Casini Raggi et al, 2002].

It was found that SSTR 2 is present in a much higher number than the other receptors in the majority of neuroblastomas, medulloblastomas, breast cancers, meningiomas, paragangliomas, renal cell carcinomas, lymphomas, hepatocellular carcinomas, and small cell lung cancers [Reubi et al, 2000; Reubi, 2003]. SSTR 1 appears in higher number in prostate cancers and in many sarcomas. SSTR 3 is found frequently in inactive pituitary adenomas [Reubi et al, 2000; Reubi, 2003]. It is possible to find several SSTRs expressed concomitantly in GH-producing pituitary adenomas (SSTR 2 and 5), pheochromocytomas, hormone-producing gastroenteropancreatic tumors, and gastric cancers. SSTR 4 was not often expressed in the human cancers tested [Jaquet et al, 2000; Reubi, 2003]. In gastroenteropancreatic neuroendocrine tumors, the expression of SSTR 2 and SSTR 5 is particularly high (Table 5). These receptors are found in approximately 90% (SSTR 2) and 80% (SSTR 5) of these tumors, which are potentially sensitive to hormonal treatment targeting these receptors [Delaunoit et al, 2005].

Some tumors, as melanomas, express the somatostatin receptors in peritumoral and intratumoral veins. Angiogenic vessels and peritumoral vessels express predominantly SSTR 2 [Reubi, 2003]. The function of somatostatin in the peritumoral vasculature may be primarily vasoconstrictive, resulting in local hypoxia and necrosis of the tumor, or with a more prolonged vasoconstrictive action, directed against metastatic tumor dissemination. There are two different mechanisms dependent on local somatostatin receptor expression: direct action on tumor cells or action on peritumoral vessels, altering the dynamics of the tumoral blood circulation and/or inhibiting angiogenesis [Reubi, 2003]. A recent study of SST receptors 1, 2, 3 and 5 expression in acoustic neuromas [Stafford et al, 2004] showed that SSTR 2 was the most prevalent
receptor in Schwann cells followed by SSTR 3, 1 and 5. The vascular expression of SSTR2 was ubiquitous with no evidence of SSTR 1, 3 or 5 expression in tumor blood vessels. SSTR 1, 2, 3 and 5 are differently expressed in acoustic neuromas and at least one subtype was expressed in all tumors studied. This was the first study to demonstrate the presence of somatostatin receptors in both the blood vessel endothelium and the Schwann cells of acoustic neuromas, suggesting that somatostatin analogs may be useful in the treatment of these tumors [Stafford et al, 2004].

<table>
<thead>
<tr>
<th>Table 5: Expression of somatostatin receptors in neuroendocrine gastroenteropancreatic tumors (%) [Öberg et al, 2004]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endocrine pancreatic tumors</strong></td>
</tr>
<tr>
<td>All tumors</td>
</tr>
<tr>
<td>Insulinoma</td>
</tr>
<tr>
<td>Gastrinoma</td>
</tr>
<tr>
<td>Glucagonoma</td>
</tr>
<tr>
<td>VIPoma</td>
</tr>
<tr>
<td>Non-functioning</td>
</tr>
<tr>
<td>Mid-gut neuroendocrine tumors</td>
</tr>
</tbody>
</table>

VIP, vasointestinal polypeptide.
*a* Using receptor subtype antibodies [32].
*b* Malignant insulinoma.

1.4.2. Somatostatin and its analogs

Despite its huge field of biological effects, the native somatostatin has been used with limited therapy applications because of its short half-life ($t_{1/2} = 3$ min). To improve this characteristic, analogs were developed, exhibiting longer half-life and increased potency.

Synthetic somatostatin analogs incorporates a Phe-(D)Trp-Lys-Thr sequence (or similar), stabilized at N- and C-terminals for clinical applications. There are three available analogs of somatostatin: octreotide [(D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr(ol); OCT], lanreotide [(D)ßNal-Cys-Tyr-(D)Trp-Lys-Val-Cys-Thr-NH$_2$; LAN ] and vapreotide [(D)Phe-Cys-Tyr-(D)Trp-Lys-Val-Cys-Trp-NH$_2$; VAP ] [Smith-Jones et al, 1999]. They showed to be effective in controlling growth of some types of cancer [Smith-Jones et al, 1999]. The first two being approved for clinical purposes were octreotide and lanreotide (Figure 12).
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Figure 12: Amino acid structures of native somatostatin (SST-14) (a) and its analogs octreotide (b) and lanreotide (c) [Grimberg, 2004]

Octreotide (Sandostatin®, SMS 201-95), the first somatostatin analog being approved, is a synthetic octapeptide analog of somatostatin, with similar effects, but a prolonged half life time ($t_{1/2} = 2h$), with high affinity for SSTR 2 and 5 [Lamberts et al., 1996]. It inhibits the release of pituitary growth hormone, suppresses the secretion of serotonin and the endocrine secretions of the pancreas, stomach, and intestine (including gastrin, vasoactive intestinal peptide, insulin, glucagon, secretin, motilin, and pancreatic polypeptide and TRH stimulated release of TSH) [Lamberts et al., 1996]. It also has a direct antiproliferative action in non-clinical models, probably by blocking the action of epidermal growth factor (EGF) [Lamberts et al., 1996]. The inhibition of gut hormones slows gastrointestinal transit time and regulates water and electrolyte transport across the gut (symptomatic benefit in patients with carcinoid syndrome and vasoactive intestinal peptide-secreting tumours). This drug is indicated to symptomatic metastatic carcinoid syndrome, symptomatic vasoactive intestinal peptide-secreting tumor (VIPoma), acromegaly, post high risk pancreatic surgery, emergency management of bleeding esophageal varices and also in functioning islet cell pancreatic cancer (CCO Formulary, 2004/2005). It can also provide benefit in other syndromes like ectopic adrenocorticotrophic hormone (ACTH) secretion with Cushing’s syndrome, oncogenic
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Introduction

osteomalacia, and hypercalcemia due to the secretion of ectopic parathyroid hormone-related peptide [Öberg et al, 2004].

**Lanreotide** (*Somatuline®, BIM 23014*) inhibits the secretion of growth hormone by the pituitary gland. This drug is extremely stable and consequently much longer acting than somatostatin. The reduction of growth hormone level which is achieved by Lanreotide permits control of the symptoms of acromegaly, but also in patients with carcinoids and endocrine pancreatic tumors. It has been introduced in some European countries a new slow-release preparation of lanreotide, *Somatuline Autogel®,* administered by deep subcutaneous injection once every four weeks [Öberg et al, 2004], compared with twice-daily injections of octreotide. Lanreotide and octreotide are equally efficacious in terms of symptom control and reduction in tumor cell markers for patients with carcinoid syndrome. Due to its simplified mode of administration, most patients prefer treatment with lanreotide [O’Toole et al, 2000].

