SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Somatostatin receptors as molecular targets in human uveal melanoma and pediatric hematological-oncological diseases

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Contents

1. Introduction

1.1. Somatostatin

Somatostatin was initially discovered as a hypothalamic neurohormone. Somatostatin receptors (SSTRs) have been shown to be expressed in several different types of cancer, including uveal melanoma and pediatric hematological-oncological disorders represent. There are two different forms of bioactive peptides which are produced in mammals. Somatostatin-14 is a cyclic peptide including 14 amino acids, and somatostatin-28 consists of 28 amino acids. Somatostatin (SST) acts mainly as an inhibitory factor in cell proliferation and hormone secretion with endocrine, paracrine and autocrine activities. Somatostatin is a widely distributed inhibitory peptide hormone that is involved in digestive, endocrine, and immune functions. The biological effects of SST are mediated by specific G protein-coupled plasma membrane receptors, which are placed in specific target cells of the gastrointestinal tract, the peripheral nervous system and various blood vessels. At least five subtypes of SSTRs have been characterized in humans (1, 2A, 2B, 3, 4, and 5), and SSTR-2 and SSTR-5 are the ones mostly studied in human cancers including primary UM. The genes for these subtypes are located on different chromosomes: SSTR-1 gene location: 14q13; SSTR-2: 17q24, SSTR-3: 22q13.1, SSTR-4: 20p11.2, and SSTR-5: 16p13.3. SSTR-2 otherwise splices to generate two isoforms named SSTR-2A and SSTR-2B, which deviate in their C-terminal sequences. They all bind to SST-14 and -28, but they have a slightly higher affinity to SST-14. Even if each tumor expresses more than one subtype of SSTRs, SSTR-2 is the most regularly observed. Somatostatin could be involved in tumor growth suppression, as confirmed by the use of SST analogs to treat neuroendocrine tumors. The antiproliferative action of SST and high expression of their corresponding SSTRs on various endocrine tumors led to the clinical application of synthetic stable analogs of SST for hormonal treatment of human malignancies such as acromegaly. Earlier in vitro and in vivo experimental studies have already demonstrated the inhibitory effect of various SST analogs and cytotoxic SST analog AN-162 in breast cancer, lung and prostate cancer, glioblastoma, and colon carcinoma, suggesting the potential application of modern powerful cytotoxic SST analogs in patients suffering from malignant tumors.

Based on structural similarity and reactivity for octapeptide and hexapeptide SST analogs, the receptor family can be divided into two subclasses: SSTR-2,3,5 react with SST octapeptide analogs and constitute members of one subgroup; SSTR-1,4 react poorly with these

compounds and fall into another subgroup. Octapeptide analogs, such as octreotide (Sandostatin®), somatuline (Lanreotide®), and octastatin (Vapreotide®) are very popular drugs and they show high affinity to SSTR-2 and SSTR-5, moderate affinity to SSTR-3 and SSTR-4, and low binding affinity to SSTR-1. It is reported that octreotide has the highest affinity to SSTR-2, then, approximately similar affinity to SSTR-5 and SSTR-3, following lower binding affinity to SSTR-1, and displaying the lowest to SSTR-4. However, Lanreotide shows the highest affinity to SSTR-2 similarly to SSTR-5 and somehow lower binding affinity to SSTR-3 and SSTR-4, and displays the lowest affinity to SSTR-1. Another novel SST analog pasireotide (SOM 230) possesses an affinity to SSTR-1, SSTR-3, and SSTR-5. In addition, synthetic SST analogs found other useful clinical applications as carrier molecules for radionuclides for tumor visualization and targeted radio- and chemotherapy of SSTR-positive tumors.

As a result of the similar origin of SST and UM cells, a possible correlation or interaction can be found. A connection between eye tissue and neurohormones has already been discovered in several studies. However, there is still limited information about SSTR expression and characterization in human UM. Therefore, we aimed to widen our research regarding the expression of SSTR types in human UM, in order to recognize specific membrane receptors as molecular targets for diagnostic and therapeutic purposes.

1.2. Uveal melanoma

Uveal melanoma is the most common primary intraocular malignancy in adults, with a yearly incidence of 4–5 cases per million. The intraocular uveal tract is comprised of the iris, choroid and ciliary body. Several histologic prognostic factors have been described for this type of cancer, such as large tumor diameter (LTD), location at onset, age at time of diagnosis, presence of epitheloid cells and involvement of the ciliary body. The reason for malignant UM formation is unknown, but various predisposition factors have been associated with the development of this quite aggressive disease. A few personal features, such as fair complexion, light irides, uveal naevi, dysplastic naevus syndrome, oculodermal and ocular melanocytosis and neurofibromatosis type 1 (NF1), have been connected with an increased chance of UM. UM most commonly affects Caucasian males. The mortality due to UM has remained relatively unchanged, in spite of earlier detection and consequently smaller primary tumor burdens. Approximately half of all patients with UM will eventually develop metastatic disease. It is

generally supposed that the primary reason for treating UM is to avoid metastatic spreading of the tumor from the eye. The liver is the most common site of metastases and once liver metastases are clinically apparent the prognosis becomes poor. One of the most significant predictors for UM-related death is loss of chromosome 3. The incidence of UM in whites is eight times higher than in blacks. The leading predictors of survival for UM are histologic cell type, largest tumor diameter, age, gender and tumor location. More unbiased classification parameters have appeared from comprehensive cytomorphometrical studies. Life expectancy of UM patients with metastatic disease depends on the rapidity of the metastatic process. In order to reduce the mortality caused by melanoma, it is essential to prevent or eliminate metastatic disease. This requires early detection and the improvement of prognostic factors. It is crucial to increase our knowledge of the mechanism of metastasis and describe reliable progression parameters as prognostic markers in primary UM. These facts inspire a constant search for new concepts to improve quality of life and extend survival of patients. For patients with UM there is no effective therapy if metastases have developed. Despite several therapeutic strategies and successful eradication of the ocular tumor, metastatic disease is almost always fatal.

