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ORIGINAL ARTICLE

Synthesis, in vitro biological activity, hydrolytic stability and docking of new analogs of BIM-23052 containing halogenated amino acids

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Abstract

One of the potent somatostatin analogs, BIM-23052 (DC-23-99) D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2, has established in vitro growth hormone inhibitory activity in nM concentrations. It is also characterized by high affinity to some somatostatin receptors which are largely distributed in the cell membranes of many tumor cells. Herein, we report the synthesis of a series of analogs of BIM-23052 containing halogenated Phe residues using standard solid-phase peptide method Fmoc/OtBu-strategy. The cytotoxic effects of the compounds were tested in vitro against two human tumor cell lines—breast cancer cell line and hepatocellular cancer cell line, as well as on human non-tumorigenic epithelial cell line. Analogs containing fluoro-phenylalanines are cytotoxic in μM range, as the analog containing Phe (2-F) showed better selectivity against human hepatocellular cancer cell line. The presented study also reveals that accumulation of halogenated Phe residues does not increase the cytotoxicity according to tested cell lines. The calculated selective index reveals different mechanisms of antitumor activity of the parent compound BIM-23052 and target halogenated analogs for examined breast tumor cell lines. All peptides tested have high antitumor activity against the HepG2 cell line ($IC_{50} \approx 100$ μM and $SI > 5$) compared to breast cells. This is probably due to the high permeability of the cell membrane and the higher metabolic activity of hepatocytes. *In silico* docking studies confirmed that all obtained analogs bind well with the somatostatin receptors with preference to ssrt3 and ssrt5. All target compounds showed high hydrolytic stability at acid and neutral pH, which mimic physiological condition in stomach and human plasma.

Keywords Peptides · Analogs of BIM-23052 · Somatostatin · Solid-phase peptide synthesis · Hydrolytic stability · Cytotoxic effects · Docking

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Introduction

Use of the peptides that can directly target cancer cells without affecting normal cells is evolving as an alternate strategy to conventional chemotherapy. Peptides possess many advantages, such as small size, simple synthesis and modification, cell-penetrating ability including tumor cells, and good biocompatibility (Thayer 2011; Borghouts et al. 2005). Apart from the use of peptide-based LHRH agonists and antagonists for treating cancer, somatostatin analogs are the only approved cancer therapeutic peptides on the market (Strowski and Blake 2008). Potent analogs of somatostatin including octreotide (sandostatin) have been developed for the treatment of acromegaly, gigantism, thyrotropinoma, diarrhea and flushing episodes associated with carcinoid syndrome, and diarrhea in patients with vasoactive intestinal peptide-secreting tumors (Saltz et al. 1993). Somatostatin (SST) is an endogenous cyclic tetradecapeptide hormone that plays an important regulatory role in a number of cellular functions, including inhibition of the insulin and endocrine secretion as well as cell proliferation. It is also a neurotransmitter in the central nervous system, reducing the amount of gastric acid, and many others (Pollak and Shally 1998; Pyronnet et al. 2008). The study of the anti-angiogenic effects of the somatostatin analogs is also of interest, because of the presence of somatostatin receptors on the membrane of endothelial cells (Torre et al. 2002). Somatostatin has the ability to affect cellular reproduction by binding to the so-called G-protein coupled somatostatin receptors (sstrs) subtype 1–5 (Reisine et al. 1995; Patel 1997, 1999; Breder et al. 1992; Meyerhof 1998). The distribution of sstrs varies widely in some tumor cells such as those present in the brain, digestive pancreatisc tract, lung, thyroid, gland, prostate, lymphatic system and ovaries (Susini et al. 2006; Reubi et al. 2001, 1995; Weckbecker et al. 2003; Patelet al. 1995; Bruns et al. 1994; Nilsson et al. 1995). Unfortunately, using of somatostatin in the medicinal practice is limited because of its very short lifetime in the human plasma < 3 min and poor absorption by the gastrointestinal tract (Dinnendahl et al. 2010; Haberfeld 2009). To overcome the disadvantages of somatostatin, a large number of analogs have been synthesized to increase their biological activity and stability. They have been shown to be effective against pancreatic (Paz-Bouza et al. 1987), breast (Rose et al. 1983), bone (Schally et al. 1984a, b) and prostate (Schally et al. 1987; Zalatnai et al. 1988) cancers. Several long-acting analogs of SST have found application for treatment of acromegaly and symptoms caused by neuroendocrine tumors, most notably carcinoid syndrome and vasoactive intestinal peptide-secreting tumors (Saltz et al. 1993).

BIM-23052 (DC-23-99) D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2 is a linear amide analog of somatostatin of particular interest. This peptide has established in vitro GH-inhibitory activity in nM concentrations (Coy and Murphy 1997). BIM 23,052 and the CGP 23,996-like compounds in vitro bind selectively to rat sstr5 versus human sstr1, mouse sstr2, mouse sstr3, and human sstr4. The linear compound BIM 23,052 displayed ~ 1000-fold lower affinity for human sstr5 than for rat sstr5. Compounds that bind potently to human sstr5, such as L-362, 855 and BIM 23,052, are relatively less potent in GH inhibition (O'Carroll et al. 1994). In addition, it is characterized by high affinity to some sstrs largely distributed into the cell membranes of many tumor cells (Shimon 2003). In a good correlation with Coy and Murphy studies, our previous investigations on a series of BIM-23052 analogs with general structure D-Phe-Phe-Phe-D-Trp-Lys-Xxx^6-Phe-Thr-NH_2, where Xxx is Aib (α-aminoisobutyric acid), Ac5c (1-aminocyclopentanecarboxylic acid), and Ac6c (1-aminocyclohexanecarboxylic acid) revealed very good activity of newly synthesized analogs against a panel of tumor cell lines (Naydenova et al. 2019).

In our previous studies a number of shortened somatostatin analogs have been synthesized and tested for their biological activity (Staykova et al. 2012a, b, c, 2015; Naydenova et al. 2018). The obtained results with the C-amide analogs of octreotide (SMS 201-995) modified at positions 5 with Orn, diaminobutanoic acid and diaminopropanoic acid and at positions 6 with the unnatural amino acids tert.-leucine, reveal concentration-dependent antiproliferative effects against the HT-29, MDA-MB-231, HepG2 and HeLa cell lines (Staykova et al. 2012a). In addition, all synthesized peptides demonstrated considerable antioxidant activity measured by HORAC and ORAC methods (Staykova et al. 2012b). Further, some modified octapeptide analogs of somatostatin with the general structure D-Phe-c(Cys-Phe-D-Trp-Xxx-Yyy-Cys)-Thr-NH_2, where Xxx is Lys or Orn and Yyy is α-aminoisobutyric acid, 1-aminocyclopentanecarboxylic acid and 1-aminocyclohexanecarboxylic acid, were prepared. The cytotoxic effects of the compounds were also tested in vitro against a panel of tumor cell lines and the compounds were the most effective to the HT-29 tumor cells (Staykova et al. 2015).