**Vapreotide** (*Octastatin®, Sanvar®, RC 160, BMY 41606*) is a somatostatin analog developed at Tulane University School of Medicine, New Orleans, USA, which holds patent rights. This is a synthetic analogue of somatostatin, a cyclic octapeptide with high affinity for SSTRs 2 and 5 and some affinity for subtype 4 [Anon, 2003]. Vapreotide has a much higher metabolic stability than natural somatostatin. Vapreotide may also inhibit growth hormone secretion, insulin secretion, glucagon secretion and prolactin secretion. It has been used in the treatment of variceal bleeding in patients with cirrhosis (portal pressure and porto-collateral blood flow without inducing the systemic vasoconstrictor effects). Vapreotide has shown fewer adverse effects than octreotide. It is also useful in the treatment of AIDS-related refractory diarrhoea [Anon, 2003].

**Pasireotide** (SOM230) is a short synthetic somatostatin peptidomimetic, designed in 2002 by Bruns and colleagues, which exhibits high affinity binding to SSTR subtypes 1, 2, 3 and 5. According to Bruns et al (2002), this drug presents a 30 to 40-fold higher affinity for SSTR 1 and SSTR 5 than octreotide, and has a long lasting inhibitory effect on GH and IGF-I release. Its terminal half-life is also substantially prolonged ($t_{1/2} = 12$ h in humans) comparing to octreotide [Ma et al, 2005]. Preliminary data from phase II clinical trials support the rationale for using pasireotide as a treatment for patients with acromegaly, Cushing’s disease and metastatic carcinoid tumors [Glusman, 2008].

The somatostatin analogs present high or intermediate affinity for SSTRs 2, 3 and 5 and very low or no affinity for SSTR 1 and 4 (Table 6) [Pawlikowski and Mélen-
Mucha, 2004]. These peptides are very interesting targeting molecules for cancer cells expressing SSTR 2, the receptor that has been detected at high levels in a wide range of human tumors [Froideveux et al, 2000].

Table 6: Binding of SST analogs to SSTRs [Pawlikowski and Mélen-Mucha, 2004]

<table>
<thead>
<tr>
<th>Compound</th>
<th>$sst_1$</th>
<th>$sst_2$</th>
<th>$sst_3$</th>
<th>$sst_4$</th>
<th>$sst_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native SST (14 amino acids)</td>
<td>2.3</td>
<td>0.2</td>
<td>1.4</td>
<td>1.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Octreotide</td>
<td>&gt;1000</td>
<td>0.6</td>
<td>34.5</td>
<td>&gt;1000</td>
<td>7</td>
</tr>
<tr>
<td>Lanreotide</td>
<td>&gt;1000</td>
<td>0.8</td>
<td>107</td>
<td>&gt;1000</td>
<td>5.2</td>
</tr>
<tr>
<td>BIM-23244</td>
<td>&gt;1000</td>
<td>0.3</td>
<td>139</td>
<td>&gt;1000</td>
<td>0.7</td>
</tr>
<tr>
<td>SOM230</td>
<td>9.3</td>
<td>1.0</td>
<td>1.5</td>
<td>&gt;100</td>
<td>0.2</td>
</tr>
<tr>
<td>Native SST (14 amino acids)</td>
<td>2.3</td>
<td>0.2</td>
<td>1.4</td>
<td>1.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Somatostatin and its analogs may contribute to cancer diagnosis and treatment through a variety of mechanisms (Figure 13). Somatostatin analogs can control hypersecretion in neuroendocrine tumors (NETs) that express somatostatin receptors and may have also antiproliferative effects [Öberg et al, 2004].

During the past 30 years, several studies have been performed to assess the tolerability and efficacy of different types and doses of somatostatin in gastroenteropancreatic neuroendocrines tumors (Tables 7 and 8).
There may be some adverse effects associated with the treatment with somatostatin analogs, as nausea, abdominal cramps, loose stools, mild steatorrhea and flatulence. However, these symptoms tend to subside within the first few weeks of treatment. At the injection site there may be local reaction, with localized pain and erythema (Table 9). In some patients, it may also occur impaired glucose tolerance or diabetes mellitus (transient inhibition of insulin secretion) [Lamberts et al, 1996] and gastric atony is a very rare side effect. Also there is a risk of developing gallstones and gallbladder sludge in patients with metastatic gut NETs or malignant islet cell tumors.

![Table 7](Image)

Table 7: Octreotide studies in Gastroenteropancreatic neuroendocrine tumors [Delaunoit et al, 2005]

<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of patients</th>
<th>Agent</th>
<th>Dosage</th>
<th>Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arnold et al, 1996</td>
<td>52</td>
<td>Octreotide</td>
<td>200 µg 3 times daily</td>
<td>0</td>
</tr>
<tr>
<td>Manton et al, 1999</td>
<td>107</td>
<td>Octreotide</td>
<td>Various doses</td>
<td>7</td>
</tr>
<tr>
<td>Koels et al, 1997</td>
<td>22</td>
<td>Octreotide</td>
<td>150-500 µg 3 times daily</td>
<td>NR</td>
</tr>
<tr>
<td>Ruszniewski et al, 1993</td>
<td>4</td>
<td>Octreotide</td>
<td>200 µg twice daily</td>
<td>NR</td>
</tr>
<tr>
<td>Eriksson et al, 1991</td>
<td>14</td>
<td>Octreotide</td>
<td>100 µg twice to 3 times daily</td>
<td>28.6</td>
</tr>
<tr>
<td>Eriksson &amp; Oberg, 1991</td>
<td>19</td>
<td>Octreotide</td>
<td>100 µg twice daily</td>
<td>NR</td>
</tr>
<tr>
<td>di Bartolomeo et al, 1996</td>
<td>38</td>
<td>Octreotide</td>
<td>500-1000 µg 3 times daily</td>
<td>3</td>
</tr>
<tr>
<td>Saltz et al, 1994</td>
<td>34</td>
<td>Octreotide</td>
<td>250 µg 3 times daily</td>
<td>0</td>
</tr>
<tr>
<td>Ranco et al, 2000</td>
<td>15</td>
<td>Long-acting octreotide</td>
<td>20 mg/mo</td>
<td>7</td>
</tr>
<tr>
<td>Shojaanmand et al, 2002</td>
<td>15</td>
<td>Long-acting octreotide</td>
<td>20-30 mg/mo</td>
<td>0</td>
</tr>
<tr>
<td>Tomasini et al, 1998</td>
<td>16</td>
<td>Long-acting octreotide</td>
<td>20 mg/mo</td>
<td>0</td>
</tr>
<tr>
<td>Rubin et al, 1999</td>
<td>26 vs 22/20/25</td>
<td>Octreotide vs long-acting octreotide</td>
<td>300-900 µg/d (total dose) vs 10/20/30 mg/mo</td>
<td>NR</td>
</tr>
</tbody>
</table>

*BR = biochemical response; NR = not reported; OR = objective response; SD = stable disease; SR = symptomatic response.
†Combined end point.