1.3. Pediatric hematological and oncological disorders

The high expression of SST-14 and somatostatin receptors in childhood neuroblastoma and medulloblastoma samples, as well as the clinical applicability of somatostatin receptor scintigraphy and radioreceptor-guided surgery have already been described. Pediatric hematological and oncological diseases represent the second most frequent cause of childhood mortality after accidents, consequently, there is an urgent need for improved methods for early detection and development of novel treatment options of these disorders. Although the presence of SST has previously been demonstrated in certain pediatric tumors, only very limited information exists about the expression and characteristics of SSTRs in pediatric hematological and oncological disorders including malignant and benign conditions.

Fibrous dysplasia (FD) of bone is a genetic, non-inherited, rare benign bone disease existing in monostotic and polyostotic forms. FD is a developmental disease of bone in which there is replacement of normal spongiosa and filling of the medullary cavity of affected bones by abnormal fibrous tissue that contains trabeculae of poorly calcified primitive bone formed by osseous metaplasia. Infantile myofibromatosis (IM) is a mesenchymal disorder described by

fibrous proliferation of the skin, bone, muscle, and viscera. Although rare, it is the most common fibrous tumor in childhood. Aggressive pediatric myofibromatosis is an autosomal recessive disease characterized by fibroblastic proliferation from cells derived from muscle-aponeurotic tissue. Its etiology is unknown, and the mean age of the reported cases is 7 years. The tumor shows rapid growth without serious pain and appears to be attached to the muscle tissue and/or bone. The treatment option is typically conservative surgical excision; however, early relapses have been reported. Teratomas are special tumors with various cellular components that contain mature (i.e., benign) or immature (malignant) proliferating pluripotent cells of more than one germ-cell layers in origin. The incidence of teratomas is 1:4000 births. The etiology of teratomas is not fully understood; however, they are likely to occur in part when individual pluripotent cells do not complete migration and continue dividing in an abnormal location, typically along the midline. Regarding their nature, teratomas can be both benign and neoplastic. Mesenchymal hamartoma is a tumor-like benign mass of disorganized tissues reflecting a local developmental malformation with a poorly understood pathogenesis. It is uncommon in older children, especially after 2 years of age. The diagnosis of this tumor is difficult because the signs and clinical symptoms may be nonspecific; therefore, a high index of suspicion is required for diagnosis and treatment. Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma among children, with an annual incidence of 4.5 cases per 1 million children and is the third most frequent extracranial solid tumor of childhood after neuroblastoma and Wilms tumor. RMS tumors are typically associated with the skeletal muscle lineage, and about 50% of cases are diagnosed in the first decade of life. Acute lymphoblastic leukemia (ALL) is the most frequent hematological neoplastic disease in children, characterized by the proliferation of transformed lymphoid cells in the bone marrow, peripheral blood, and other organs. The age-adjusted incidence rate of ALL in the United States is 1.38/100,000 individuals per year with an estimated 5930 new cases and 1500 deaths in 2019. ALL accounts for 75–80% of acute leukemias in children. Hodgkin lymphoma (HL) is a highly curable form of childhood cancer, with estimated 5-year survival rates exceeding 98% after treatment with chemotherapy alone or combined with radiotherapy (RT). Hereditary spherocytosis (HS) is the most frequent congenital red blood cell membrane defect in which abnormalities of red blood cell structural proteins lead to loss of erythrocyte membrane surface area, resulting in spherically shaped, hyperdense, poorly deformable red blood cells accompanied by hemolysis. Incidence of HS is 1/5000 among individuals of European descent and in the United States. Immune thrombocytopenia (ITP) is one of the most common bleeding disorders during childhood, occurring in ~5 to 10 per 100,000 children per year. ITP in childhood is often a self-

limited condition. Granulocytopenia is a heterogenous group of disorders accompanied by a decrease in peripheral blood granulocytes below the lower limit of normal range (<0.5 x 10^9/l). Chronic benign neutropenia is the most common neutropenia in childhood. Neutropenia can be considered chronic if it does not improve after 8 weeks. In most children, neutropenia may persist for a few years and then resolve spontaneously. Children with chronic benign neutropenia are generally healthy and have a normal physical exam.

2. Aims of the study

In the present study, our goal in the experimental work was to examine the expression of mRNA for somatostatin receptor types-1, -2, -3, -4 and -5 (SSTR-1–5) in human UM cell lines and tissue samples. We also aimed to study the presence and binding characteristics of SSTR protein by Western blot and ligand competition assays. An additional goal was to compare our results with clinicopathological data in order to better understand their diagnostic and therapeutic significance.

In addition, in the current study, we aimed to investigate the expression of mRNA for somatostatin receptor subtypes in a cohort of pediatric hematological and oncological specimens using RT-PCR and the binding characteristics of SSTR protein by ligand competition assays. In addition, we analyzed the correlation among the expression pattern of SSTR subtypes and clinicopathological characteristics of the pediatric patients. To the best of our knowledge, this is the first report showing the expression of SSTRs in various childhood tumors and hematological disorders. Our results may open up a new avenue for potential diagnostic and therapeutic applications of somatostatin analogs in pediatric oncology.

The research aims of the PhD thesis were to set the following specific goals:

- Investigate the expression of mRNA for SSTR subtypes in human primary UM tissue samples by qRT-PCR
- Evaluate the presence and binding characteristics of SSTR protein in human UM specimens by ligand competition assays
- Investigate the expression of mRNA for SSTR subtypes in two human UM cell lines by qRT-PCR
- Western blot analyses of SSTR protein in human UM cell lines
- Correlation analysis among the expression of mRNA for SSTR-1, -2, -3, -4, -5 receptors in OCM-1 and OCM-3 cell lines
- Investigate the expression of mRNA for SSTR subtypes in human pediatric solid tumors and hematological diseases by RT-PCR
- Study the presence and binding characteristics of SSTR protein in pediatric hematological and oncological specimens by ligand competition assay
- Evaluate the receptor binding affinity of SST analogs to membrane receptors of human pediatric cancer cells
- Correlation analyses among SSTR expression pattern and clinical outcome or clinicopathological characteristics of pediatric hematological and oncological cases
- Patient follow-up

3. Materials and methods

3.1.1. UM cell lines and culturing conditions

OCM-1 and OCM-3 human primary UM cell lines were kindly provided by the Department of Biophysics and Cell Biology, University of Debrecen, Hungary. In all our in vitro experiments as a positive control CACO-2 (human Caucasian colon adenocarcinoma) cell line was used, which was kindly provided by the Department of Pharmaceutical Technology, University of Debrecen, Hungary. OCM-1 and OCM-3 cell lines were cultured in RPMI 1640 medium supplemented with L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin in a humidified chamber in 5% CO_2 at 37 °C. CACO-2 cell line was grown in Dulbecco's Modified Eagle's Medium, supplemented with 3.7 g/L $NaHCO_3$, 10% (V/V) heat-inactivated fetal bovine serum (FBS), 1% (V/V) non-essential amino acids solution, 1% (V/V) L-glutamine, and 100 IU/mL penicillin/streptomycin in a saturated humidified chamber in an atmosphere of 5% CO_2 at 37 °C. Cells were subcultured every 3 days using a standard trypsinization procedure.