Coy and Murphy in their studies showed that the optimization of hydrophobicity of the analogs of somatostatin increased their biological activity (Coy and Murphy 1997). Halogenation is a useful strategy to modulate the properties of biologically active molecules. Fluorine is extensively used in the pharmaceutical industry to improve the pharmacokinetics of drugs. It is a halogen element of periodic table with extreme electronegativity. The chemical bond between carbon and fluorine in organic compounds is polarized in the opposite direction to carbon–hydrogen bond, and is both

more stable, and less polarizable than a C–H bond. The van der Waals radius of fluorine is only slightly greater than that of hydrogen, so fluorine is often considered isosteric with hydrogen. Moreover, introduction of ^{18}F in the place of hydrogen atom is widely used in nuclear medicine for manufacturing radiotracers in the diagnosis of cancer (Pająk et al. 2016). On the other hand, chlorine is also a halogen element, and similarly to fluorine has several electron pairs which are able to form strong hydrogen bonds in the active centers of enzymes as well as to realize stronger interactions with different receptors.

Based on the conclusions of Barrie et al. that inhibition of angiogenesis by somatostatin and somatostatin-like compounds is structurally dependent (Barrie et al. 1993) as well as the investigations of Coy and Murphy (1997), and taking into account some specific properties of fluorine and chlorine, the aim of this work was to synthesize analogs of the potent linear analog of SST, BIM23052, including differently halogenated Phe in the position 1, 2 and 3 (Fig. 1).

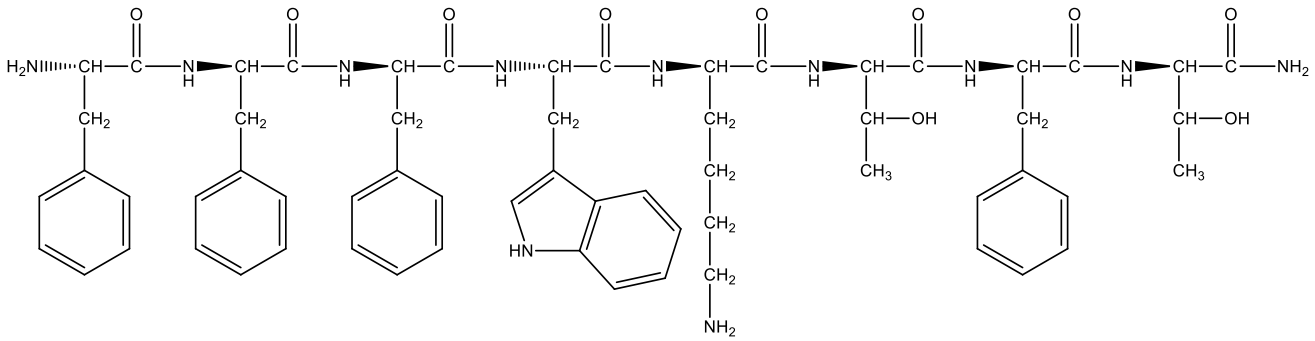
Thus, peptides with a general formula D-Phe(H or X)-Phe(H or X)-Phe (H or X)-D-Trp-Lys-Thr-Phe-Thr-NH_2, where X is fluorine or chlorine, were successfully obtained. In this context, the second objective in this study was in vitro evaluation of antiproliferative effects of these peptides against two human tumor cell lines (MDA-MB-231 and HepG2). To estimate their selectivity, the non-tumorigenic epithelial cell line MCF-10A was used. Finally, we also examined the hydrolytic stability of the resulting peptide analogs under physiological conditions such as: physiological pH value of 2 (stomach), 7.4 (blood plasma) and 9.0 (small intestine) and temperature 37 °C.

Materials and methods

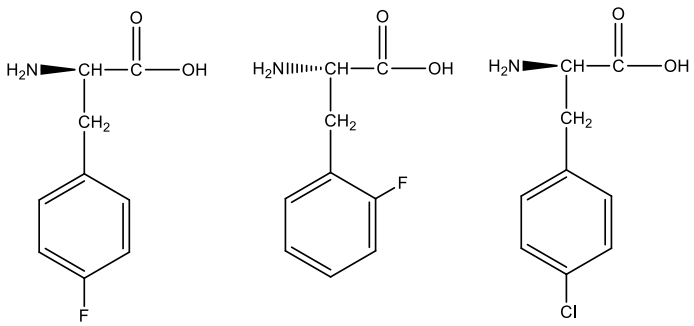
Synthesis

The protected amino acids and Fmoc-Rink Amide MBHA Resin were purchased from Iris Biotech (Germany). All other reagents and solvents were analytical or HPLC grade and were purchased from Merck (Germany).

The LC/MC spectra were recorded on an LCMS-8045 triple quadrupole liquid chromatograph mass spectrometer (LC–MS/MS), Shimadzu, (Shimadzu Corporation). Optical rotations were recorded on an MCP200 modular circular polarimeter (Anton Paar Opto Tec GmbH, Seelze, Germany).



BIM-23052



A B C

Fig.1 Structure of original BIM-23052 and the halogenated phenylalanines used in the current work: **a** L-4-F-Phe; **b** D-2-Phe; **c** L-4-Cl-Phe