![Table 8](Image)

Table 8: Lanreotide studies in gastroenteropancreatic neuroendocrine tumors [Delaunoit et al, 2005]

<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of patients</th>
<th>Agent</th>
<th>Dosage</th>
<th>Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eriksson et al, 1997</td>
<td>19</td>
<td>Lanreotide</td>
<td>4 mg 3 times daily</td>
<td>5</td>
</tr>
<tr>
<td>Imaizumi et al, 1997</td>
<td>8</td>
<td>Lanreotide</td>
<td>4 mg 3 times daily</td>
<td>0</td>
</tr>
<tr>
<td>Faiss et al, 1999</td>
<td>30</td>
<td>Lanreotide</td>
<td>5 mg 3 times daily</td>
<td>6.7</td>
</tr>
<tr>
<td>Rucci et al, 2000</td>
<td>25</td>
<td>Prolonged-release lanreotide</td>
<td>30 mg every 14 d</td>
<td>8</td>
</tr>
<tr>
<td>Schenke et al, 1994</td>
<td>18</td>
<td>Prolonged-release lanreotide</td>
<td>30 mg every 10-14 d</td>
<td>NR</td>
</tr>
<tr>
<td>Tomasini et al, 1998</td>
<td>18</td>
<td>Prolonged-release lanreotide</td>
<td>30 mg every 10 d</td>
<td>0</td>
</tr>
<tr>
<td>Ruszniewski et al, 1996</td>
<td>39</td>
<td>Prolonged-release lanreotide</td>
<td>30 mg every 14 d</td>
<td>0</td>
</tr>
<tr>
<td>Wyra et al, 1999</td>
<td>55</td>
<td>Prolonged-release lanreotide</td>
<td>30 mg every 14 d</td>
<td>6</td>
</tr>
</tbody>
</table>

*A = abdominal pain; BR = biochemical response; D = diarrhea; F = flushing; NR = not reported; OR = objective response; SD = stable disease; SR = symptomatic response.

Role of somatostatin receptors in angiogenesis 18
1.4.3. Somatostatin and its analogs in the inhibition of angiogenesis

In 2004, Adams et al studied the effects of octreotide and pasireotide in primary HUVECs expressing SSTR 1, 2 and 5. The authors observed that pasireotide significantly inhibited proliferation in VEGF-stimulated HUVECs that were unaffected by octreotide (46% at $10^{-6}$ – $10^{-7}$M). In this study, the authors suggest that the high affinity of pasireotide for SSTR 1, 2, 3 and 5, makes this drug an effective antiangiogenic agent regardless of variations in endothelial SSTR expression. This study and another one from 2005 [Adams et al] are the only two published works evaluating the effect of somatostatin analogs (pasireotide and octreotide) on primary HUVECs, but they only assess this effect on cell proliferation.

The peritumoral vascular system is a very attractive target of somatostatin action in tumor development. A recent study suggested that the expression of somatostatin receptors in peritumoral veins is a general phenomenon. All medullary thyroid carcinomas, colonic, and gastric cancers express somatostatin receptors in peritumoral veins; a majority of parathyroid adenomas, renal cell cancers, melanomas, sarcomas, breast cancers, and prostate cancers have somatostatin receptors in peritumoral veins, whereas gastroenteropancreatic tumors or ovarian cancers rarely do so [Reubi, 2003].

Combination therapies have been evaluated in clinical trials, including somatostatin analogs in combination with interferon-alpha. This combination, according to the results obtained by Faiss et al (2003), does not present higher antiproliferative effect than the obtained with monotherapy using a somatostatin analog in patients with progressive and metastatic gastroenteropancreatic neuroendocrine tumors, although they found that hormone-related symptoms were significantly better controlled with the combination therapy.

<table>
<thead>
<tr>
<th></th>
<th>Octreotide</th>
<th>Lanreotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction of diarrhea [33]</td>
<td>50%</td>
<td>45%</td>
</tr>
<tr>
<td>Reduction in flushing [33]</td>
<td>68%</td>
<td>54%</td>
</tr>
<tr>
<td>Most common adverse events</td>
<td>Gastrointestinal disorders, biliary disorders, injection site pain</td>
<td>Gastrointestinal disorders, biliary disorders, injection site pain</td>
</tr>
<tr>
<td>Availability of short-acting formulation</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Frequency of administration</td>
<td>Every 4 weeks</td>
<td>Every 2-4 weeks</td>
</tr>
</tbody>
</table>

Table 9: Comparative features of octreotide and lanreotide [Öberg et al, 2004]
Carcinoid heart disease is a rare but important cause of intrinsic tricuspid and pulmonary valve disease which leads to significant morbidity and mortality caused by right heart failure. Somatostatin analogs have provided symptomatic improvement and have improved survival in patients with this disease [Fox and Khatar, 2005]. Octreotide binds to SSTRs having the direct effect of reducing the vasoactive peptides that provoke the carcinoid syndrome [Fox and Khatar, 2005]. In this study, 70% patients obtained symptomatic relief from diarrhoea and flushing with a decrease in measurable 5-hydroxyindole acetic acid urinary secretion and serum 5-hydroxytryptamine concentrations [Fox and Khatar, 2005].

**1.5. Aims**

The purpose of this work was to explore the role of somatostatin receptors in angiogenesis, by evaluating the effects of somatostatin analogs in endothelial cell viability, migration, cell death and apoptosis, and expression of somatostatin receptors. Thus, the specific aims were:

- Evaluate the influence of somatostatin analogs on HUVECs’ growth along time and at different concentrations by the exclusion method of tripan blue;
- Evaluate the influence of somatostatin analogs on cell migration by wound-healing assay;
- Evaluate the effects of somatostatin analogs on cell death by TUNEL assay and on apoptosis by Annexin V-FITC;
- Develop an RT-PCR protocol to analyse SSTR expression and assess SSTR expression before and after somatostatin analogs exposure.
2. **Material and methods**

2.1. **Human umbilical endothelial cells (HUVEC)**

HUVECs were gently offered by Professor Ruis Reis, leader of the 3B’s Research Group Biomaterials, Biodegradables and Biomimetics, University of Minho. HUVECs were harvested by collagenase digestion, according to the method of Jaffe *et al* (1973), with minor modifications. Tests carried out to confirm the cell type were immunocytochemistry (immunostaining for 2 surface and 1 internal antigens/markers: CD54 and CD31 at the cell membrane surface, and von Willebrand factor, intracellular) and morphology evaluation under light microscopy (cells in confluent culture have polygonal cobblestone-like sheet shape typical of endothelial cells cultured in static conditions – **Figure 14**).

![Figure 14: HUVEC in monolayer culture, x200](image)
2.2. Somatostatin analogs

Somatostatin analogs, Octreotide (SMS 201-995) and Pasireotide (SOM 230), were kindly provided by Novartis™. Both drugs were dissolved in deionized water and stock solutions were prepared with concentrations ranging from $10^{-4}$ to $10^{-10}$M, based on previous studies (Adams R et al., 2004 and Adams R et al., 2005).

2.3. HUVEC growth conditions

HUVECs were cultured in Medium 200 (ref. M-200-500) supplemented with Low Serum Growth Supplement kit (fetal bovine serum 2% v/v, hydrocortisone 1µg/ml, human EGF 10ng/ml, bFGF 3ng/ml with heparin 10µg/ml and PSA, ref. S-003-K), purchased from Cascade Biologics™. HUVECs were grown at 37°C with 5% of CO$_2$ in a humidified cell culture incubator (Figure 15) and assayed at passage three, in a laminar flow biological safety cabinet (Figure 16).