3.1.2. Preparation of uveal melanoma tissue samples from patients

Specimens of primary human UM were obtained from 46 patients at the time of initial surgical treatment at the Department of Ophthalmology, University of Debrecen, Debrecen, Hungary. The local Institutional Ethics Committee approved the collection and use of these specimens for the current study and informed consent was obtained from these patients. Tumor tissues were immediately frozen in liquid nitrogen and stored at -80 °C until further processing. Histopathological examination of each specimen was undertaken to confirm the presence of cancer with minimal mixed non-malignant tissue. All cancer samples were primary tumors and without metastases.

3.1.3. RNA isolation and reverse transcription PCR

Homogenization of the UM tissue samples were performed with Tissue Ruptor (IKA®-WERKE GmbH, Staufen im Breisgau, Germany). Total RNA from tumor tissues and OCM-1 and OCM-3 cells was isolated with NucleoSpin DNA/RNA/Protein Kit (Macherey-Nagel,

Düren, Germany) according to manufacturer's protocol. Quantitative and qualitative assays for RNA were performed using a NanoDrop spectrophotometer (ND-1000, Bioscience, Budapest, Hungary).

Two hundred fifty nanograms of RNA from each sample were reverse-transcribed into cDNA using Tetro cDNA Synthesis Kit (Bioline Reagents, London, UK). Reaction was performed according to the manufacturer's instructions. RT-PCR reaction was performed in 25 μL reaction volume with gene specific primers. The reaction consisted of 35 cycles (95 °C for 15 s, 60 °C for 30 s, 72 °C for 10 s) and lasted for 2 min extension at 72 °C. PCR was performed with gene-specific primers for SSTR-1, SSTR-2, SSTR-3, SSTR-4 and SSTR-5 using PCR MyTaq Master Mix (Bioline Reagents). PCR reaction was performed in a C1000 TM Thermal Cycler RT-PCR system (Bio-Rad, Hercules, CA, USA). For SSTR-1, -2, -3, -4, -5 and β-actin an initial denaturing step at 94 °C for 30 s was followed by 30 PCR cycles consisting of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 15 s. β-actin was used as a positive internal control. PCR product was separated in a 1.5% agarose gel containing GelRed and detected in UV light, digitalized with AlphaDigiDoc™ RT (Alpha Innotech, Santa Clara, CA, USA). To determine the size of DNA, 50 bp DNA marker (Bioline Reagents) was used.

3.1.4. Quantitative real-rime PCR

Total RNA (1000 ng) was reverse transcribed into cDNA using Tetro cDNA Synthesis Kit (Bioline Reagents) according to the manufacturer's instruction. To quantity mRNA for SSTR-1, -2, -3, -4 and SSTR-5 real-time RT-PCR method was performed with iTaq™ Universal SYBR® Green Supermix (Bio-Rad) in CFX96 Touch TM Real-Time PCR detection System (Bio-Rad) with a 20 μL final reaction mixture. The reaction was carried out at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s. β-actin was used as an endogenous reference gene. All real-time amplifications were measured in triplicates. Relative mRNA expression for SSTRs was measured by ΔΔCt method with threshold cycle times of each target SSTR and β-actin. Template-free and reverse transcription-free controls excluded non-specific amplification and DNA contamination.

3.1.5. Western blot analysis

For western blot analysis adherent cells were harvested in M-PER lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease inhibitor (Sigma-Aldrich, St. Louis, IL, USA). Total protein concentration was measured by the Pierce BCA protein assay kit (Thermo Fisher Scientific). SDS-PAGE gel electrophoresis was performed under reducing conditions using 10% polyacrylamide gels. Equal amounts of proteins (40 µg) were separated and then transferred to PVDF membrane using wet transfer. After blocking with 5%TBST-milk, the membranes were incubated (overnight, 4 °C) with primary antibodies SSTR-5: anti-somatostatin-receptor-2-rabbit, 1:1000 dilution (ab134152 rabbit monoclonal; Abcam, Cambridge, UK) and anti-somatostatin-receptor-5-rabbit, 1:1000 dilution (ab109495 rabbit monoclonal; Abcam). As a housekeeping gene anti-HPRT was used, 1:1000 dilution (PA-22281 rabbit polyclonal; Cell Signaling Technology, Danvers, MA, USA). After the washing steps, the membrane was incubated with alkaline phosphatase conjugated polyclonal rabbit-anti-mouse secondary antibody, 1:3000 dilution (sc-2771; Santa Cruz Biotechnology Inc., Dallas, TX, USA). Proteins were detected with Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad).

3.1.7. Radioligand binding studies

Radioiodinated derivatives of SST analog RC-160 were prepared using the chloramine-T method and purified using reverse phase high performance liquid chromatography (RP-HPLC) as described earlier. Somatostatin receptor binding studies were performed as previously reported with some minor modifications using in vitro ligand competition assays based on binding of [^{125}I]RC-160 as radioligand to uveal melanoma membrane fractions. This radioiodinated ligand was well characterized as reported earlier and demonstrated high affinity binding to SSTR-2 and SSTR-5. Tumor membrane homogenates were incubated with 50,000–70,000 cpm of radioiodinated RC-160 and 10^{-12}–10^{-6} M of nonradioactive peptides as competitors. After 2 h of incubation the binding reactions were terminated, and the bound ligand was separated then counted in a gamma-counter. The LIGAND-PC computerized curve-fitting software of Munson and Rodbard was used to identify the type of receptor binding, dissociation constant (K_d), and the maximal binding capacity of the receptors (B_{max}).

