Effects of somatostatin analog SOM230 on cell proliferation, apoptosis, and catecholamine levels in cultured pheochromocytoma cells

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Abstract

Surgery is the primary therapy for pheochromocytoma (PHEO), a catecholamine-producing tumor. Benign and malignant PHEO could develop recurrences, and the intraoperative risk of recurrent PHEO is an important unresolved issue. Non-surgical treatments of PHEO recurrence would therefore better prepare patients for reintervention as well as provide them with palliative management. We investigated the effects of the new somatostatin analog (pasireotide) SOM230 versus octreotide (OCT) in primary PHEO cell cultures (Pheo-c). Pheo-c from six benign surgical samples were set up and characterized by immunocytochemistry. Real-time PCR, using both PHEO tissues and Pheo-c, showed different levels of somatostatin receptor1–5 mRNA expression. Cells treated with various doses of OCT or SOM230 for 48 and 72 h were analyzed to assess their effects on cell proliferation and apoptosis and catecholamine levels. Even if reduction of cell viability was observed in Pheo-c treated for 48 h with either OCT or SOM230 and this effect increased after 72 h, a more significant inhibition of cell growth as well as a significantly higher induction of apoptosis was seen in Pheo-c treated with SOM230 versus OCT. In particular, apoptosis in Pheo-c was detected after 48 h and was associated with increased expression and activation of caspase-3 and cleaved poly(ADP-ribose) polymerase. OCT 10−6 M and SOM230 10−7 M significantly reduced catecholamine levels. Our results indicate that while both OCT and SOM230 modulate cell growth and apoptosis and catecholamine levels in Pheo-c through specific receptors, SOM230 is more effective. This improves our knowledge on the mechanism of SOM230 action in PHEO and supports a possible therapeutic use in benign PHEO recurrence.

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Introduction

Pheochromocytoma (PHEO) is a catecholamine-producing and -secreting tumor derived from neuroectoderm arising from the adrenal medulla or extra-adrenal neural crest. PHEO, which has an annual incidence of 2–8 per million population (Pacak et al. 2007, Scholz et al. 2007), is generally treated surgically. Both benign and malignant PHEO are at the risk of recurrence, and patients presenting with recurrent disease and repeated interventions are at high risk of perioperative complications (Plouin et al. 2001, Mannelli 2006, Chrisoulidou et al. 2007, Pacak et al. 2007, Scholz et al. 2007). There is no generally effective systemic treatment for PHEO recurrence. Although a considerable proportion of the patients respond to (131)I-meta-iodobenzylguanidine ((131)I-MIBG) radiotherapy or cytotoxic chemotherapy (Chrisoulidou et al. 2007, Pacak et al. 2007), novel agents and approaches are greatly needed to increase the efficacy of current treatment options to better prepare patients for reintervention or to provide them with palliative management. Somatostatin (SS), which acts on both endocrine and non-endocrine tissues, is a key regulator of the release of various hormones and growth factors (Patel 1999). SS exerts its effects by binding to all five SS receptor (sst) subtypes, sst1–5. SS analogs have been used in the detection and treatment of different neuroendocrine tumors. For example, octreotide and lanreotide are somatostatin-like oligopeptides that are widely employed in the therapy of somatotropin-secreting pituitary adenomas and neuroendocrine tumors of the gastroenteropancreatic system. In radio-labeled forms (usually 111In-pentetreotide), these analogs are also useful for somatostatin receptor imaging (SRI) (Kalsas et al. 2001, Chrisoulidou et al. 2007); however, they bind with high affinity to only sst2.
and sst$_5$ subtypes (Patel 1999). Although it has been shown that PHEO tissues express more than one sst receptor (Kubota et al. 1994, Mundschenk et al. 2003, Unger et al. 2004, Kolby et al. 2006), only a small amount of conflicting data on the role of OCT for PHEO tumor treatment exists (Invitti et al. 1993, Plouin et al. 1995, Tenenbaum et al. 1995, Kopf et al. 1997, Koriyama et al. 2000, Lamarre-Cliche et al. 2002, Zatelli et al. 2003), and knowledge of the mechanistic action of SS analogs on PHEO remains incomplete. The poor effectiveness of OCT, for example, might be due to only one (sst$_2$) of the several sst subtypes expressed in PHEO tissues binding with high affinity to standard somatostatin analogs. Furthermore, immunohistochemical staining has shown that greater than 60% of PHEO cells express sst$_3$ (Unger et al. 2004). Thus, these tumors might represent a potential target for new synthetic analogs active on sst$_{1-3}$ and sst$_5$, such as SOM230 (Weckbecker et al. 2002). Whether a subgroup of SRI-positive patients will benefit from new somatostatin analogs with an improved affinity for sst3, such as SOM230, remains to be elucidated. The purpose of this study was to define sst expression in both tissues and cells using an in vitro system of primary cultured PHEO cells derived from surgically removed PHEO tissue of patients affected by benign PHEO, and to investigate the effects of OCT and SOM230 treatment on cell growth control and apoptosis.