Figure 15: Humidified cell culture incubator

Figure 16: Laminar flow biological safety cabinet prepared to work with cell culture
2.4. **Influence of different somatostatin analog concentrations on HUVEC growth**

HUVECs were seeded into six-well plates at a density of $10^5$ cells/well. After 24 hours (doubling time), drugs were added in fresh medium, to achieve the following final concentrations: $10^{-6}$ M, $10^{-8}$ M, $10^{-10}$ M and $10^{-12}$ M. HUVECs were incubated with the drugs either for 24 hours or 48 hours. Cells were then trypsinized (0.5% Trypsin, 5.3 mM EDTA 4Na, 10x, ref. 15400-054, Invitrogen) and counted in a haemocytometer chamber using tripan blue stain (0.04% solution) under a bright-field microscope (Figure 17) in order to distinguish live (unstained) from dead cells (blue staining).

Results are from 3 independent experiments and are given as mean ± standard deviation.

![Figure 17: Bright-field microscope](image)

2.5. **Influence of somatostatin analog exposure time on HUVEC’s growth**

HUVECs were seeded into six-well plates at a density of $10^5$ cells/well. After 24 hours, drugs were added in fresh medium, in order to achieve a $10^{-6}$ M concentration. The HUVECs were incubated with the drugs for 3h, 6h, 12h, 18h and 24h. Cells were then trypsinized and counted in a haemocytometer chamber using tripan blue stain (0.04% solution) in order to distinguish live (unstained) from dead cells (blue staining).

The results are from 3 independent experiments and are given as mean ± standard deviation.
2.6. **Influence of somatostatin analog exposure on cell migration**

In order to study the effects of drug exposure on cell migration during the exposure to octreotide and pasireotide, the *in vitro* scratch assay was used. This method mimics cell migration during wound-healing *in vivo* according to Liang et al (2007). Briefly, HUVECs were grown on a 6 well-plate to 90% confluence. An artificial gap (“scratch”) was created on the monolayer and the images were captured at the beginning of the process and at regular intervals (6, 12 and 24h) during cell migration to close the wound. The images acquired for each sample were analyzed quantitatively using the ImageJava, a free-ware program downloaded from http://rsb.info.nih.gov/ij/. The images were compared from time zero to the last time established to obtain the distance of each wound closure measured (Magnification x200).

2.7. **Cell death and apoptosis assays**

HUVECs were seeded into six-well plates at a density of $10^5$cells/well and were treated with the drugs for six hours.

2.7.1. **In situ cell death detection (TUNEL assay)**

Cleavage of genomic DNA may yield single stranded breaks (“nicks”) in high molecular weight DNA (*Figure 18*). Those DNA stranded breaks were identified by terminal deoxynucleotidyl transferase-mediated deoxurydine triphosphate nick end labeling (TUNEL) with the In Situ Cell Death Detection Kit, Fluorescein (Cat.no 11 684 795 910, Roche Applied Sciences, Mannheim, Germany). Propidium iodide was used for counter staining.

**Optical counting by fluorescence microscopy:** HUVECs were grown on cover slips under pre-determined conditions and after 90% confluence were rinsed three times in PBS to remove the medium. Cells were fixed with a freshly prepared fixation solution (2% p-HCHO/1xPBS) for 20 min at room temperature and, after washing with PBS, incubated in permeabilization solution (0.1% Triton X-100 in 0.1% SDC) for 2 minutes at 4°C. Each slide was washed with PBS, covered with TUNEL mixture (enzyme solution, TdT and nucleotide mixture) and incubated for 60 minutes at 37°C in a humidified
chamber, in the dark. After incubation, slides were embed with antifade medium (Vectashield® Mounting Medium) and protected with coverslides to be analyzed by epifluorescence (excitation wavelength in the range of 450-500nm and detection in the range of 515-565nm). A total number of 900 cells were counted in order to establish a reliable relationship between the different conditions.

**Automatic counting by flow cytometry:** Fluorescein (FITC) labels incorporated in nucleotides polymers were detected and quantified also by flow cytometry (flow cytometer Coulter Epics). HUVEC grown at pre-established conditions and exposed 6h to drugs were centrifuged at 2000 rpm during 5 minutes. The cell pellet was washed 3 times in PBS, resuspended and the fixation solution was added for 20 minutes at room temperature, on a shaker to avoid excessive clumping of the cells. Cells were then centrifuged at 1200 rpm during 10 minutes and the fixative removed by suction. HUVECs were washed once with PBS, centrifuged at 1200 rpm for 10 minutes to remove the buffer and the pellet was resuspended in permeabilisation solution for 2 minutes at 4°C. After washing the cells twice with PBS (by centrifugation at 1200 rpm, 10 min), cells were resuspended in 50 µl with TUNEL reaction mixture. The preparation was incubated for 60 minutes at 37°C in a humidified chamber, in the dark. After incubation, cells were washed twice in PBS and transferred into a tube to a final volume of 500 µl in PBS. The cell suspension was introduced in the flow cytometer for automatic reading.

![Figure 18](image-url): DNA stranded breaks labeled by TUNEL reaction and incorporation of fluorescein in nucleotide polymers for detection and quantification by flow cytometry and fluorescence microscopy. (Author Isis Alonso)
2.7.2. **Annexin V-FITC/PI**

Phosphatidylserine molecules, translocated to the outer layer of the intact plasma membrane during the apoptotic process (Figure 19), were identified by binding annexin V labeled with fluorescein isothiocyanate (FITC). To discard cells in later apoptosis or in necrosis (the plasma membrane is no longer intact), propidium iodide was used simultaneously.

HUVECs were treated under pre-determined conditions and, after being collected by centrifugation, were resuspended in Annexin V binding buffer (10mM HEPES/NaOH, pH7.4, 140mM NaCl, 2.5mM CaCl2). Annexin V-FITC (Apodetect™ AnnexinV-FITC kit, cat no 33-1200, Zymed® laboratories) and propidium iodide (20µg/ml) were added to the suspension and the mixture was incubated in the dark, for 5 min, at room temperature. The cell suspension was placed on a glass slide and covered with a coverslide. The preparation was readily observed under an epifluorescence microscope (filters for FITC and rhodamine).

![Figure 19: Exteriorization of phosphatidylserine molecules during apoptosis and Annexin V-FITC labeling (Author Isis Alonso)](image-url)
2.8. Analysis of SSTR expression

2.8.1. Primers design and PCR conditions

Human SSTRs 1-5 oligonucleotide forward and reverse primers were designed using Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3). Primer sets were submitted to a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm their uniqueness (Table 10).

According to previous studies (Adams et al., 2004 and Adams et al., 2005), human genomic DNA was used as the positive control for all somatostatin receptors. For optimization of the conditions required, PCR reactions were performed in a final volume of 25 µl, with the following composition: buffer 1x (Bioron™, Germany); 1.75mM MgCl$_2$ (Bioron™, Germany); 200µM of dNTPs mix (Fermentas™, USA); 0.3µM of each primer ((MWG Biotech™, Germany); 1 U of Taq polymerase (Bioron™, Germany); 0.5µl of genomic DNA. The mix was treated in a Biorad™(USA) thermocycler with the following protocol: denaturation at 96°C for 10 minutes, 36 cycles of: denaturation (96°C), primer annealing (SSTRs 1, 2, 4, 5: 56.4°C; SSTR 3: 51.3°C) and extension (72°C) for 45 seconds and a final extension cycle (72°C) for 10 minutes.