3.1.8. Statistical analysis

Correlation analysis was carried out among the expression of mRNA for SSTR-1, -2, -3, -4, -5 receptors in OCM-1 and OCM-3 cell lines with the use of GraphPad Prism 7—one way ANOVA test to assess the significance of the expression of SSTR-1–SSTR-5 genes of OCM-1 and OCM-3 cell lines (GraphPad Software Inc., La Jolla, CA, USA).

3.2.1. Pediatric hematology and oncology specimens

The other part of my research work we examined 15 pediatric hematological and oncological specimens. Average age of patients was 8.03 years (range: 9 months–15 years). Human samples were collected from patients treated at the Department of Pediatric Hematology-Oncology, University of Debrecen, Hungary. Seven patients had solid tumors, eight children had malignant or benign type of hematological disorders. Seven samples were bone marrow aspirates, one hematological specimen was obtained from peripheral blood. Solid tumor tissues were obtained at the time of primary surgery. All the samples were processed for routine histopathological examination and the pathological diagnosis was confirmed by a local pathologist. For molecular biology analysis, human bladder tumor tissue was used as an SSTR-positive control. Local Institutional Ethics Committee approved the collection and use of these specimens for the current study and informed consent was obtained. Tumor tissues were immediately frozen in liquid nitrogen and stored at -80 °C until further processing. All diagnostic interventions were performed based on suspected neoplastic conditions.

3.2.2. Histology

Histopathological examination of the 15 human pediatric hematological and oncological samples involved in our study revealed that five patients were suffering from ALL. Two samples were classified as RMS and one sample each was characterized histologically as teratoma, IM, FD, HL, hamartoma, ITP, HS, and chronic benign neutropenia.

3.2.3. RNA isolation

Homogenization of the samples was performed with Tissue Ruptor (IKA®-WERKE GmbH). Total RNA was isolated with an RNA Isolation Kit (Macherey-Nagel) according to the manufacturer's protocol. Isolated total RNA was redissolved in RNAs-free water and quantity and quality were measured by a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific Inc.). Whenever enough material was available, experiments were performed in triplicate.

3.2.4. RT-PCR analysis of total RNA samples

Total RNA was analyzed using a MMLV kit (Promega Co., Madison, WI, USA). The reverse transcriptase reaction mix (25 mM $MgCl_2$; 10 x PCR buffer [500 mM KCl, 100 mM Tris-HCl, pH 8.3], distilled, autoclaved water, 1 mM dinucleotides, 2.5 U/µL MuLV reverse transcriptase, 1 U/µL RNase inhibitor, and 2.5 µM oligo dT) was added together with 200 ng RNA template. The total reaction volume of 20 µL was incubated at 23 °C for 10 min and 42 °C for 15 min, followed by 99 °C for 5 min.

RT-PCR reaction was performed in 25 µL reaction volume with gene-specific primers. The primers for PCR amplification were designed using the published sequences for the respective genes. Primer sequences with as small as possible homology among SST receptor subtypes were selected as described before.

For PCR reaction mixture 1 x PCR buffer, 1 U of Taq Polimerase (Invitrogen), 1.5 mM of MgCl2, 200 µM of dNTP (Fermentas), 0.5 µM of each of the gene specific primers (SSTR-1, SSTR-2, SSTR-3, SSTR-5), (Invitrogen), and 3 µL cDNS template was used in 25 µL reaction volume. For SSTR-4 RT-PCR reaction 300 µM dNTP (Fermentas) was added and 3 µL cDNS template was used in 25 µL volume as well.

PCR reaction was performed in a C1000 TM Thermal Cycler RT-PCR system (Bio-Rad Laboratories, Inc.). To run the RT-PCR, the following PCR protocol was used:

- for SSTR-1, SSTR-2, SSTR-4, SSTR-5: denaturation (3 min at 94 °C), 40 cycles (94 °C for 45 s, 60 °C for 30 s and 70 °C for 90 s);
- for SSTR-3: denaturation (3 min at 94 °C) was followed by 40 cycles (94 °C for 45 s, 63 °C for 30 s and 70 °C for 90 s).

The PCR reaction was finished by a prolonged extension time of 72 °C for 10 min.

PCR products were separated in a 1.5% agarose gel containing GelRed and detected under UV light, digitalized with AlphaDigiDoc™ RT (Alpha Innotech). To determine the size of the DNA, a 50 bp DNA marker (Bioline) was used. To test the quality of the RT-PCR, β-actin was used as a positive internal control for each of the transcribed RNA samples.

3.2.5 Radioligand binding studies

The radioiodinated derivatives of the RC-160 SST analogue were prepared by the chloramine-T method and purified by reverse phase high-performance liquid chromatography (RP-HPLC). Somatostatin receptor-binding assays were performed as described previously with some minor modifications, using in vitro ligand competition assays based on the binding of [^{125}I] RC-160 as a radioligand to membrane fractions of the pediatric samples.

This radioiodinated ligand has been well characterized and reported previously and has high affinity for SSTR-2 and SSTR-5. Tumor membrane homogenates were incubated as competitors with 50,000–70,000 cpm radioiodinated RC-160 and 10^{-12}–10^{-6} M nonradioactive peptides. After incubation for 2 h, the binding reactions were stopped, and the bound ligand was separated and counted in a gamma counter. The LIGAND-PC computer curve-fitting software of Munson and Rodbard was used to determine the type of receptor binding, the dissociation constant (K_d), and the maximal binding capacity of SSTRs (B_{max}). The binding potencies of SST analogs and cytotoxic SST analog AN-162 to SSTRs were also determined by displacement of [^{125}I]-RC-160 binding. The final binding affinities were expressed as IC_{50} values. Protein concentration was determined by the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc.).

4. Results

4.1. Expression of SSTRs in human uveal melanoma tissue samples

In our study 46 human UM tissue samples were investigated. Twenty nine samples were obtained from men and 17 samples from women. More than half of the patients (24, 52%) were over 66 years of age, while the remaining samples were equally distributed among the different age groups.

Histopathological classification of these samples revealed that 10 of them belonged to spindle type. Seven samples were determined to be epithelioid type and 2 samples were mixed of spindle and epithelioid types. Two samples were not classified histologically. Due to the limited amount of good quality RNA isolated from UM tissue samples. 46 specimens were studied for the expression of mRNA for SSTR-2 and nine specimens for SSTR-5.