**Materials and methods**

**Cell culture**

Tissue explants were obtained from six patients (aged 25–57 years) after the achievement of pre-surgical diagnosis of sporadic benign PHEOs by clinical, hormonal, and morpho-functional evaluation. Patients who underwent surgical removal of the adrenal mass gave consent for surgery and for genetic and research studies on tissue specimens. Tissue samples were minced and placed in the lid of a small culture dish with 3 ml dissociation solution: 2.6 mg/ml collagenase type IV, 3 mg/ml BSA, 0.15 mg/ml DNase I, and 0.15 mg/ml hyaluronidase I-S in Hanks’ Balanced Salt Solution (HBSS) at room temperature. Medulla chunks and the solution were recovered and transferred into a Falcon tube using a glass Pasteur pipette. The tube was placed in a thermostatic bath at 37°C for 30 min. The solution was gently resuspended every 10 min with a Pasteur pipette. Over the last 5 min, the solution was continuously resuspended until the medullary tissue became not visible by eye, then stopped to avoid damage of the cells. To stop the enzymatic reaction, cold HBSS was added. The tube containing the digested tissue was placed in the rotor of a pre-cooled (4°C) refrigerated centrifuge and spunned at 313 g for 10 min. The pellet was resuspended in pre-warmed Nutrient Mixture F12 Ham/Dulbecco’s modified essential medium (dMEM) (vol/vol) supplemented with t-glutamine, 10% fetal bovine serum (FBS), and antibiotics (GibcoBrl, Life Technologies). To assess the quality of chromaffin cells cultures, the cells were stained with trypan blue solution (0-2%) and counted in a hemocytometer chamber to estimate both the yield and the cellular viability. The purity in chromaffin cells of our cultures was estimated by staining the cells with neutral red, a dye that selectively reacts with monoamine-containing cells. Pheo-c was used for experimental protocols at the first and second culture passages, and the experiments were repeated at least thrice.

**Immunocytochemistry of Pheo-c**

Cells were grown on four-chamber tissue-culture treated glass slides (Falcon Becton Dickinson, Labware, NJ, USA) pre-coated with poly-L-lysine to enhance cell attachment at a density of 5000 cells per well. Cultured cells were rinsed with PBS, and finally fixed in alcohol.

Immunostaining was performed using the linked streptavidin–biotin horseradish peroxidase technique (LSAB-HRP). Briefly, after hydration, the slides were treated with 0.3% H$_2$O$_2$ for 15 min to quench endogenous peroxidase. Antigen retrieval was performed using microwave heating of the slides immersed in 10 mM citrate buffer (pH 6), firstly for 3 min at 650 W, and a second and third time for 3 min at 350 W. The slides were then blocked for 60 min with 1-5% bovine serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS buffer before incubating with primary antibody (Ab). Mouse monoclonal Ab against chromogranin A (AB356M – Biogenex, San Ramon, CA, USA) and mouse monoclonal Ab against Neuron Specific Enolase (Dako, Dako, Denmark) were diluted 1:100 with 0.05 M Tris–HCl buffer (pH 7.4) containing 1% BSA and were incubated with samples for 120 min at 24°C. Negative control slides without primary antibody were included for each staining condition. After two washes with PBS, the slides were treated with biotinylated species-specific secondary antibodies and streptavidin horseradish peroxidase reagent (LSAB-HRP, Dako, Denmark), which was followed by color development in 3,3’-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer’s hematoxylin, and mounted using xylene-based mounting medium. Evaluations of the immunocytochemical staining were performed separately by two observers with at least 10 high power fields using optical microscopy.