To assess the quality of amplification, PCR products were separated by electrophoresis on 2% agarosis gel stained with ethidium bromide and visualized under ultra-violet light. Images were captured by an Alpha Innotech FluorChem 8800 (Alpha Innotech Co. CA).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Sequence</th>
<th>Melting Temperature (°C)*</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSTR1</td>
<td>Forward</td>
<td>TGAGTCAGCTGTGGGTACATC</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACACTGTAGCCACGGCTCTTT</td>
<td>59.4</td>
</tr>
<tr>
<td>SSTR2</td>
<td>Forward</td>
<td>CCCCTCACCCATCTACGTCTCT</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGTGAGGACCACGCAAAAG</td>
<td>59.4</td>
</tr>
<tr>
<td>SSTR3</td>
<td>Forward</td>
<td>TCTGCTACCTGCTACGTG</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTGAAGCGGTAGGAGGAGAAG</td>
<td>57.3</td>
</tr>
<tr>
<td>SSTR4</td>
<td>Forward</td>
<td>CACCAGCGTCTCTGCTCTCA</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGGGGAGGTGACCAACAG</td>
<td>59.4</td>
</tr>
<tr>
<td>SSTR5</td>
<td>Forward</td>
<td>TCATCTGCGTGTCTGCTACCTG</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAGAGGATGACCACGAGA</td>
<td>59.4</td>
</tr>
</tbody>
</table>

* Table 10: Somatostatin receptors 1-5 (SSTR1-5) oligonucleotide forward and reverse primers; $T_m=4(G+C) +2(A+T)$
2.8.2. **Extraction and quantification of RNA from HUVEC**

Extraction of RNA from HUVECs was performed by the Trizol® method [adapted from Chomczynski and Sacchi, 1987].

HUVECs treated under predetermined conditions were collected by centrifugation (1200 rpm, 5 min), homogenized and lysed in Trizol® (Invitrogen™, USA) reagent by repetitive pipetting (1 ml Trizol®/5-10 x 10⁶ cells). After vigorous mixing, samples were homogeneized for 5 minutes at room temperature (complete dissociation of nucleoprotein complexes), chloroform (0.2 ml/ 1 ml Trizol®) was added, the mix was shaken for 15 seconds and incubated for 5 minutes. Following incubation, samples were centrifuged at 13,000 rpm for 15 minutes at 4°C in order to separate the mixture into a lower red phenol-chloroform phase and a colorless upper aqueous phase, which contains the RNA. This aqueous phase was transferred onto a new sterile tube, isopropyl alcohol (0.5 ml / 1 ml Trizol®) was added and samples were incubated at room temperature for 10 minutes (precipitation of the RNA). Following incubation, samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol (1 ml / 1 ml Trizol®), with vigorous mixing (10 seconds), following centrifugation at 8,000 rpm for 5 minutes at 4°C. The supernatant was then carefully discarded and the RNA pellet was briefly air-dried and dissolved in RNase-free water (20µl). Extracted RNA was quantified by spectrophotometry (Nanodrop®) and stored at -80°C.

2.8.3. **Synthesis of complementary DNA (cDNA) for Reverse Transcriptase-PCR**

To assess the expression of the receptors semi-quantitatively, exponential amplification via reverse transcription polymerase chain reaction was used, following the manufacturer’s instructions for the kit SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen™, USA). The extracted RNA (5 µg), random primer (1 µl) and DEPC-water (necessary volume to complete a total of 11µl) mixture was prepared in a PCR tube. Samples were incubated for 10 minutes at 70°C, followed by incubation for 5 minutes at 4°C. A second mix (8µl) was added to the samples: PCR buffer 10x (2µl), 25mM MgCl2 (2µl), 10mM dNTP mix (1 µl), 0.1 M DTT (2 µl) and RNase out (1 µl).
Samples were incubated for 5 minutes at 42°C, then the SuperScript® RT enzyme (1 µl) was added and this mixture was incubated for 50 minutes at 42°C, followed by incubation for 15 minutes at 70°C for 15 minutes. At the end of this step, RNase H (1µl) was added and the samples incubated for 20 minutes at 37°C. The final product (cDNA) was stored at -20°C.

The PCR conditions used to the amplification of the resulting cDNA samples and the primers used were described in Section 2.8.1. For semiquantitative comparison, HPRT was used as the house keeping gene. PCR products were separated by electrophoresis on 2% agarosis gel stained with ethidium bromide and visualized under ultra-violet light. Images were captured by an Alpha Innotech FluorChem 8800 (Alpha Innotech Co. CA).

2.9. Statistical analysis

Results are expressed as Mean±SD of three independent experiments with three replicates each. Data were analysed using the statistical functions of Microsoft Excel version 2007, from Microsoft Home Student 2007. Data were compared by Student’s t-test. *P*-values lower than 5% were considered statistically significant.
3. Results and Discussion

3.1. Influence of different somatostatin analog concentrations on HUVEC’s growth

The concentration-response effect of both drugs was determined after selected exposure times. Treatment of HUVEC with $10^{-12}$ to $10^{-6}$ M octreotide for 24 and 48 h resulted in a concentration-dependent decrease in the percentage of viable cells (Figure 20 A). A significant decrease in the percentage of viable cells relative to control ($p<0.05$) was observed in incubations with $10^{-6}$ M (48h) and $10^{-8}$ M (24 and 48h) octreotide. A significant and more evident decrease ($p<0.01$) was observed with $10^{-6}$ M octreotide at 24h. In general, the percentage of viable cells at 24 hours was lower than at 48 hours, although not significant. HUVEC treated with $10^{-12}$ to $10^{-6}$ M pasireotide for 24 and 48h presented also a concentration-dependent decrease in the percentage of viable cells (Figure 20 B). A significant decrease in viability ($p<0.05$) was observed in incubations with $10^{-6}$ M (48h) and $10^{-8}$ M (24 and 48h) of pasireotide. A significant and more evident decrease in cell viability ($p<0.01$) was found with $10^{-6}$ M of pasireotide at 24h.

For both drugs the same pattern was obtained: the number of viable cells for both times was dose-dependent, meaning that the number of viable cells decreased with increasing concentrations of the drugs. From these findings and in agreement with Adams et al. (2005), octreotide and pasireotide were used at a concentration of $10^{-6}$ M in subsequent assays. In the referred study, HUVECs were exposed to octreotide and pasireotide for 21h ($10^{-10}$M to $10^{-6}$M for both drugs) and dose-response curves were constructed. Octreotide significantly inhibited HUVEC proliferation at the concentration range of $10^{-10}$M to $10^{-6}$M and pasireotide at the concentration range of $10^{-9}$M to $10^{-6}$M, in a dose-dependent manner. Inhibition of proliferation was significantly higher for $10^{-6}$M octreotide and $10^{-6}$M pasireotide.
### 3.2. Influence of somatostatin analog exposure time on HUVEC’s growth

Once the most effective concentration was determined, it was necessary to select the most effective drug exposure time in order to establish the subsequent working conditions.