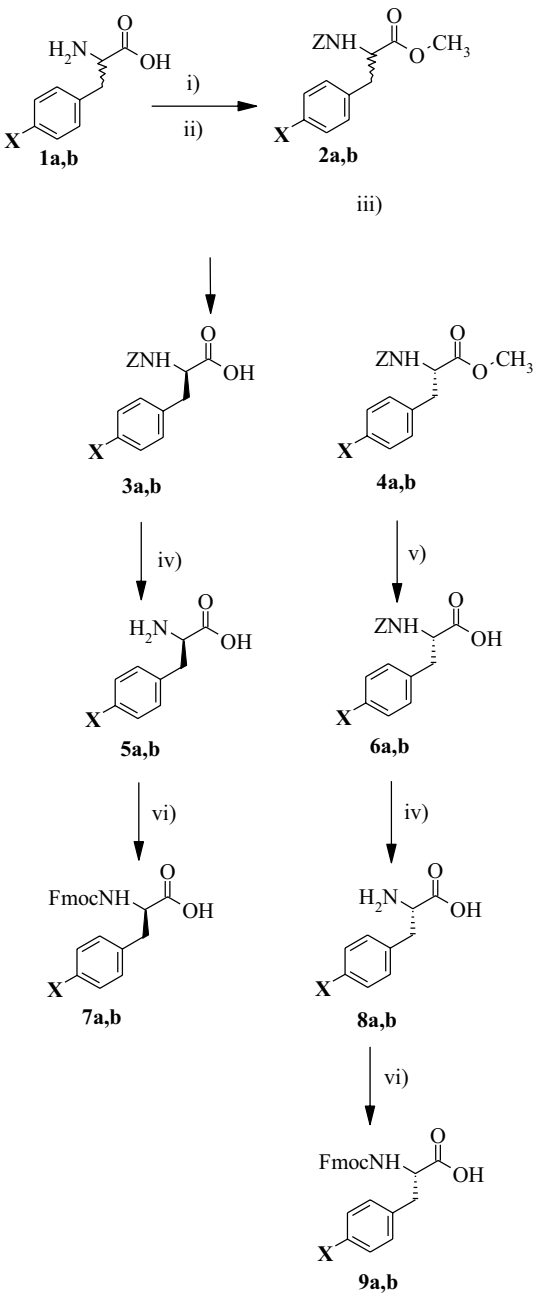
The conventional solid-phase peptide synthesis based on Fmoc (9-fluorenylmethoxycarbonyl) chemistry was employed to synthesize a series of new analogs of BIM-23052. Rink-amide MBHA resin and HBTU (3-[Bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate) or DIC (*N*,*N*′-Diisopropylcarbodiimide) were used as solid-phase carrier and coupling reagents, respectively.

The halogenated amino acids Fmoc-Phe(4-Cl)-OH, Fmoc-Phe(4-F)-OH, and Fmoc-D-Phe(2-F)-OH were synthesized at the Institute of Molecular Biology, BAS, according to the procedures previously described by Pajpanova (Scheme 1) (Pajpanova 2000). Separation of the racemates **2a, b** was achieved using alkaline protease from *Bacillus subtilis DY* strain according to Aleksiev et al. (1981). As well as the high level of enantiomeric discrimination involved with enzymatic processes, the workup procedure after the use of protease is usually particularly straightforward since the unchanged D-amino acid derivatives **4a, b** can be extracted from the reaction mixture using a water—immiscible solvent, but the N_{α}-L-protected enantiomers **3a, b**—after acidifying the reaction mixture. In our present experiments, we achieved good resolution of the both racemic derivatives. The N_{α}-benzyloxycarbonyl protected L-enantiomers **3a**, **b** recrystallized from appropriate alcohol in good yields (up to 93%) were used for catalytic hydrogenation step to obtain the final halogenated L-phenylalanines **5a**, **b**. The Z-protecting group was removed by hydrogenolysis with 10% palladium on charcoal in methanol using formic acid as a hydrogen donor.

Further, Fmoc-OSu was used as reagent for the synthesis of the corresponding Fmoc-derivatives **7a, b** because of monitored high yields under mild conditions. The optimum developed procedure uses a 10% excess of the corresponding halogenated amino acids over Fmoc-OSu, a minimum volume of dioxane to aqueous phase (~ 1:10 by volume) and twofold excess of sodium carbonate over the amino acid component. The reaction was most efficient when the reactants were stirred vigorously at room temperature. The preparation of Fmoc-L-4-F-Phe **7a** and Fmoc-L-4-Cl-Phe **7b** was accomplished in 84–85% yields and very good purity.

The synthesis of Fmoc-D-2-F-Phe was accomplished in a similar manner as outlined in Scheme 1. The chiral purity for all halogenated Phe derivatives was confirmed by LE-CE (Koidl et al. 2005) and HPLC (Kučerová et al. 2013). The chiral analysis of halogenated amino acids by LE-CE was performed using the Cu(II) complexes of L-4-Hypro dissolved in 5 mM phosphoric acid and adjusted to pH 4.5 by adding ammonia. The LOQ (limit of quantitation) for the minor enantiomer was estimated to be about 0.1%.

All HPLC measurements were performed on teicoplanin-based CSPs (Astec Chirobiotic T) column. The mobile phase was composed of MeOH/20 mM ammonium acetate buffer, pH 4.00, 70/30 (v/v) volume ratios. In the D-forms, 0.05% of the unwanted enantiomer can be detected.



Scheme 1 Chiral separation of racemic D,L-4-F-Phe **1a** and D,L-4-Cl-Phe **1b**: (i) Z-Osu/Na_2CO_3,H_2O/acetone, 3 h; (ii) DMAP/EDCI, MeOH, 0 °C, 2 h; (iii) alkaline protease from *Bacillus subtilis DY* strain, DMF/H_2O, $NaHCO_3$, pH 9, 37 °C, 4 h; (iv) 10% Pd/C, MeOH, HCOOH; (v) 1 N NaOH, MeOH; (vi) Fmoc-Osu, Na_2CO_3,dioxane/H_2O, RT

Three-functional amino acids were protected as follows: N^{α}-Fmoc-Thr(tBu)-OH, N^{α}-Fmoc-Lys(Boc)-OH, N^{α}-Fmoc-D-Trp(Boc)-OH. The coupling reactions were performed using for amino acid/TBTU/HOBt/DIEA/resin a molar

ratio 3/3/3/9/1, or amino acid/DIC/HOBt/resin a molar ratio 3/3/3/1, respectively. The Fmoc-$^{\alpha}$NH-group was deprotected by treatment with 20% piperidine solution in dimethylformamide. Coupling and deprotection reactions were monitored by the standard Kaiser and chloranyl tests. Final cleavage of the synthesized peptide from the resin was done, using a mixture of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) and 2.5% water.

The peptide was obtained as a filtrate in TFA and precipitated with cold dry ether. The precipitate was filtered, dissolved in water and lyophilized to obtain the crude peptide.

The peptide purity was monitored by RP-HPLC and ESI-MS analysis on Shimadzu Nexera X2, Column: Agilent Infinitylab Poroshell 120 (100 × 4.6 mm), 2.7 um particle size, C 18 stationary phase, elution flow: 0.30 mL/min; temperature of column: 40 °C using a linear binary gradient of phase A: H_2O (10% AcCN; 0.1% HCOOH) and phase B: AcCN (5% H_2O; 0.1% HCOOH) as follows.

Time (min)	0.01	10.00	15.00	15.50	22.00
m.ph. A (%)	80	5	5	80	80
m.ph. B (%)	20	95	95	20	20

The optical rotation was measured at $c = 1$ in methanol. The analytical data for the synthesized peptides are summarized in Table 2.