The expression of mRNA for SSTR-2 was detected in 30 of the 46 human UM specimens (65.2%). Nine human UM tissue samples were tested for SSTR-5 expression and we found that 66.6% (6 specimens) of these samples were positive for SSTR-5. Among the positive cases for SSTR-2, 60.0% were men and 40.0% women. The distribution of SSTR-2-positive samples by age group, showed the following findings: age 30–45 years: 62.5%; age 46–55: 75.0%; age 56–65: 66.7%; age above 66 years: 66.7%. There was no correlation between the expression of different SSTR-types and age groups. 40% of the samples positive for SSTR-2 were spindle, 36.7% were epithelioid and 10% were mixed histological type. Four SSTR-5-positive samples were spindle (66.7%) and two specimens were epithelioid (33.3%).

The presence of SSTRs, the characteristics of these SST binding sites, and the specific binding of radioiodine to RC-160 membrane receptors on human UM samples were determined by ligand competitive assays. Of the 20 samples tested, 14 (70%) showed SSTR binding. Receptor binding affinity and the concentration of SSTRs in the membranes of tumor samples were measured by displacement experiments. Analysis of typical displacement curves for [^{125}I] RC-160 using the same unlabeled peptide (RC-160) showed that the one-site model could provide the best fit. Based on these results, the presence of one class of high affinity SSTR in crude membranes derived from human UM samples was indicated. The computerized non-linear curve-fitting program and the Scatchard plot analyses of the SST receptor binding data in 14 receptor positive tumor samples showed that the single class of binding sites had a mean dissociation constant (K_d) of 7.14 nM (range 3.18–11.8 nM), with a mean maximal binding

capacity (B_{max}) of 604.0 fmol/mg membrane protein (range 260–1052 fmol/mg protein). Biochemical specifications and parameters crucial for characterizing specific binding sites were also defined. Thus, the binding of [^{125}I]RC-160 was detected to be reversible, temperature and time-dependent, and linear with protein concentration in the human UM specimens examined. Competitive binding studies also demonstrated the specificity of SST binding sites using numerous peptides structurally related or unrelated to SST. The binding of radiolabeled RC-160 was displaced completely by increasing concentrations (10^{-12}–10^{-6} M) of SST-14, whereas none of the structurally and functionally different and unrelated peptides analyzed, such as luteinizing hormone-releasing hormone (LHRH), growth hormone-releasing hormone (GHRH), epidermal growth factor (EGF), [Tyr^4]bombesin, and insulin-like growth factor I inhibited the binding of radioiodinated RC-160 at concentrations as high as 1 μM.

4.2. Expression of SSTRs in human uveal melanoma cell lines

Our RT-PCR and qRT-PCR results clearly show that SSTR-1-5 were expressed in both human UM cell lines examined. CACO-2 cell line as a positive control also showed the expression of all five SSTRs examined. The highest expression of all the investigated SSTRs was observed in OCM-3 cell line. However, in both cell lines, the expression of SSTR-2 and -5 was stronger than those of SSTR-1, -3 and -4. All five SSTRs examined displayed significantly ($p < 0.0001$) higher expression in OCM-3 UM cell line than in OCM-1. qRT-PCR results show that SSTR-1, -2, -3, -4, -5 are expressed in both human UM cell lines examined. CACO-2 cell line was used as a positive control. The p value obtained by GraphPad Prism—one-way ANOVA test was considered to be significant (* $p < 0.0001$).

Western blot analysis also confirmed the presence of SSTR-2 and SSTR-5 receptor protein in OCM-1 and OCM-3 human UM cell lines. Similarly to our findings on receptor mRNA, higher expression levels of SSTR-2 and SSTR-5 receptor protein were found in OCM-3 tumor cell line, than in OCM-1 cell line detected by western blot.

In cell membranes of OCM-1 and OCM-3 human UM cell lines, ligand competition studies also revealed a single class of high affinity binding sites for SST with a mean dissociation constant (K_d) of 5.34 and 6.72 nM, respectively. The concentration of SSTRs was 433 fmol/mg membrane protein in OCM-1 cells, while OCM-3 cells showed a markedly higher receptor level (981 fmol/mg membrane protein).

Western blot analysis confirmed the presence of SSTR-2 and SSTR-5 receptor proteins in OCM-1 and OCM-3 human UM cell lines. CACO-2 human Caucasian colon adenocarcinoma cell line was used as a positive control. Anti-HPRT (1:1000 dilution) was used as a housekeeping gene.

4.3. Expression of SSTRs in human pediatric solid tumors and hematological diseases

We performed subtype-specific RT-PCR analyses on 15 pediatric hematological and oncological samples to investigate the mRNA expression of SSTR subtypes. The expected size of PCR products amplified with gene-specific primers was 216 bp for SSTR-1, 168 bp for SSTR-2, 188 bp for SSTR-3, 222 bp for SSTR-4, and 191 bp for SSTR-5, respectively. We found that the pediatric samples investigated highly expressed mRNA for the five subtypes of somatostatin receptors. The mRNA expression of SSTR-2 subtype was detected in all (15/15) of the samples independently from their histological type. Of the 15 specimens, only a HL sample expressed mRNA for all five SSTR subtypes and IM, FD, and ITP showed only one receptor subtype, SSTR-2. The expression of SSTR-5 was detected in two malignant solid tumor specimens (case 4, HL, and case 5, RMS). Of the 15 pediatric hematological and oncological specimens, PCR products for SSTR-3 could be detected only in malignant samples, two solid tumors (HL and RMS) and two cases (ALL and HS) expressed SSTR-3. Subtypes SSTR-3 and SSTR-5 were expressed in lower number of the specimens, found only in four and two samples, respectively. The incidence of SSTR-1 and SSTR-4 was similar (9/15, 60%) in the 15 specimens analyzed. Among all nine SSTR-4-positive samples, two were rhabdomyosarcomas, two specimens were ALL, one was HL, one was teratoma type, one histologically was defined as hamartoma, one as HS, and one was chronic benign neutropenia. Both RMS samples in our study mostly showed positivity for SSTR-1, SSTR-2, and SSTR-4. Both solid benign tumor samples showed SSTR-2 receptor expression, but none of them expressed SSTR-3 and SSTR-5.

Template-free and reverse transcriptase-free controls excluded nonspecific amplification and DNA contamination. PCR amplification with specific primers for β-actin produced a single product in every sample, confirming no RNA degradation in the samples. As a positive control, human pituitary samples were used.