As shown in Figure 21 A, a significant decrease in viability ($p<0.01$) was observed from 3 to 18h exposure to $10^{-6}$M octreotide. The lowest percentage in viable cells was obtained after 6 hours of exposure. After 24h of incubation, cell viability was lower in treated cells compared to control but this difference as not significant. In cells exposed to pasireotide, a significant decrease in cell viability was achieved from 6 to 24h of treatment ($p<0.01$, Figure 21 B). The percentage of viable cells at 6h of exposure exhibited the lowest registered value.

Both drugs presented a time-dependent decrease in viability with a tendency to recover with time, being 6h the most effective time of exposure. All subsequent experiments were performed with $10^{-6}$M drug concentration and 24 h exposure time.
3.3. Cell migration in vitro in the presence of somatostatin analogs

Wound-healing assay is a reliable method to analyze cell migration according to Liang et al (2007). The hypothesis tested is that in the presence of these somatostatin analogs the migration capacity of the cells would be decreased. As shown in Figure 22 and 23, after incubation with $10^{-6}$M octreotide and $10^{-6}$M pasireotide for 6h (maximal drug effect), a non-significant decrease in the distance between the edges of the “scratch” (wound) was observed. After 12h of drug treatment, this distance significantly diminished ($p<0.05$ compared to control), corresponding to the period of time in which a weaker effect of both drugs on cell viability was observed. In the absence of drugs, the migration capacity of the cells was intense, represented by a significant decrease in the distance between the edges of the wound at 6 and 12h of incubation in control cells ($p<0.01$ compared to control at 0h). In comparison to control cells the presence of both analogs inhibited migration significantly after 6 and 12h of incubation ($# p<0.01$). After 24h of incubation, all samples were confluent, forming a perfect monolayer. These results translated the time-dependent negative influence of the presence of octreotide and pasireotide on HUVEC migration, suggesting that this particular behaviour might be observed in vivo, preventing during a certain period of time the formation of new blood.
vessel through the inhibition of proliferation and migration of the endothelial cells, which was postulated as one of the remarkable functions for somatostatin and its analogs [Woltering et al, 1997; Adams et al, 2004].

![Cell migration analysis in wound-healing assay](image)

**Figure 22:** Cell migration analysis in the presence of drugs at a concentration of 10^{-6}M. The distances between the edges of the wound decreased significantly in control cells with time (**p<0.01**); migration was not significant in treated cells after 6h but the distance between the edges decreased significantly in treated cells after 12h (**p<0.05**). In comparison to control cells the presence of both analogs inhibited migration significantly after 6 and 12h incubation (# p<0.01). Data are from 3 independent experiments and are expressed as mean±SD.
Figure 23: Cell migration *in vitro* in the presence of somatostatin analogs. Representative images of control cells (untreated) and cells after exposure to $10^{-6}$M octreotide and $10^{-6}$M pasireotide for 0, 6, 12 and 24h. Magnification x200.
3.4. Evaluation of cell death and apoptosis after exposure to somatostatin analogs

TUNEL assay was used to assess cell death and Annexin V-FITC was used to detect early apoptosis upon HUVECs exposure to somatostatin analogues.

3.4.1. In situ cell death detection (TUNEL assay)

After 6h of incubation (Figures 24 and 25), cells exposed to $10^{-6}$M octreotide and $10^{-6}$M pasireotide presented a significantly higher percentage of cell death in comparison with control cells ($p<0.01$), as evaluated by optical counting. Additionally, cells exposed to $10^{-6}$M pasireotide presented a significantly higher percentage of cell death comparing to cells exposed to $10^{-6}$M octreotide ($p<0.05$).

In order to confirm and validate the results obtained with the optical counting, flow cytometry reading was used. As shown in Figure 26, a significant increase in the percentage of death after 6h exposure to $10^{-6}$M pasireotide and $10^{-6}$M octreotide was observed ($p<0.01$ compared to control). The percentage of death is significantly higher for samples treated with $10^{-6}$M pasireotide than for those treated with $10^{-6}$M octreotide ($p<0.05$). These results are in agreement with the ones obtained by optical counting. Automatic cell counting by flow cytometry is less subject to human error, leading to more reliable results. With this methodology, all cells are quantified independently of viability, while when counting cells on slides, only adherent cells are visualized, which might explain the difference in the relative percentage of cell death between the two assays. However, cells grown on slides allow the acquisition of images of the labeled cells.

Nevertheless, with both methods, the same pattern of results was observed: the presence of both drugs promotes a very significant increase in cell death, being $10^{-6}$M pasireotide more efficient than $10^{-6}$M octreotide. In a previous study [Pan et al, 2004], a significant percentage (30.53%) of cell death was achieved with the native molecule somatostatin and increasing concentrations resulted in increased percentage of cell death. Somatostatin analogs present most of the characteristic actions of the native molecule, including the induction of cell death [Pironnet et al, 2008]. Other authors have already described the significant decrease in the number of viable cells when exposed to both somatostatin analogs [Adams et al, 2004; Adams et al, 2005; Schmid, 2008].
Results and Discussion

**Figure 24:** Percentage of dead cells detected by the TUNEL assay, after 6h hours of exposure to somatostatin analogs (optical counting). A significant increase in cell death was observed after treatment with 10^{-5}M octreotide and pasireotide (***p<0.01 compared to control). Percentage of cell death is significantly higher for samples treated with 10^{-6}M pasireotide than for those treated with 10^{-5}M octreotide (#p<0.05). Data are from 3 independent experiments and are expressed as mean±SD.

**Figure 25:** Images of TUNEL assay showing total cell population labeled with propidium iodide (left) and dead cells labeled with fluorescein (right), after 6 hours of exposure to somatostatin analogs. Magnification x200, Olympus epifluorescence microscope.
Results and Discussion

Figure 26: Evaluation of cell death (TUNEL assay) after 6h of exposure to somatostatin analogs by flow cytometry A. A significant increase in cell death was observed after treatment with 10⁻⁶ M octreotide and 10⁻⁶ M pasireotide (***p<0.01 compared to control cells). Cells exposed to 10⁻⁵ M pasireotide presented a significantly higher percentage of cell death comparing with cells exposed to 10⁻⁶ M octreotide (##p<0.05). Data are from 2 replicates and are expressed as mean±SD; B, C, D. Flow cytometry chart for control cells (untreated, B), cells treated with 10⁻⁵ M octreotide (C) and 10⁻⁶ M pasireotide (D), respectively.

3.4.2. Annexin V-FITC/PI

In this assay, the index of early apoptosis was assessed. The values were obtained by optical counting in an epifluorescence microscope after 6h of exposure to 10⁻⁶ M pasireotide and 10⁻⁶ M octreotide (Figure 27). A significant increase in the percentage of cells marked only with FITC was observed in cells treated with both drugs (p<0.01 compared to control). These cells presented the phosphatidyserine molecules in the outer layer of the intact plasma membrane, indicative of the earlier process of apoptosis. The difference in percentages of cells in early apoptosis between cells exposed to 10⁻⁶ M...
octreotide was significantly higher than to cells exposed to 10^{-6}M pasireotide (p<0.05). Comparing with the results obtained in the previous section with TUNEL technology, where 10^{-6}M pasireotide was responsible for a higher number of dead cells than octreotide, it is probable that pasireotide induced apoptosis earlier than octreotide. This particular characteristic was not addressed by any of the authors of the referred publications. However, this might be an interesting issue to be further explored.