Cytotoxic effect

The cytotoxicity was evaluated by colorimetric assay based on tetrazolium salt MTT. The yellow MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is metabolized in viable cells to insoluble purple formazan crystals, later dissolved in lysis solution. The assay was performed on MDA-MB-231 (breast cancer cell line), HepG2 (human hepatocellular cancer cell line) and MCF-10A (non-tumorigenic epithelial cell line). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Austria) supplemented with 10% fetal bovine serum (Gibco, Germany), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco, USA) under a humidified 5% CO_2 atmosphere at 37 °C. The complete medium for MCF-10 cells was additionally supplemented with insulin, hydrocortisone, and EGF (epidermal growth factor) according to the protocol recommended by Sigma-Aldrich.

For experiments, cells in exponential phase of growth were seeded into 96-well flat-bottom plates after treatment with Trypsin–EDTA, at a density of 2×10^4 cells/well, at final volume 100 µl/well. Cells were incubated overnight and treated with the compounds of investigation and further incubated for 24 h. The peptides were all dissolved in DMSO and tested in a wide concentration range (400–40–4–0.4–0.04 µM). The final concentration of DMSO did not interfere with the cells' viability. After 24 h incubation, MTT solution was added and cells were incubated for 3.5 h. Later, the medium was removed and 100 µl solution DMSO/ethanol (v/v) was added to each well. The plates were placed in a plate shaker at room temperature till complete dissolving of purple formazan.

ELISA plate reader (Bio-Tek) was used for reading the results. Optical density (OD) was determined at a wavelength of 550 nm and a reference wavelength of 630 nm. Cell viability determined by MTT assay was calculated as:

$$\% \text{ viability} = ((\text{OD sample} - \text{OD blank control})/(\text{OD control} - \text{OD blank control})) \times 100.$$

Cytotoxicity was expressed as IC_{50} values (the concentration that causes 50% cell death), calculated using non-linear regression analysis (GraphPad Prizm 6 Software). The statistical analysis included application of one-way ANOVA, followed by Bonferroni's post hoc test. $p < 0.05$ was accepted as the lowest level of statistical significance. All results are presented as mean ± SD.

Docking studies

Docking of ligands

Somatostatin receptors were modeled as previously described (Naydenova et al. 2019). 3D structures of the ligands were modeled in Avogadro (https://www.chemcomp.com). Ligands were protonated at physiological pH 7.4.

Docking was carried out with GOLD 5.2 software. It uses generic algorithm and considers full ligand conformational flexibility and partial protein flexibility. The binding site for sstrs, in literature (Møller et al. 2003), was defined as residues Ser-Gln-Leu-Ser (305–308) for sstr1, Phe-Asp-Phe-Val (294–297) for sstr2, Tyr-Phe-Leu-Val (295–297) for sstr3, Asn-His-Val-Ser (293–296) for sstr4, and Tyr-Phe-Phe-Val (286–289) for sstr5. For the docking, we use the first residues from the sequences and the space within 10 Å radius of them. ChemScore scoring function of GOLD was used. The conformations of the ligands with the best scoring functions were selected and parameters of the scoring function were used to find correlations between them and the obtained in vitro results. Molegro Molecular Viewer (https://molegro.com/index.php) was used for generating figures.

To find the relationship between sets of data derived from in vitro assay and docking results, we tried to predict it with the help of Pearson's correlation, using GraphPad Prism 3.0.

Hydrolytic stability

Selected pH values mimic human pH in the stomach, blood plasma and small intestine. Buffers used for determination of hydrolytic stability of the target compounds are prepared according to the European Pharmacopoeia, 6th Edition, as follows:

(i) Buffer with pH 2.0—6.57 g KCl is dissolved in water (CO_2 free) and 119.0 mL 0.1 mol/L HCl is added. The obtained solution is completed to 1000.0 mL with dH_2O.
(ii) Buffer with pH 7.4—2.38 g Na_2HPO_4, 0.19 g KH_2PO_4 and 8.0 g NaCl are dissolved in dH_2O. The obtained solution is completed to 1000.0 mL with dH_2O.
(iii) Buffer with pH 9.0—1000.0 mL of solution I is mixed with 420.0 mL of solution II. Solution I: 6.18 g H_3BO_3 is dissolved in 0.1 mol/L KCl and it is completed to 1000.0 mL with the same solvent; Solution II: 0.1 mol/L NaOH.

An HPLC gradient method was developed as follows: HPLC Shimadzu LC20AD chromatographic system equipped with PDA (photodiode array detectors) detector, column: Infinity Lab Poroshell 120 EC-C18 150 × 4.6 mm, 4 µm; mobile phase: A: water:AcCN:TFA = 90:10:0.1, and B: water:AcCN:TFA = 5:95:0.1; elution rate 0.7 mL/min, column temperature 40 °C; scanning wave length 280 nm, internal standard Trp is added for quantification; injected volume: 20 µL. The elution was performed in gradient mode as follows.

Time (min)	0	10	10.1	16.5
m.ph. A (%)	80	5	80	80
m.ph. B (%)	20	95	20	20

Results and discussion

To optimize the physicochemical properties (stability) and to enhance the anti-tumor potential of the parent BIM-23052 molecule D-Phe^1-Phe^2-Phe^3-D-Trp^4-Lys^5-Thr^6-Phe^7-Thr^8-NH_2, a series of halogenated analogs were synthesized. To study the structure–activity relationships, Phe at position 2 and 3 was successively or simultaneously replaced with Phe, and halogenated at position 4 in the aromatic ring. The influence of the type of halogen atom in the aromatic ring of Phe was determined using Phe (4-Cl) and Phe (4-F). Also, the D-Phe at position 1 was replaced with Phe (2-F). All analogs were synthesized as C-terminal amides to increase plasma half-life (Table 1).

We synthesized the SST analog BIM-23052 (compound S1) as a standard to compare its in vitro cytotoxic activity toward the selected cell lines to the other compounds.

Halogenated amino acids Fmoc-Phe(4-Cl)–OH, Fmoc-Phe(4-F)–OH, and Fmoc-D-Phe(2-F)–OH were synthesized using self-developed laboratory procedure illustrated in Scheme 1. The key intermediate **5b** was obtained according to the procedures previously described by Pajpanova (2000).

The peptides were synthesized by standard solid-phase peptide chemistry methods—Fmoc (fluorenylmethoxycarbonyl)-strategy, using TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) or DIC (*N*, *N*′-diisopropylcarbodiimide) an efficient peptide coupling reagent. The Fmoc-Rink-amide MBHA resin was used as solid-phase carrier to obtain the C-terminal amide derivatives. The cleavage of the synthesized peptides from the resin was done, using TFA. The yields of each peptide were over 90%. HPLC analysis revealed a purity of > 95% for all obtained analogs. The compounds were checked by LC–electrospray ionization mass spectrometry and the optical rotation was measured in methanol. The data are summarized in Table 2.