4.4. Radioligand Binding Studies

The presence of SSTR protein, characteristics of these SST-binding sites and specific binding of radioiodinated SST analog RC-160 to membrane homogenates of human pediatric solid tumor samples were determined using ligand competition assays. Of the seven specimens examined, five malignant tumors and two benign tumor samples displayed SSTR binding. Receptor binding affinities and concentrations of SSTRs in tumor membranes were also studied. Analyses of the typical displacement of [^{125}I]RC-160 and the Scatchard plots of the specific binding data showed that the one-site model could provide the best fit. Based on these receptor binding results, the presence of one class of high affinity SSTR in crude membranes derived from human pediatric samples was indicated. The computerized nonlinear curve-fitting program and the Scatchard plot analyses of the SST receptor binding data in seven receptor-positive tumor samples showed that the single class of SSTRs had a mean dissociation constant (K_d) of 6.00 nM (range 4.02–8.12 nM), with a mean concentration of SSTRs (maximal binding capacity, B_{max}) of 391.1 fmol/mg membrane protein (range 255.1–760.8 fmol/mg protein). Biochemical specifications and parameters crucial to characterize specific binding sites were also defined. Thus, the binding of [$_{125}$I]RC-160 was detected to be specific reversible, temperature- and time-dependent, and linear with protein concentration in the human pediatric tumor specimens examined. The specificity of SST binding sites was also demonstrated in competitive binding assays. Various peptides structurally related or unrelated to SST were used in these studies. The binding of radioiodinated RC-160 was displaced completely by increasing concentrations (10^{-12}–10^{-6} M) of SST-14, whereas none of the structurally and functionally different and unrelated peptides examined, such as epidermal growth factor (EGF), luteinizing hormone-releasing hormone (LHRH), growth hormone-releasing hormone (GHRH), [Tyr^4]bombesin, and insulin-like growth factor I inhibited the binding of radiolabeled SST octapeptide analog RC-160 at concentrations as high as 1 μM.

The expression of mRNA for SSTR subtypes was accompanied by ligand binding in all pediatric tumor specimens examined. Comparative analysis of the results of radioreceptor assays and SSTR subtype mRNA studies revealed that the expression of SSTR-2 and/or SSTR-5 subtypes was 100% consistent with the presence of specific binding sites for [^{125}I] RC-160. The binding affinity of SST analogs and cytotoxic SST analog AN-162 to membrane receptors of human pediatric cancer cells (HL and RMS) expressing SSTRs was also investigated by ligand competition assay. Displacement of [^{125}I] RC-160 as a radioligand by the unlabeled SST

analogs as competitor was determined. Our results show that SST octapeptide analogs RC-160 (vapreotide) RC-121 (as carrier peptide of AN-162) and cytotoxic SST analog AN-162 could effectively bind to SSTRs at low nanomolar concentration. Nevertheless, the cytotoxic SST conjugate AN-162 had only slightly lower binding affinity to specific SSTRs than the free peptide carrier RC-121. Our results demonstrated that the high binding affinity of the carrier peptide RC-121 to tumoral SSTRs was fully preserved in the targeted cytotoxic analog of SST, AN-162.

4.5. Patient follow-up

In the group of samples investigated, 5 children died within 5 years (2 patients with RMS, one patient with teratoma, and two with ALL). The male to female ratio in the group investigated was 2:1 (10 boys, 5 girls). In the solid tumor group, four patients were still alive after 5 years, three children died, and the male to female ratio was 1.33:1 (4 male and 3 female patients). In the hematological diseases group, six patients were still alive after five years, two children with ALL died within 5 years and the male to female ratio was 3:1 (6 male and 2 female patients). In our study, among the five deceased patients, only two children showed relapse (both were ALL patients) that caused the death of these patients. We did not detect any significant correlation among somatostatin receptor expression pattern and clinical outcome or clinicopathological characteristics.

5. Discussion

Uveal melanoma is the most frequently diagnosed primary malignant intraocular tumor in adults, and the second most common form of melanoma after cutaneous melanoma. Over the past 50 years, despite advances in diagnosis and effective local therapies, stage-specific UM mortality rates have remained essentially unchanged and continue to be associated with significant mortality. The liver is the most common and important target of metastasis, and liver failure due to metastasis is the immediate cause of death in most patients. Patients with UM face a gloomy prognosis, as about 45% die due to metastasis, irrespective of the fact that the tumor is most frequently diagnosed and locally cured before any signs of clinical distributed disease appear. This led to the theory that micrometastases are already present early in the disease process, but they remain inactive for many years before a clinically noticeable macrometastasis develops.

The systemic treatment of UM is very limited. Adjuvant systemic therapy is primarily used in patients at high risk of metastasis or developed metastatic patients, but the response rates for classic chemotherapeutic agents remain between 7% and 25%. Systemic chemotherapy for the treatment of UM is ineffective. In spite of new insights into the genetic and molecular background of metastatic UM, satisfactory systemic treatment approaches are currently lacking. There are currently no effective systemic therapies available that could be used for the primary tumor. Despite the improvement in the diagnosis and therapy of primary UM, the number of metastatic deaths has not been significantly reduced over the past 20 to 30 years. Therefore, innovative therapeutic methods are urgently awaited.

Because of the short plasma half-life of SST-14 (3 min), more stable and more potent synthetic SST analogs have been developed, including octreotide (Sandostatin®), vapreotide (RC-160, Octastatin®), and lanreotide (Somatulin®). The plasma half-life of SST analogs is 120 min, and these peptide analogs are about 50 times more potent than SST in inhibiting growth hormone release from the pituitary. It has been well published that SST and its octapeptide analogs exert their effects through specific G-protein coupled membrane receptors. While native SST shows similar high affinity to SSTR-1-5, the synthetic octapeptides such as octreotide, RC-160, and RC-121 bind especially to SSTR-2 and SSTR-5, present a moderate affinity to SSTR-3, and a low affinity to SSTR-1 and SSTR4. Somatostatin analogs, including RC-160, have been shown to function as effective tumor growth suppressors in experimental models of various cancers. To increase therapeutic efficacy, new analogs have been prepared

which contain the cyclohexapeptide, designated SOM230 (pasireotide), which binds with high affinity to SSTR-1, -2, -3 and SSTR-5. The presence of somatostatin receptors, mainly SSTR-2 on tumors, allows the localization of certain primary tumors and their metastases using scintigraphic techniques. Radiolabeled analogs of SST, such as [^{111}In-DTPA-D-Phe1]-octreotide (OctreoScan®) are used clinically for the localization of tumors expressing receptors for somatostatin. Targeted radiotherapy, in which somatostatin analogs are linked to numerous radionuclides such as ^{68}Gallium or ^{90}Yttrium, is also advancing clinically.