![Graph: Control, Octreotide, Pasireotide](image)

**Figure 27:** Evaluation of early apoptosis in cells exposed to somatostatin analogs. A. Percentage of apoptotic cells (Annexin V-FITC positive) after 6 hours of exposure to somatostatin analogs. A significant increase in the percentage of cells in early apoptosis was observed after treatment with 10^{-6}M octreotide and 10^{-6}M pasireotide (**p<0.01**). The difference of percentages of cells in early apoptosis between cells exposed to 10^{-6}M octreotide was significantly higher than to cells exposed to 10^{-6}M pasireotide (*p<0.05*). Data are from 3 independent experiments and are expressed as mean±SD. B, C, D. Representative images of the three conditions: control (untreated cells, B), 10^{-6}M octreotide (C) and 10^{-6}M pasireotide (D).
3.5. **Analysis of SSTR expression upon exposure to somatostatin analogs**

G protein-linked receptors, as SSTRs, are rapidly internalized after agonist-induced receptor phosphorylation which translates in a decrease in the expression of these receptors [Pierce *et al.*, 2002; Liu *et al.*, 2005]. The significance of the decrease in SSTR expression after exposure to drugs is directly related to the efficiency in binding to SSTR [Liu *et al.*, 2005].

3.5.1. **Optimization of amplification conditions**

The analysis of SSTR expression (SSTR 1, 2, 3, 4 and 5) was obtained by Reverse-Transcriptase PCR and was quantified by comparison with a reference gene (HPRT) expression. After primer design, several reaction conditions were tested using genomic DNA was used as positive control. The results obtained are represented in **Figure 28**. The annealing temperature of 56.4°C was efficient to amplify SSTR 1, 2, 4, 5 and 51.3°C for SSTR 3. Nevertheless, SSTR3, 4 and 5 presented weak bands and a slighted higher concentration of MgCl$_2$ (1.75mM) was used. The results obtained were satisfactory and are represented in **Figure 29**. The final conditions are described in the **Material and Methods** section.

![Figure 28](image1.png)

**Figure 28**: PCR optimization of SSTR amplification in genomic DNA, at different annealing temperatures. (+) – positive control; (-) – negative control

![Figure 29](image2.png)

**Figure 29**: Optimization of SSTR amplification in genomic DNA with higher MgCl$_2$ concentration. (+) – positive control; (-) – negative control
3.5.2. Expression of SSTR 1

As illustrated in Figure 30, the SSTR 1 expression in control cells (untreated) after 6h of incubation was relatively weak. Nevertheless, in cells treated with $10^{-6}$M pasireotide, the expression of SSTR1 was significantly lower compared with the value obtained for control cells ($p<0.05$). After 6h hours, cells exposed to $10^{-6}$M octreotide did not present a significant difference in SSTR1 expression when compared to control cells.

According to Bruns et al (2002), pasireotide presents a 30 to 40-fold higher affinity for SSTR1 than octreotide, promoting a more extensive number of ligand-receptor interactions and thus decreasing the number of externalized receptors. This means that in proliferating human endothelial cells expressing SSTR1, continuous exposure to pasireotide would probably result in a decrease of SSTR1 expression, as obtained in this work. Activation of SSTR 1 by SST and its analogs is involved in the inhibition of cell proliferation through a cytostatic effect [Roosterman et al, 1998; Florio et al, 1999, 2000; Susini and Buscali, 2006] and in inhibition of endothelial of cell migration [Buchan et al, 2002]. According to Adams et al (2004), SSTR 1 is one of the somatostatin receptors that have an important role in the mediation of antiproliferative effects of pasireotide.

![Figure 30: Expression of SSTR 1 after somatostatin analog exposure.](image)

A. After 6h of incubation, expression of SSTR1 in cells treated with $10^{-6}$M pasireotide was significantly lower than control cells ($p<0.05$), while in cells treated with $10^{-6}$M octreotide the difference was not significant when compared to control cells. B. Image of agarosis gel representative of the SSTR1 expression from each condition (in triplicate); (L) – Ladder 100 bp. C. Image of agarosis gel representative of the HPRT expression (reference gene) in the different samples.
3.5.3. **Expression of SSTR 2**

After 6h of incubation, the expression of SSTR 2 in cells treated with $10^{-6}$M pasireotide was significantly lower than control cells ($p<0.05$). Similar results were obtained for cells exposed to $10^{-6}$M octreotide. **(Figure 32)**

According to Adams *et al* (2005), SSTR 2 is preferentially expressed in proliferating endothelial cells, as the HUVECs used in this work. As described by Pawlikowski and Mélen-Mucha (2004), pasireotide and octreotide present high binding affinity for SSTR 2. These data support the results obtained in this analysis of SSTR 2: the expression of externalized SSTR 2 in proliferating endothelial cells decreases significantly after exposure to pasireotide and octreotide. SSTR 2 is the most widely distributed SSTR subtype in both normal and tumoral tissues being the most targeted SSTR pharmacologically [Susini and Buscail, 2006].

![Figure 31: Expression of SSTR 2 after somatostatin analog exposure. A. After 6h of incubation, expression of SSTR 2 in cells treated with $10^{-6}$M pasireotide and $10^{-6}$M octreotide was significantly lower than control cells (*$p<0.05$). B. Image of agarosis gel representative of the SSTR 2 expression from each condition (in triplicate); (L) – Ladder 1000bp. C. Image of agarosis gel representative of the HPRT expression (reference gene) in the different samples.](image-url)
3.5.4. **Expression of SSTR 3**

After 6h of incubation, a significant increase of expression of SSTR 3 in cells treated with $10^{-6}$M octreotide cells was observed ($p<0.05$ compared to control; **Figure 33**). Although not expected, this was a consistent result in all independent assays performed for this receptor, and might reflect a compensation mechanism of HUVECs, since octreotide has a low affinity for this receptor compared to SSTR2 and SSTR5. Additionally, it has been described a transient increase in SSTR 3 expression before a significant decrease to basal levels in another cell line upon exposure to native somatostatin, showing that in some tissues the cellular response may be biphasic [Moller et al, 2003]. This particular HUVEC line might have presented that kind of biphasic response to octreotide and the increase in SSTR 3 expression observed would be the first phase. However, further studies will be necessary to confirm this hypothesis.

HUVECs exposed to pasireotide present residual expression of SSTR 3, lower than control cells, although not significantly different (**Figure 33**).

![Figure 32: Expression of SSTR 3 after drug exposure. A. After 6h of incubation, expression of SSTR 3 in cells treated with $10^{-6}$M octreotide was significantly higher than control cells ($^*p<0.05$). B. Image of agarosis gel representative of the SSTR 3 expression from each condition (in triplicate). C. Image of agarosis gel representative of the HPRT expression (reference gene) in the different samples.](image-url)
3.5.5. **Expression of SSTR 4**

The relative expressions of SSTR 4 obtained in all samples in comparison with HPRT expression are residual (Figure 34) and have to be considered with reservation. Further studies will have to be performed to confirm the low expression of SSTR 4, for example using real time PCR and Western Blot (protein analysis). However, our results are in accordance with a previous study, [Adams *et al.*, 2005], in which the authors did not observe SSTR 4 expression in any HUVECs samples used. They then assumed that there may be an individual variability related to primary culture cells from different donors, since the presence of this receptor has been already reported in primary endothelial cells [Curtis *et al.*, 2000].