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Real-time quantitative RT-PCR

Total RNA was isolated from tissues and primary cell cultures at early passages (first to second) using TRIZOL (Invitrogen). Before RNA extraction, we washed tissues twice in PBS and macroscopic fibrosis were cut off to avoid contamination by blood cells and to reduce stromal cells. Residual DNA was removed by RNase-free DNase I treatment (Promega). RNAs were reverse transcribed with 5 μg total RNA and reverse transcriptase (Superscript-BRL-200 U) as previously described (Sinisi et al. 1997, Pasquali et al. 2000, 2004). We include a total RNA sample that was not reversed transcribed and a no-cDNA control to monitor for genomic DNA contamination or exogenous contamination respectively. The expression of ssts in Pheo-c and PHEO was quantified using the SYBR green reagent (2 × SYBR Green Supermix, Bio-Rad Laboratories) following the manufacturer’s instructions on a Bio-Rad iCycler. PCR was performed in triplicate under optimized conditions: 95°C denaturation for 3 min, followed by 40 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C using the following primers: sst1 (5’-tgatgtcatcttcggctct-3’ (sense) and 5’-tgagaggtgagcatgccgt-3’ (antisense)); sst2 (5’-ctttgttgtctctccct-3’ (sense) and 5’-gagagg- cagcttgataaggc-3’ (antisense)); sst3 (5’-tagggcctacttcc- caaggt-3’ (sense) and 5’-ttggcagaccttgtca-3’ (antisense)); sst4 (5’-tcctttcaaaatccaaaa-3’ (sense) and 5’-gcatgatccgacagctca-3’ (antisense)); sst5 (5’-cattttggctctctca-3’ (sense) and 5’-gcccccagctccgagc-3’ (antisense)). In these experiments, the size of the product with the size expected from the housekeeping gene, β2-microglobulin, and subsequently to an internal control. The following primers used were: sense, 5’-tcctttcaaaatccaaaa-3’ (antisense), 5’-gtgaagatgcatctgta-3’. No other products were amplified because melting curves showed only one peak in each primer pair and only one specific product was observed on agarose ethidium bromide gel for each primer pair. The identity of PCR products was confirmed by comparing the size of the product with the size expected from the gene sequence. Fluorescence signals were measured over 40 PCR cycles. The cycle number was recorded when the signals crossed a threshold set within the logarithmic phase. Data were expressed as the amount of specific PCR products from each gene in cells and in tissues, and were normalized based on the housekeeping gene product β2-microglobulin (which showed no significant differences between samples). The efficiency of amplification of each pair of primers was determined by serial dilutions of cDNA templates and all were larger than 0.9. Experiments were repeated at least thrice and represented as folds of expression.

Cell proliferation assay

Cell proliferation was evaluated with the tetrazolium salt (MTT) method as previously described (Pasquali et al. 2000). The MTT Cell Proliferation Assay is a quantitative colorimetric method used to determine cell proliferation. It utilizes the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide), which is metabolized by mitochondrial succinic dehydrogenase activity of proliferating cells to yield a purple formazan reaction product. The MTT assay is an alternative to the thymidine incorporation test, which measures cell proliferation by determining the amounts of incorporated thymidine into freshly synthesized DNA. Conversely, when metabolic events lead to apoptosis or necrosis, MTT measures the reduction in cell viability. Briefly, cells were plated in 96-well plates at a density of 2000 cells per well, starved for 24 h in MEM without FBS, then they were incubated in 1% FBS-supplemented medium with OCT or SOM230 at 10⁻⁶ M, 10⁻⁷ M, and 10⁻⁸ M or solvent (control cells) for 48 and 72 h, and then processed according to the manufacturer’s instructions. Absorbance was read at 550 nm using a microtitre plate reader.