In this study, the expression of mRNA for SSTR-2 was studied in 46 UM specimens, and SSTR-5 in 9 UM tumor samples. Primary tumors were also tested at the protein level. In addition to detecting SSTRs, OCM-1 and OCM-3 UM cell lines were also analyzed for receptor mRNA with quantitative real-time RT-PCR. Thirty-one (65.2%) samples showed SSTR-2 positivity and 6 of nine samples (66.6%) were SSTR-5 positive. Conversely, Ardjomand et al. identified SSTR-2 expression in almost all of the 25 tested UM samples. Furthermore, using ligand competition assay we investigated the binding of radioiodinated RC-160 to membrane fractions of 20 UM specimens. We found that 70% of the human UM samples studied displayed specific SSTRs with a mean K_d of 7.14 nM and with a mean B_{max} of 604 fmol/mg membrane protein. It is also important to note that all receptor-positive tumor samples expressed a well-detectable amount of the SSTR-2 or SSTR-5 receptor gene.

Histological distribution of the analyzed samples showed that more than half of the samples (56.1%) were spindle cells, 36.6% were epithelioid, and the remaining 7.3% were mixed histologically. Our findings suggest that the patient's gender and age are not predicting factors. Our results demonstrate that SSTR-2 and SSTR-5 are expressed in human UM specimens. qRT-PCR results show that SSTR-2 and SSTR-5 are also highly expressed in both UM cell lines investigated. In this study, we provide evidence for the existence of SSTRs in two human UM cell lines and demonstrate that OCM-3 cells express SSTRs at a significantly higher level than OCM-1 cells ($p < 0.0001$). The receptor protein encoded by mRNA for SSTR-2 or SSTR-5 was also demonstrated by western blot in both UM cell lines.

Targeted therapy of cytotoxic peptide analogs consisting of a cytotoxic group, such as a doxorubicin, conjugated to peptide carriers should be more effective and less toxic than conventional systemic chemotherapy. The only major side effect of these analogs is myelosuppression caused by the infrequent chemical cleavage of the cytotoxic radical doxorubicin. The high incidence of SST receptors in UM suggests that this type of tumor might be a good candidate for therapy with SST analogs including the targeted cytotoxic peptide AN-

162. Since therapy for UM is not effective, our work helps to identify specific molecular targets for the prevention of metastasis or further proliferation of disseminated metastases.

Despite stunning improvements since the mid-1990s, hematological and oncological diseases are still among the leading causes of childhood mortality. As compared to adult oncology, at this moment, targeted oncotherapies play a much less important role among children with cancer. Moreover, combined chemotherapy protocols cannot be intensified any further without an unacceptable increase in acute toxicity and late complications. Thus, there is an urgent need for improved methods of early detection, accurate follow-up, and development of novel, effective treatment approaches based on recently identified molecular targets.

Common treatment approaches include chemotherapy, surgery, radiation therapy, and stem cell transplantation. Innovative treatment modalities such as immunotherapy and targeted small molecular drugs become more and more important elements of first-line treatment in pediatric oncology. Tumor-associated peptides provide attractive properties for treatment strategies due to easy access, convenient purification and storage. Furthermore, they are less immunogenic than antibody-based immunotherapies, have high tissue penetration and high affinity to specific cellular targets that influence cancer cell survival, proliferation, and differentiation. They are characterized by a rapid clearance from the body and are prominent candidates for straightforward conjugation strategies.

Based on our best knowledge, there are no definitive or conclusive data available in the literature showing the potential activation of other receptors by these SST octapeptide analogs. In addition, it has been demonstrated that SST analogs could be effectively used as carriers of radionuclides for visualization and targeted radio- or chemotherapy of SSTR-positive tumors. Many tumor cells of various origins, such as well-differentiated gastroenteropancreatic neuroendocrine neoplasms as well as other cancers show overexpression of SSTR, which can serve as the molecular basis of targeted diagnostic and treatment methods.

Based on these findings, SST peptide analogs and SSTRs may have great diagnostic and therapeutic potential in pediatric hematology and oncology. Similarly to adult oncology, radiolabeled somatostatin analogs might offer an effective tool for identifying the localization and extent of tumors in children. Very limited data are available on somatostatin analogs in childhood tumors. Most of these data are not about malignancies, and almost all data were obtained with octreotide. In addition, Dishop and Kuruvilla reported that SST analogs are also used therapeutically to reduce the symptoms and side-effects of chemotherapy and to induce

differentiation in pediatric oncology. It has not been established whether SSTRs could be used for the localization and treatment of hematological and oncological disorders in children and the previous findings available are incomplete and inconclusive. Therefore, in our present study, we aimed to investigate the expression of mRNA for SSTR subtypes (SSTR-1–5) in samples from human pediatric solid tumors as well as bone marrow aspirates and peripheral blood of children with hematological diseases. We also studied the binding characteristics of SSTR protein by radioreceptor assay. Moreover, we analyzed the potential correlation among the expression of the SSTRs, their binding characteristics, and clinicopathological data of the pediatric patients.