![Expression of SSTR 4 after drug exposure. A. After 6h of incubation, expression of SSTR 4 was residual for all samples. B. Image of agarose gel representative of the SSTR 4 expression from each condition (in triplicate). C. Image of agarose gel representative of the HPRT expression (reference gene) in the different samples.](image)

*Figure 33: Expression of SSTR 4 after drug exposure. A. After 6h of incubation, expression of SSTR 4 was residual for all samples. B. Image of agarose gel representative of the SSTR 4 expression from each condition (in triplicate). C. Image of agarose gel representative of the HPRT expression (reference gene) in the different samples.*
3.5.6. **Expression of SSTR 5**

The relative expressions of SSTR 5 obtained for all samples in comparison with HPRT expression are residual (Figure 35). Adams et al (2005) described in their results that 3 of 9 (33.3%) different primary HUVEC (from different donors) were negative for SSTR5, which might explain the results obtained in the current work. In the referred work, the authors concluded that SSTR 2 and 5 were preferentially expressed in proliferating endothelium. Nevertheless, the expression of the different SSTRs was not assessed after treatment with pasireotide and octreotide and it was not possible to conclude if the inhibition of cell proliferation would be caused by activation of either or both receptors (2 and 5), since both drugs have considerable affinities for them. However, further studies would have to be done to assess SSTR 5 expression using real time PCR and Western-blotting (protein analysis).

The results obtained with the current work suggest that SSTR 2 may represent a very important role in the inhibition of primary endothelial cell proliferation. Our results indicate that the residual expression of SSTR 5 is not impeditive for success in the inhibition of cell proliferation if the cells express other SSTRs, especially SSTR 2 for which the somatostatin analogs used present high affinity.

![Image of agarosis gel representative of the HPRT expression](image)

**Figure 34:** Expression of SSTR 5 after drug exposure. A. After 6h of incubation, expression of SSTR 5 was residual for all samples. The value registered for expression in cells treated with 10^{-6}M octreotide was not significant. B. Image of agarosis gel representative of the SSTR 5 expression from each condition (in triplicate). C. Image of agarosis gel representative of the HPRT expression (reference gene) in the different samples.
4. **Concluding remarks**

Since decades the importance of angiogenesis in the maintenance and development of tumors has been highlighted by many investigators. Dr Judah Folkman has been the guide leader in the pursuit of different ways to control the mechanisms for inhibit this process [Folkman, 1971; Folkman, 2003].

The current work was based in the fact that proliferating endothelial cells express somatostatin receptors [Adams et al, 2005], representing suitable targets for antiangiogenic therapy. Somatostatin analogs have been explored as potential antiangiogenic drugs besides their action in the control of hormone-release [Woltering et al, 1997]. In the present study, octreotide, the first SST analog approved by FDA, and pasireotide, a multi-receptor ligand SST analog, yet in phase II of clinical trials [Glusman, 2008] were used.

In the present work, HUVECs presented similar behaviour as described in earlier studies [Adams et al, 2004; Adams et al, 2005]. In cells exposed to pasireotide, the decrease in cell viability and the increase in cell death were significantly marked in comparison to cells exposed to octreotide. The evaluation of cell death showed that pasireotide was responsible for a higher number of dead cells than octreotide, however, a significant higher apoptotic index was obtained for cells exposed to octreotide than to pasireotide. Comparing with these results, one could hypothesise that pasireotide induced apoptosis earlier than octreotide. This particular characteristic was not explored by any of the publications referred in this work. This might be an interesting issue to study further, but the most important is that both drugs could induce apoptosis significantly. Cell migration was analised by wound-healing assay and it was observed that the presence of both analogs inhibited migration significantly after 6 and 12h of incubation. These results suggest that this particular behaviour might be observed in vivo, preventing during a certain period of time the formation of new blood vessel through the inhibition of proliferation and migration of the endothelial cells, which was postulated as one of the remarkable functions for somatostatin and its analogs.

There were no reports on the behaviour of SSTRs expression after endothelial cell exposure to octreotide and pasireotide. Adams et al (2005) concluded that SSTR 2 and 5 were preferentially expressed in proliferating endothelium, however, the different SSTRs’ expressions were not assessed after treatment with the somatostatin analogues and it was
not possible to conclude if the inhibition of cell proliferation would be caused by activation of either or both receptors (2 and 5), once both drugs have considerable affinities for them.

In this work, a significant decrease in the expression of SSTR 1 in cells treated with pasireotide was observed, while cells exposed to octreotide did not present a significant difference in SSTR 1 expression. The expression of SSTR 2 in cells treated with pasireotide and octreotide was significantly lower comparing with control cells. SSTR 3 expression was not the expected with an apparent upregulation of the receptor in cells exposed to octreotide, while in untreated cells and in cells treated with pasireotide the expression was residual. Expressions of SSTR 4 and 5 were residual under the conditions tested. The results obtained are probably related to the pattern of SSTR expression in the HUVECs used. Pasireotide is a multi-receptor ligand, exhibiting high affinity binding to SSTR 1, 2, 3 and 5 which means that this particular analog is more effective in cells that present SSTR variety. Octreotide presents only high affinity binding to SSTR 2 and 5, reducing the choices in cells expressing different SSTRs. According to Schonbrunn (2008), differences in the actions of SST analogs are a consequence of the variability in the nature and concentration of SSTRs subtypes and effectors expressed in different targets and Adams et al (2004) suggest that there is a variation in the temporal expression of SSTRs in HUVEC cultures from different umbilical cords. This variation was also reported in a wide range of normal and neoplastic tissues, being of particular importance in the clinical application of somatostatin analogs in different individuals [Hofland and Lamberts, 2001; Reubi et al, 2001].

The results obtained with this current work add a new contribution to the study of the inhibitory action of somatostatin analogs in vessel-forming endothelial cells, as HUVECs: SSTR 2 may represent a very important role in the inhibition of primary endothelial cell proliferation, indicating that the residual expression of SSTR 5 is not impeditive for success in the inhibition of cell proliferation if the cells express other SSTRs, especially SSTR 2 and the somatostatin analogs used present high affinity for this receptor.
Future perspectives

The results obtained in this thesis, paved the way for future studies in the field of utilization of somatostatin analogs in the control of angiogenesis. Thus, a number of experiments which would complement the work herein presented, could be undertaken:

- Evaluation of the influence of treatment with somatostatin analogs in SSTR expression either at the RNA level, by real time PCR (more accurate method than semi-quantitative), or at the protein level by Western-blotting, in a significant number of primary HUVEC from umbilical cords of different donors.

- Evaluation of cell death and apoptosis under the influence of somatostatin analogs within a wider time range using other methods, for example by evaluating the level of caspase-3 expression.

- Assessment of the role of SSTRs in the control other types of receptors (for example, EGFR, VEGFR, etc) in different tumor tissues (breast cancer, prostate cancer, etc), in order to explore the potentialities of the use of somatostatin analogues as rational approach for treatment in these types of tumors.
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