Apoptosis detection

From among the many ways of detecting apoptosis at different stages, we used the most common method called TUNEL (terminal deoxynucleotidyl transferase biotin d-UTP nick end labelling), which identifies apoptotic cells in situ by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to strand breaks of cleaved DNA after the activation of Ca/Mg-dependent endonucleases in individual apoptotic cells. Cells were plated at 2×10⁶ cells/100 mm Petri dish. The cell monolayers were grown directly on sterilized slides (Superfrost; Carlo Erba, Milan, Italy), starved for 24 h in DMEM without FBS, and then incubated in 1% FBS-supplemented medium with OCT or SOM230 at 10⁻⁷ M, or solvent (control cells) for 48–72 h. Using the in situ Cell Death Detection Kit (La Roche, Italy), samples were processed and analyzed as described previously (Pasquali et al. 2000).

Caspase-3 activity

Caspase-3 enzymatic activity was measured using Apo-ONE Homogenous Caspase-3 Assay (Promega). Briefly, Pheo cells were seeded in 96-well plates (50 000 cells/well) and treated with or without octreotide, and SOM230 (at 10⁻⁸, 10⁻⁷, 10⁻⁶ M) for 48 h at 37°C, in DMEM (Sigma Chemical) supplemented with 10% FCS. The timing of treatment was determined on the basis of preliminary experiments carried out at different time intervals showing the maximal stimulation at 48 h. Experiments were repeated at least twice and each determination was done in quintuple.
Electrophoresis and western blot analysis

Lysates of Pheo-c treated with 10^{-7} M OCT or SOM230 for 48 h were subjected to gel electrophoresis followed by immunoblotting using polyclonal antibody anti-caspase-3 (H-277), and mouse monoclonal IgG 200 μg/ml anti-poly ADP-ribose polymerase (PARP)-1 (F-2) (Santa Cruz Biotechnology, Inc.) at working concentrations of 1:200 and 1:500 respectively. Western blot analysis was done as reported previously (Pasquali et al. 2000).

Determination of intracellular catecholamine levels

Catecholamine levels were determined in medium collected from cells, starved for 24 h in dMEM without FBS, and in the absence (control) or presence of OCT and SOM230 10^{-8} M, 10^{-7} M, 10^{-6} M for 24, by high-pressure liquid chromatography (HPLC) with electrochemical detection. A 12.5 cm long, 5 μm C-18 reverse phase column (Waters Chromatography Division, USA) was connected to a carbon electrode set at a potential of +0.75 V relative to the Ag/AgCl reference electrode, in turn connected to an LC-4 amperometric detector (Bioanalytic System Inc., West Lafayette, IN, USA) in the HPLC system. The detection limit for catecholamines, defined as a peak height to noise ratio greater than 2, was 40 pg. The mobile phase consisted of 1.75 g heptanesulfonic acid, 0.1 g disodium EDTA, 3.5 ml triethylamine, 4 ml phosphoric acid, and 40 ml acetonitrile made up to 1 l in distilled water and was filtered and degassed just prior to use. External standards of dopamine and norepinephrine were dissolved in 0.1 M perchloric acid and run simultaneously with each experiment. The protein content of the tissue pellets solubilized in 0.5 M NaOH was assayed by the method of Lowry.

Statistical analysis

The data were reported as mean ± S.E.M. from at least three separate experiments performed in triplicate. The means were compared using ANOVA. Differences in expression of sst_{1-5} in PHEO tissues or Pheo cells evaluated by real-time PCR were determined using one-way ANOVA followed by a Neuman–Keuls post hoc analysis.