Cytotoxic effect

All newly synthesized compounds were tested against two tumor cell lines of human origin (MDA-MB-231 and Hep-G2) and one human non-tumorigenic epithelial mammary

Table 1 Structure of the synthesized analogs

No.	Code	Amino acid sequence
1	S1	D-Phe^1-Phe^2-Phe^3-D-Trp^4-Lys^5-Thr^6-Phe^7-Thr^8-NH_2 **(BIM-23052)**
2	S2	D-Phe^1-**Phe(4-Cl)**^{2}-Phe^3-D-Trp^4-Lys^5-Thr^6-Phe^7-Thr^8-NH_2
3	S3	D-Phe^1-**Phe(4-Cl)**^{2}-**Phe(4-Cl)**^{3}-D-Trp^4-Lys^5-Thr^6-Phe^7-Thr^8-NH_2
4	D1	D-Phe^1-**Phe(4-F)**^{2}-Phe^3-D-Trp^4-Lys^5-Thr^6-Phe^7-Thr^8-NH_2
5	D2	D-Phe^1-**Phe(4-F)**^{2}-**Phe(4-F)**^{3}-D-Trp^4-Lys^5-Thr^6-Phe^7-Thr^8-NH_2
6	D5	**D-Phe(2-F)**^{1}-Phe^2-Phe^3-D-Trp^4-Lys^5-Thr^6-Phe^7-Thr^8-NH_2

Table 2 Characteristics of the synthesized analogs

Code	General formula	Mw_{exact}	$[MH]^+$	t_R (min)	M.p (°C)	α_{546}^{20} [a] (°)	Chromatographic purity (%)
S1	$C_{61}H_{75}N_{11}O_{10}$	1121.57	1122.57	6.02	132–135	− 20.00	88
S2	$C_{61}H_{74}ClN_{11}O_{10}$	1155.53	1156.76	7.58	140–143	− 26.00	95
S3	$C_{61}H_{73}Cl_2N_{11}O_{10}$	1189.49	1191.65[c]	7.27; 7.97 and 8.42	119–123	− 14.00	96[d]
D1	$C_{61}H_{74}FN_{11}O_{10}$	1139.56	1140.55	6.07	118–120	− 26.00	87
D2	$C_{61}H_{73}F_2N_{11}O_{10}$	1157.55	1158.55[c]	5.87; 6.11 and 6.3[b]	117–119	− 26.00	96[c]
D5	$C_{61}H_{74}FN_{11}O_{10}$	1139.56	1140.55	6.97	117–120	− 14.00	92

[a]Optical rotation in methanol ($c = 1$) at 20 °C

[b]All three peaks have the same molecular mass and are isomers of the same compound

[c]$[MH]^+$ value is for the main peak

[d]Chromatographic purity is calculated as a sum for all three peaks

cell line MCF-10A by using the standard MTT test. After incubation of the examined tumor cells with the new compounds for 24 h, the percent of vitality was calculated relative to untreated controls (100% viability). All compounds exerted concentration-dependent cytotoxic effects after 24 h exposure, which enabled the construction of concentration–response curves (Fig. 2).

Their cytotoxic effect was expressed in μM concentration. The data are summarized in Table 3.

Our previous investigations on the octreotide analogs including unnatural amino acids Dap (diaminopropanoic acid), Dab (diaminobutanoic acid) and Orn (Staykova et al. 2012a) on the same cell line showed that compound (D-Phe-c(Cys-Phe-D-Trp-Dap-Tle-Cys)-Thr-NH_2 has IC_{50} at 0.03 mM concentration against MDA-MB-231 cells. In addition, better activity even in nM range of IC_{50} shows analogs of BIM-23052 with sterically restricted amino acids Aib (α-aminoisobutyric acid), Ac5c (1-aminocyclopentanecarboxylic acid), Ac6c (1-aminocyclohexanecarboxylic acid) and Tle (tert-leucin) (Naydenova et al. 2019). Herein, the obtained results for IC_{50} values reveal lower activity but it is combined with better stability for newly synthesized analogs of BIM-23052.

Docking

The results from docking were analysed in Molegro Molecular Viewer, where the total energies of the obtained ligand–receptor complexes were calculated (Table 4).

In previous our papers, we reported the synthesis of modified analogs of BIM-23052 containing unnatural hindered amino acids (α-aminoisobutyric acid, 1-aminocyclopentanecarboxylic acid and 1-aminocyclohexanecarboxylic acid) to elucidate the influence of the modification to the in vitro cytotoxic activity (Naydenova et al. 2019). To continue our investigation on the structure–activity relationship of analogs of BIM-23052, herein we report the synthesis and evaluation on peptide analogs containing halogenated amino acids, especially differently fluorinated as well as chlorinated phenylalanines. It is important to mention that reactions of condensation of halogenated amino acids into the amino acid sequence required several repetitions of the procedure, as well as with changing of activation agent HBTU with DIC.

The activity of newly synthesized compounds was evaluated against two tumor cell lines (MDA-MB-231 and HepG2), and normal human cell line MCF-10A. The obtained results are collected after 24 h of incubation. The revealed values for IC_{50} for compounds **S2** and **S3** (fluorinated analogues) as well as **D1** and **D2** (chlorinated analogues) show that accumulation of more than one halogenated Phe residue does not lead to increase of cytotoxic activity (Table 3). Monohalogenated Phe(4-F or Cl) analogs have better activity against HepG-2 cell line than double substituted analogs. In addition, IC_{50} value for analog containing Phe(2-F) **D5** shows better selectivity against HepG-2 tumor cell line. According to MDA-MB-231 cell line, chlorinated analogs do not show activity in the tested concentration range, but they show good activity against HepG-2 cells. The fluorinated analogs show also good activity, better against HepG-2 tumor cell line. The obtained IC_{50} values reveal lower effect of newly synthesized compound against non-tumorigenic epithelial cell line (MCF 10). Selectivity index (ratio between IC_{50} of MCF-10/IC_{50} of tumor cell line) was observed at MDA-MB-231 treated with compounds S1 and D2. The calculated selectivity index (SI) for S1 and D2 were, respectively, 1.75 and 1.66. In addition, S1 was tested at MCF-7 breast cancer (luminal adenocarcinoma type A). The calculated IC_{50} was 120 ± 6.5 μM and SI = 4.9. The big difference between SI and S1 for MDA-MB-231 and MCF-7 shows a different mechanism of antitumor activity for examined breast tumor cell lines.