Our results show that the 15 pediatric samples investigated highly expressed mRNA for all five subtypes of SSTRs with various patterns. No sample was found without the expression of at least one of the SSTR subtypes. It is noteworthy that only a HL sample expressed mRNA for all five SSTR subtypes. Similarly, only SSTR-2 was observed in IM, FD, and ITP. Two malignant solid tumor specimens showed the expression of SSTR-5 and PCR products for SSTR-3 could be detected only in malignant samples. The incidence of SSTR-1 and SSTR-4 was similar (9/15, 60%) in the 15 specimens analyzed. Among the nine SSTR-4 positive samples, two were RMS, two ALL, one was HL, one teratoma-type, one histologically was defined as hamartoma, one as HS, and one as chronic benign neutropenia. Among the five ALL samples, only the sample of the female patient was positive for SSTR-3, and none of the four samples from male children expressed mRNA for SSTR-3. The very limited amount of the biological samples did not allow us to perform Western blot or immunohistochemistry. However, in seven cases we were able to prepare crude membrane protein fractions for radioligand binding studies to demonstrate the presence of specific SSTR binding sites. Radioligand binding studies also confirmed the presence of specific, high affinity SSTRs in pediatric solid tumors investigated with a mean dissociation constant (K_d) of 6.00 nM and mean maximal binding capacity (B_{max}) of 391.1 fmol/mg protein. Molecular biology analyses and radioligand binding studies clearly demonstrated that the expression of mRNA for SSTR-2 and SSTR-5 subtypes was 100% consistent with the presence of specific receptors for radiolabeled SST octapeptide analog RC-160.

Although the size of the investigated cohort is rather small and its composition is heterogenous, it is worth to note that in the examined group, five children with cancer died during the 5-year follow-up period. Three of the five ALL patients were alive, but two children died within 5 years. Patients diagnosed with rhabdomyosarcoma and teratoma showed the worst

outcome in this cohort. Correlation between SSTR expression and clinical data was not observed. The high binding affinity of the synthetic octapeptide analogs to SSTR in malignant pediatric samples suggests that children with SSTR-positive cancers might be good candidates for therapy with SST analogs, including the targeted cytotoxic SST analog AN-162.

Our results demonstrated for the first time that somatostatin receptors are highly expressed in childhood benign and malignant solid tumors and in samples of pediatric hematological disorders. These findings might open up a new avenue for a potential application of peptide hormone analogs for the detection and treatment of pediatric oncological and hematological disorders. However, to clarify the therapeutic and clinical significance of SST receptors in pediatric oncology and hematology, further studies are needed. Our results may also help to better understand the exact mechanism of pediatric hematological and oncological disorders and provide better approaches for the early detection of these malignancies. Our findings may facilitate the potential clinical application of synthetic analogs of somatostatin or its radionuclide or cytotoxic derivatives for diagnostic or therapeutic purposes.

6. Summary

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults, with an incidence of 4–5 cases per million. The prognosis of UM is very poor. Hematological and oncological disorders represent leading causes of childhood mortality. Neuropeptide somatostatin (SST) has been previously demonstrated in various pediatric tumors, but limited information exists on the expression and characteristics of SST receptors (SSTR) in hematological and oncological disorders of children.

In the present study, our aim was to investigate the expression of mRNA and protein for SSTR-2 and -5 in human UM tissue samples and in OCM-1 and OCM-3 human UM cell lines by qRT-PCR, western blot and ligand competition assay. We also aimed to investigate the expression of mRNA for SSTR subtypes (SSTR-1–5) in 15 pediatric hematological/oncological specimens by RT-PCR. The presence and binding characteristics of SSTRs were further studies by ligand competition assay.

The mRNA for SSTR-2 showed markedly higher expression in UM tissues than SSTR-5. The presence of SSTRs was demonstrated in 70% of UM specimens using ligand competition assay and both human UM models displayed specific high affinity SSTRs. Among the five SSTRs, the mRNA investigated for SSTR-2 and SSTR-5 receptors was strongly expressed in both human UM cell lines, SSTR-5 showing the highest expression. The presence of the SSTR-2 and SSTR-5 receptor proteins was confirmed in both cell lines by western blot. Our results show that the pediatric tumor samples highly expressed mRNA for the five SSTR subtypes with various patterns. The mRNA for SSTR-2 was detected in all specimens independently of their histological type. A Hodgkin lymphoma sample co-expressed mRNA for all five SSTR subtypes. SSTR-3 and SSTR-5 were detected only in malignant specimens, such as rhabdomyosarcoma, Hodgkin lymphoma, ALL, and a single nonmalignant condition, hereditary spherocytosis. The incidence of SSTR-1 and SSTR-4 was similar (60%) in the 15 specimens investigated. Radioligand binding studies demonstrated the presence of specific SSTRs and high affinity binding of SST analogs in pediatric solid tumors investigated. In summary, the expression of SSTRs in human UM specimens and in OCM-1 and OCM-3 human UM cell lines suggests that they could serve as a potential molecular target for therapy of UM using modern powerful cytotoxic SST analogs targeting SSTR-2 and SSTR-5 receptors. The high incidence of SSTRs in hematological and oncological disorders in children supports the merit of further investigation of SSTRs as molecular targets for diagnosis and therapy.

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List of publications related to the dissertation

1. **Harda, K. M.**, Szabó, Z., Juhász, É., Dezső, B., Kiss, C., Schally, A. V., Halmos, G.: Expression of Somatostatin Receptor Subtypes (SSTR-1-SSTR-5) in Pediatric Hematological and Oncological Disorders.
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   Gyógyszertár. 14 (4), 10-12, 2015.

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8. Published abstracts

Lecture at a professional international conference:

Professzorok az Európai Magyarországért Egyesület, XIX. Nemzetközi Tudományos Konferenciája, Budapest, 2019. 11. 14. Titel: Szomatosztatin receptorokon alapuló, célzott daganatterápia lehetősége.

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MedPECS: Medical Conference for PhD Students and Experts of Clinical Sciences, Pécs 2019. november 09. Poster presentations: Investigation of somatostatin receptor expression in renal tumors. Harda Kristóf, Szabó Zsuzsanna, Szabó Erzsébet, Szegedi Krisztián, Flaskó Tibor, Halmos Gábor.

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Participation in an international professional conference, published abstract:

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XIX. Klinikai Farmakológus Továbbképző Kongresszus és GCP napok, Debrecen, 2017. 12. 7-9. Poster presentations: Szomatosztatin receptorok, mint lehetséges terápiás célpontok a

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XIX. Klinikai Farmakológus Továbbképző Kongresszus és GCP napok, Debrecen, 2017. 12. 7-9. Poster presentations: Intratumor heterogenitás és epigenetikai faktorok szerepe a világossejtes vesecarcinóma kialakulásában? Szabó Zsuzsanna, Szegedi Krisztián, Bereczky Zsuzsanna, Kállai Judit, Sipos Éva, Oláh Gábor, Dobos Nikoletta, Flaskó Tibor, Harda Kristóf, Halmos Gábor.

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