Results

Immunocytochemical identification of PHEO cells in primary culture

Ultrastructural analysis of PHEO shows that the cells contain numerous neurosecretory-type dense-core granules containing norepinephrine and epinephrine. Immunohistochemical analyses of PHEO tissues always show reactivity for chromogranin, neuron-specific enolase, as well as catecholamines, catecholamine-synthesizing enzymes, synaptophysin, and opioid peptides. We therefore used specific monoclonal antibodies designed for the immuno-localization of chromogranin A and neuron-specific enolase (NSE). PHEO cells in primary culture (Pheo-c), which were processed for immunocytochemical analysis, stained positively in a granular pattern with the antibody against chromogranin A (Fig. 1A), and NSE (Fig. 1B). Morphological assessments by pathologists and the strong immunoreactivities of the specific neuroendocrine markers have convinced us that the cells cultured are selected chromaffin cells, and we thus excluded the possibility of contaminating material.
In order to further characterize our various tissue samples and the in vitro cell system used, we studied the expression pattern of sst, which would be useful for explaining potential differences of SOM230 and OCT action on Pheo-c. Transcripts of the ssts family were differentially expressed in PHEO tissues and Pheo-c cells. In all tissues and cultures studied, the sst2 and sst5 mRNAs were found (Table 1). However, sst1, sst3, and sst4 were absent in PHEO5, PHEO1, and PHEO3 respectively and their corresponding cultured cells, demonstrating the heterogeneity which can exist in these neuroendocrine cells (Table 1). Furthermore, real-time PCR analysis using PHEO tissue samples and Pheo-c cultures showed that sst2 was the dominant receptor expressed in these samples (Fig. 2).

**PHEO tissue and primary cultured cell expression of sst genes**

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**In vitro effects of octreotide and SOM230 on cell viability**

Cells were seeded at 2000 cells per well, and treated with $10^{-8}$ M, $10^{-7}$ M, and $10^{-6}$ M OCT or SOM230 for 48 and 72 h. Pheo-c cell growth was inhibited after 48-h treatment with $10^{-7}$ and $10^{-6}$ M SOM230 ($P<0.05$ and $P<0.001$ respectively), while only a significant reduction in cell viability was seen using $10^{-6}$ M OCT (Fig. 3A). This effect was even greater after 72-h exposure to either compound. As shown in Fig. 3B, OCT and SOM230 at $10^{-7}$ M increased the TUNEL-positive process in cultured cells after 48-h treatment (8% and 16%, respectively). Induction of the apoptotic-related event was significantly higher in Pheo-c treated with SOM230 versus OCT ($P<0.01$).

**Caspase-3 and poly (ADP-ribose) polymerase levels**

We studied the effect of OCT and SOM230 on the cellular apoptotic protein markers caspase-3 and proteolytic cleavage products of PARP-1. Western blot analysis of cells that were treated with $10^{-7}$ M OCT or SOM230 for 48 h revealed a significant increase in the expression of the 32 kDa caspase-3 protein (Fig. 4A) and activity (Fig. 5). In cultured cells obtained from all PHEO tissues, OCT and SOM230 induced a dose-dependent increase in caspase-3 activity that was detected at concentrations higher than $10^{-8}$ M (data not shown) and maximal at $10^{-7}$ M (178±20% and 180±20% respectively versus basal) (Fig. 5). PARP-1 is a nuclear enzyme involved in DNA repair and is specifically digested into 89 and 24 kDa fragments by caspase-3 during the apoptotic process (Soldani & Scovassi 2002). After treatment of Pheo-c with $10^{-7}$ M OCT or SOM230 for 48 h, an increase was seen in 113 kDa PARP-1 protein compared with control cells, and in addition, 89 kDa fragment was present (Fig. 4B).

### Table 1 Somatostatin receptor (sst) subtype mRNA expression in six pheochromocytoma tissues and in corresponding primary cell cultures

<table>
<thead>
<tr>
<th>Tissue/Culture</th>
<th>sst1</th>
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PHEO 1, 2, 3, 4, 5, and 6, pheochromocytoma tissues; Pheo-c 1, 2, 3, 4, 5, and 6, pheochromocytoma cell cultures. +, mRNA present; --, mRNA absent.

![Figure 2](https://example.com/fig2.png)  
**Figure 2** Evaluation of the expression level of sst 1–5 in three PHEO tissues and primary cell cultures of pheochromocytoma. Real-time PCR products of sst1-5 were normalized to a housekeeping gene product in each experiment (β2-microglobulin). Data are the mean ± s.e.m. (n=6).
Thus, the apparent enzymatic digestion of PARP-1 shown here is a further evidence for the caspase-dependent apoptotic induction by these compounds.

Effects of octreotide and SOM230 on intracellular catecholamine levels

PHEO cells were treated with octreotide and SOM230 at different doses for 24 h to examine the intracellular catecholamine levels by HPLC. As shown in Table 2, $10^{-8}$ M octreotide and SOM230 had no effect on intracellular catecholamine levels. At $10^{-7}$ M and over, SOM230 significantly inhibited the intracellular dopamine and norepinephrine levels ($P<0.05$ and $P<0.01$ respectively). Octreotide induced a significant reduction of catecholamine levels only at the higher doses.