All peptides tested have high antitumor activity against the HepG2 cell line ($IC_{50} \approx 100$ μM and SI > 5) compared to breast cells. This is probably due to the high permeability

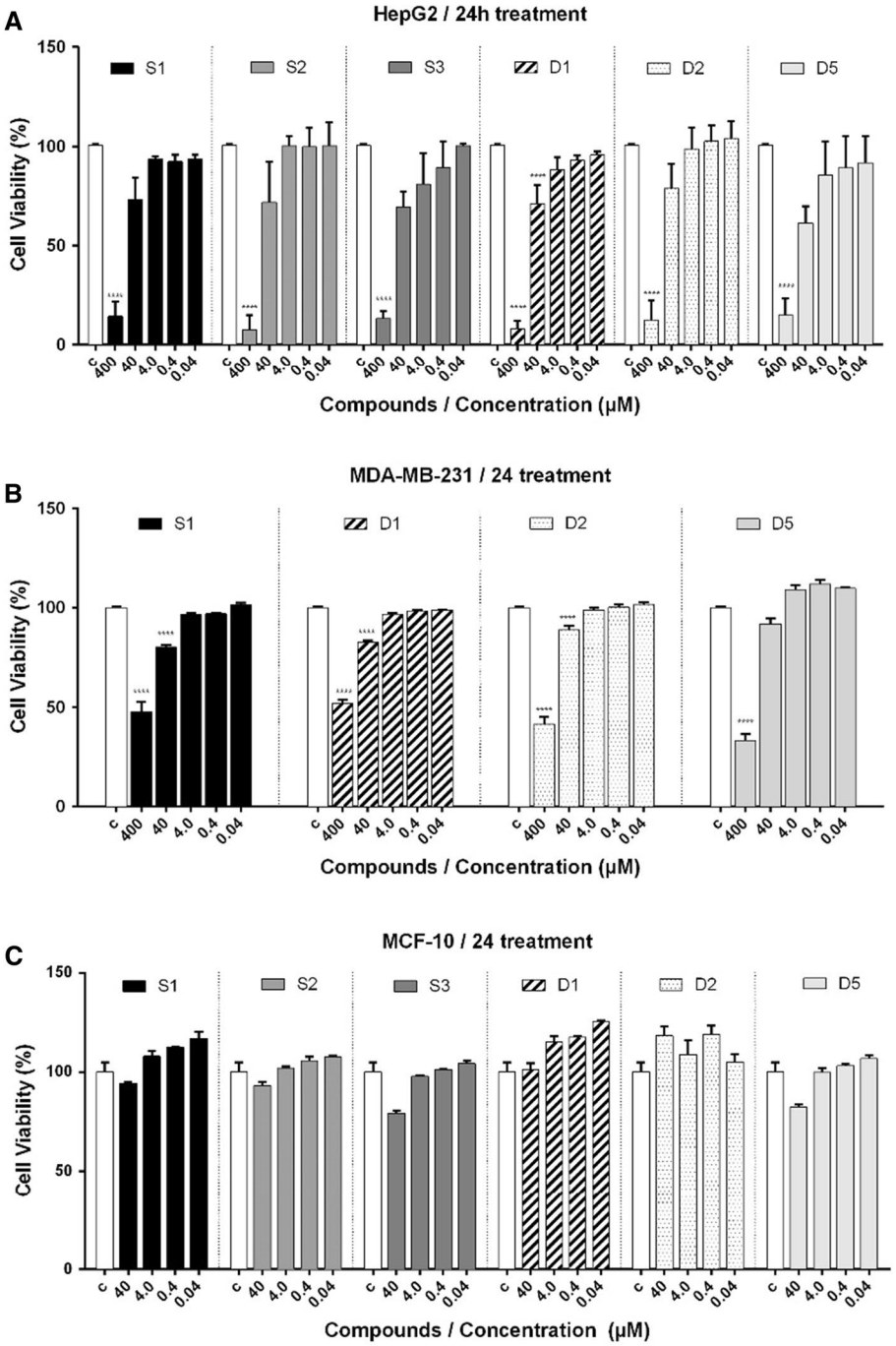


Fig.2 Comparative graph of cytotoxic effects of the tested compounds after 24 h on: **a** HepG2 cells; **b** MDA-MB-231; **c** MCF-10

of the cell membrane and the higher metabolic activity of hepatocytes.

With an attempt to explain the results of biological activity of the analogs of BIM, we performed docking with previously obtained structures of all five somatostatin receptors. A review study of Møller et al. (2003) has shown that residues Ser-Gln-Leu-Ser (305–308) for sstr1, Phe-Asp-Phe-Val (294–297) for sstr2, Tyr-Phe-Leu-Val (295–297) for sstr3, Asn-His-Val-Ser (293–296) for sstr4, and Tyr-Phe-Phe-Val (286–289) for sstr5 are important residues for ligand recognition. The binding site was defined as residues within 10 Å radius of the first residue in those sequences. Docking was performed with five chosen models and six ligands.

Total energies show how strong the ligand binds the receptor. We assume that they represent the affinity of the compound to the respective receptor type. The results of the docking show that the tested analogs bind at different degrees to different receptor types (one-way ANOVA, Kruskal–Wallis test, p value 0.0003).

Table 3 IC_{50} values of newly synthesized compounds after 24 h treatment of cells

Code	IC_{50} of Mean ± SD (μM)		
	MDA-MB-231	HepG2	MCF-10A
S1	334.8 ± 15.4	105.6 ± 1.48	> 400
S2	–	79.18 ± 1.56	> 400
S3	–	232 ± 2.91	> 400
D1	356.7 ± 1.11	78.14 ± 1.41	334.3 ± 1.9
D2	277.4 ± 1.06	106 ± 1.34	> 400
D5	> 400	85.48 ± 2.00	320.2 ± 15.35

The majority of tumors express sstr 2, followed by sstr 1, sstr 5 and sstr 3, while sstr 4 is expressed in minority of tumors (Susini and Buscail 2006; Reubi et al. 2001; Weckbecker et al. 2003). This determines the need of highly selective SST analogs to SST receptors. The data in Table 4 show that all analogs bind most strongly to ssrt3, followed by ssrt5 and ssrt4 and they bound weakly to ssrt1 and ssrt2. The ligands form a larger number of hydrogen bonds and interact (electrostatic, hydrophobic) with ssrt3 and sstr5 than with ssrt1 (Fig. 3). This is due to the fact that in the first case, the binding is between the alpha helices and a greater number of amino acid residues are involved, while in the second case it is on the surface of the receptor.

A correlation was found between the biological effect of the analogs with respect to HepG2 and the total energy

Table 4 Total energies of the complexes between ligands and five models of sstrs

Code	Total energy of the complex with sstr1, kJ	Total energy of the complex with sstr2, kJ	Total energy of the complex with sstr3, kJ	Total energy of the complex with sstr4, kJ	Total energy of the complex with sstr5, kJ
S1	– 175.289	– 159.547	– 322.739	– 234	– 283.73
S2	– 165.076	– 169.339	– 313.005	– 150.473	– 178.57
S3	– 260.445	– 179.97	– 317.304	– 209.099	– 232.318
D1	– 288.472	– 150.622	– 293.568	– 214.973	– 263.308
D2	– 183.272	– 250.963	– 364.883	– 205.41	– 259.6
D5	– 141.026	– 135.881	– 337.411	– 249.643	– 215.059

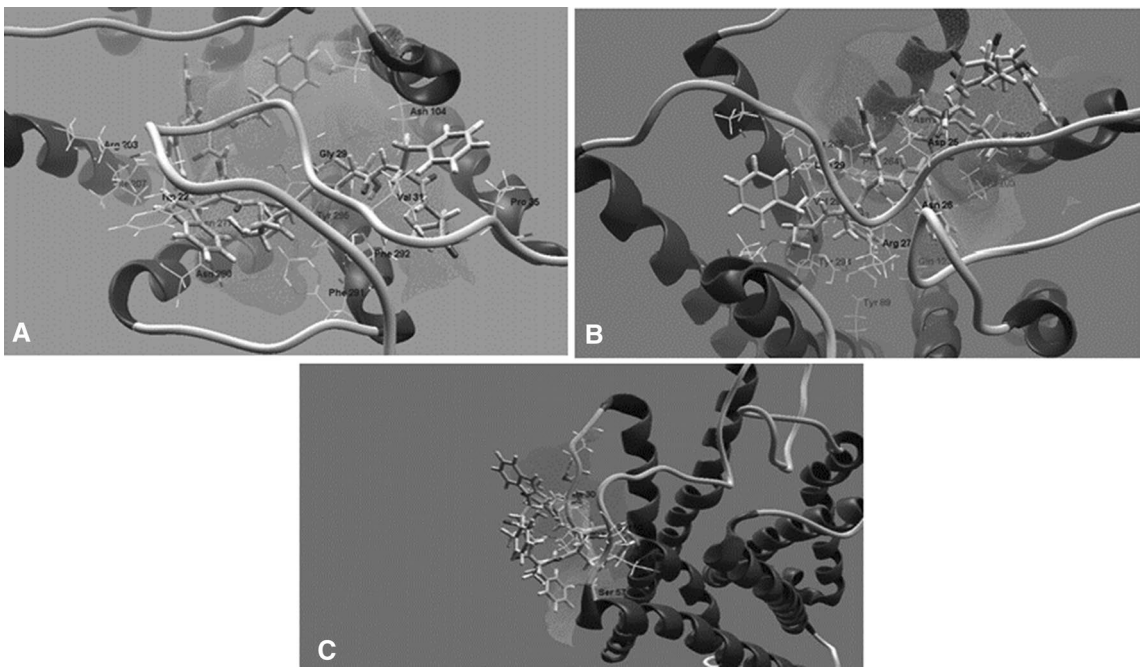


Fig. 3 **a** Binding of D1 with sstr3—binding is between the alpha helices and a greater number of amino acid residues are involved. **b** Binding of D2 with sstr5—binding is between the alpha helices and a greater number of amino acid residues are involved. **c** Binding of D5 with sstr1—binding on the surface of the receptor (picture was generated by Molegro)

values of the complexes with sstr5 (Pearson r 0.85, p value 0.01). Positive dependence has a biological meaning, and it shows that as a more stable complex is formed with the receptor, the effect is stronger. Another implication of this result is that in the cell tested line, the biological effect is likely to be due to the action of the analogs on sstr5, i.e. probably the highest percentage of sstr5 is expressed there.

Finally, the hydrolytic stability of newly synthesized BIM analogs was investigated.

The hydrolytic stability of peptides is one of the most important properties in terms of their application in practice. Pre-information on stability is essential for the pharmacokinetic behavior in the body, storage conditions, the occurrence of toxic effects associated with their degradation products and others.

The work solutions are prepared with a concentration of 0.04 mg/mL in the corresponding pH buffer. Samples are incubated at 37 °C for 96 h. Samples are injected every 1 h in the chromatographic system with addition of internal standard Trp. Detection is done at 280 nm by monitoring the absorbance of the indole cycle of Trp residue. The obtained results show that all compounds are stable at 96 h at pH 2.0, except compound S3 (containing two chlorinated Phe), where minor hydrolysis till 93.55% is monitored. At pH 7.0, all compounds show good hydrolytic stability for 96 h, except both chlorinated analogs (compounds S2 and S3), where 85.11% and 69.92%, respectively, of starting product is monitored for 8 h. All compounds are hydrolyzable at pH 9.0 with different rates of hydrolysis except compound D5 (containing 2-F-D-Phe) which is stable till 96 h (Table 5).

The pharmacokinetic investigation on octreotide reveals that the elimination half-life is 100 min when the compound is applied subcutaneously; after intravenous injection, the substance is eliminated in two phases with half-lives of 10 and 90 min, respectively (Dinnendahl et al. 2010; Haberfeld 2009). Our obtained data for hydrolytic stability show the following structure–stability relationships:

- All newly synthesized halogenated analogs are stable at pH 2.0. Introduction of second Phe(4-Cl) residue leads to decrease in hydrolytic stability and the appearance of hydrolysis at the 96th hour.
- The analog containing Phe(2-F) residue with good selectivity according tumor cell line of breast cancer (MDA-MB-231) shows good hydrolytic stability at all pH values during 96 h.
- Introducing of one Phe(4-Cl) residue does not slow down the hydrolysis process at neutral and alkaline pH, even in neutral pH it is faster than with starting compound BIM-23052.
- Introducing of one Phe(4-F) residue (the most active analog) slows down the hydrolysis at alkaline pH, but the incorporation of a second Phe(4-F) residue leads to acceleration of the hydrolysis process.
- All synthesized analogs are quite stable in acid and neutral pH and they can be hydrolyzed in the organism in the small intestine.

Table 5 Hydrolytic stability of newly synthesized peptides at pH 9.0

Code	Hour	% Starting product
S1	24	19.43
S2	24	21.84
S3	24	9.89
D1	24	77.73
D2	8	56.40
D5	96	100.00

Conclusions

It was shown that changes in any single position of the peptide may affect other residues at all other positions in the parent peptide. Therefore, each single modified peptide variant may lead to different activity and stability.