Discussion

In order to gain further mechanistic insights into the responses of PHEO to compounds which could interact via somatostatin receptors (sst), this study was aimed at
have proved to be a widespread histological marker for polypeptide-producing cells, the adrenal medulla, and tumors derived from them. The largest of these polypeptides is chromogranin A; the plasma concentration of this substance is usually elevated in PHEO, a feature of great diagnostic utility. The enolase enzymes comprise five different forms, each of which is composed of three homodimers and two hybrids. The cultured PHEO cells showed the presence of both chromogranin A and NSE (Fig. 1).

The first point emerging from our data is that both PHEO and Pheo-c expressed sst subtypes with large heterogeneity similar to that observed by previously published data (Kubota et al. 1994, Mundschken et al. 2003, Zatelli et al. 2003, Unger et al. 2004, Kolby et al. 2006). PHEO tissues and Pheo-c from the same patient did not show different pattern of sst, in spite of blood vessels, fibroblasts, and other stromal cells that could be present in tissues. This could be explained considering the extreme accuracy in the collection of the sample immediately after surgery; a further possible explanation could be that each tumor has its own pattern of sst, which is the same in all compartments.

The most remarkable result of our study is that both OCT and SOM230 affect cell growth and apoptosis and catecholamine levels, but SOM230 was more effective than OCT. For example, SOM230 at concentrations of $10^{-6}$ M and $10^{-7}$ M caused inhibition of cell growth in Pheo-c after 48 h, while only the higher dose of OCT ($10^{-6}$ M) was effective; this inhibition persisted after 72-h treatment with either compound. Moreover, SOM230 induced a significant increase in apoptotic cells compared with OCT. Even if concentrations of OCT and SOM230 ($10^{-6}$ and $10^{-7}$ M) necessary to affect cell proliferation and apoptosis could be considered high, they were the same doses used for functional studies with OCT in our previous in vitro cell culture studies (Pasquali et al. 2000). Furthermore, we showed that SOM230 markedly suppressed intracellular catecholamine levels (Table 2), suggesting that it could inhibit catecholamine biosynthesis.

PHEO, which belongs to a heterogeneous group defined as neuroendocrine tumors (Barakat et al. 2004), is generally treated surgically. Although prognosis after tumor resection is excellent, a significant proportion of PHEOs may recur, some as metastases. Thus, appropriate follow-up is mandatory, and recurrence of PHEO is a key problem needing particular attention. Treatment with metyrosine with or without phenoxybenzamine might help to control blood pressure and alleviate symptoms, but it has no effect on tumor size (Steinsapir et al. 2003, Zatelli et al. 2004). Palliative management comprising of chemotherapy and/or therapeutic doses of MIBG has only limited effects on tumor growth, and patients with malignant PHEO might not take up MIBG (Sisson & Shulkin 1999, D PASQUALI and others 269).
Interestingly, sst3 mRNA, which is reported to be very expression after 48-h treatment with either 10^−8 M OCT or SOM230, showing that SS analogs can induce the activation of both apoptotic pathways. Moreover, the duration and the efficacy of SOM230 on cell growth inhibition, apoptotic induction, and reduction of catecholamine levels were markedly stronger than the effects elicited by OCT. SOM230 at 10^−7 M was able to inhibit Pheo-c cell growth significantly after 48-h treatment, and by even greater levels after 72 h. This short term as well as the chronic efficacy of SOM230 is probably due to both its improved pharmacodynamic and pharmacokinetic properties.

In conclusion, our results confirm the remarkable heterogeneity of sst expression in neuroendocrine cells. They also demonstrate that OCT and SOM230 are both capable of reducing cell viability and inducing programmed cell death via caspase-3 and PARP-1 apoptotic pathways; however, SOM230 appears to be more effective than OCT. Data in this study add further to the mechanistic insights of SOM230 in PHEO, suggesting a potential role in the diagnostic procedures (as radiolabeled form) and management of the recurrence of this tumors especially in patients at high risk for reintervention.

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