Taking into account all obtained results, we can conclude that introduction of fluorinated Phe has good anti-proliferative effect against tested cancer cell lines (MDA-MB-231) and (HepG2) at μM concentration. Moreover it is higher than those of parent compound BIM-23052. In addition, these analogs affected weaker the viability of the non-tumor epithelial cell line (MCF-10A), which also shows certain selectivity toward the tumor cells.

The analog of BIM-23052 containing Phe(2-F) residue shows better selectivity according to HepG2 cell line. Chlorinated analogs are also selective for HepG2 and show good activity, but they do not show activity against breast cancer cell line. The results obtained for the cytotoxic effect of the newly synthesized compounds on these cell lines indicate that increasing number of halogenated residues in the peptide structure does not lead to better activity.

Compounds S1 and D2 showed closed SI according to tumor cell line MDA-MB-231, respectively, 1.75 and 1.66. In addition, parent compound S1 showed SI = 4.9 according to MCF-7 breast cancer which reveals a different mechanism of antitumor activity for the examined breast tumor cell lines.

All peptides tested have high antitumor activity against the HepG2 cell line ($IC_{50} \approx 100$ μM and $SI > 5$) compared to breast cells. This is probably due to the high permeability of the cell membrane and the higher metabolic activity of hepatocytes.

In silico calculations show that the best complex, newly synthesized molecules form with sstr3 and sstr5. Hydrolytic stability investigations demonstrate good stability at acid

and neutral pH for all newly synthesized BIM analogs. They are more sensitive to hydrolytic degradation in an alkaline environment, which showed a different hydrolysis rate.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with animals or humans performed by any of the authors.

Informed consent All authors are aware of the details of their research work that are published in the current paper and give their consent to their publication.

References

Aleksiev B, Shamlian P, Widenov G, Stoev S, Golovinsky E (1981) Verwendung von alkalischer Protease des Bacillus-subtilis-Stammes DY zur Hydrolyse von Aminosäure und Peptid-Esterm. Hoppe Seyler's Z Physiol Chem 362:1323–1329

Barrie R, Woltering EA, Hajarizadeh H, Mueller C, Ure T, Fletcher WS (1993) Inhibition of angiogenesis by somatostatin and somatostatin-like compounds is structurally dependent. J Surg Res 55:446–450

Borghouts C, Kunz C, Groner B (2005) Current strategies for the development of peptide-based anti-cancer therapeutics. J Pept Sci 11(11):713–726

Breder CD, Yamada Y, Yasuda K, Seino S, Saper CB, Bell GI (1992) Differential expression of somatostatin receptor subtypes in brain. J Neurosci 12:3920–3934. https://doi.org/10.1523/JNEUROSCI.12-10-03920.1992

Bruns C, Weckbecker G, Raulf F, Kaupmann K, Schoeffter P, Hoyer D, Lubbert H (1994) Molecular pharmacology of somatostatin-receptor subtypes. Ann NY Acad Sci 733:138–146

Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr D Biol Crystallogr 66:12–21. https://doi.org/10.1107/S0907444909042073

Coy DH, Murphy WA (1997) Linear somatostatin analogs. US Patent, Patent Number 5633263, 27 May 1997. https://www.lens.org/images/patent/US/5633263/A/US_5633263_A.pdf

Dinnendahl V, Fricke U (2010) Arzneistoff-Profile 8 (23), Govi Pharmazeutischer Verlag, Eschborn, Germany

Haberfeld H (2009) Austria-Codex 2009/2010 ed. Österreichischer Apothekerverlag, Vienna

Koidl J, Hödl H, Schmid MG, Pantcheva S, Pajpanova T, Gübitz G (2005) Chiral separation of halogenated amino acids by ligand-exchange capillary electrophoresis. Electrophoresis 26(20):3878–3883

Kučerová G, Vozka J, Kalíková K, Geryk R, Plecitá D, Pajpanova T, Tesařová E (2013) Enantioselective separation of unusual amino acids by high performance liquid chromatography. Sep Purif Technol 119:123–128

Laskowski RA, MacArthur MW, Moss D, Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst 26:283–291

Meyerhof W (1998) The elucidation of somatostatin receptor functions: a current view. Rev Physiol Biochem Pharmacol 133:55–105

Molecular Operating Environment (MOE), 2012.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2012. Available at https://www.chemcomp.com

Møller LN, Stidsen CE, Hartmann B, Holst JJ (2003) Somatostatin receptors. Biochim Biophys Acta 1616:1–84

Naydenova ED, Wesselinova DW, Staykova STS, Goshev IG, Vezenkov LT (2018) Synthesis, cytotoxicity and antioxidant activity of new analogs of RC-121 syntheticderivatives of somatostatin. Anti-Cancer Agents Med Chem 18:1417–1424. https://doi.org/10.2174/1871520618666180417164344

Naydenova ED, Wesselinova DW, Staykova STs, Danalev DL, Dzimbova TA, (2019) Synthesis, in vitro biological activity and docking of new analogs of BIM-23052 containing unnatural amino acids. Amino Acids 51(9):1247–1257

Nilsson S, Reubi JC, Kalkner K-M, Laissue JA, Horisberger U, Olerud C, Westlin J-E (1995) Metastatic hormone-refractory prostatic adenocarcinoma expresses somatostatin receptors and is visualized in vivo by [111In]-labeled DTPA-D-[Phe1]-octreotide scintigraphy. Cancer Res 55:5805–5810

O'Carroll AM, Raynor K, Lolait SJ, Reisine T (1994) Characterization of cloned human somatostatin receptor SSTR5. Mol Pharmacol 46(2):291–298

Pająk M, Rałka K, Winnicka E, Kańska M (2016) Synthesis of halogen derivatives of L-tryptophan, L-tyrosine anad L-phenylalanine labeled with hydrogen isotopes. J Label Compd Radiopharm 59:4–8

Pajpanova T (2000) A simple and convenient procedure for the preparation of 3,5-halogenated tyrosine derivatives useful in peptide synthesis. C R Acad Bulg Sci 53(8):53–56

Patel YC (1997) Molecular pharmacology of somatostatin receptor subtypes. J Endocrinol Invest 20:348–367. https://doi.org/10.1007/BF03350317

Patel YC (1999) Somatostatin and its receptor family. Front Neuroendocrinol 20:157–198. https://doi.org/10.1006/frne.1999.0183

Patel YC, Greenwood MT, Panetta R, Demchyshyn L, Niznik H, Srikant CB (1995) The somatostatin receptor family. Life Sci 57:1249–1265

Paz-Bouza JI, Redding TW, Schally AV (1987) Treatment of nitrosamine-induced pancreatic tumors in hamsters with analogs of somatostatin and luteinizing hormone-releasing hormone. Proc Natl Acad Sci USA 84(4):1112–1116

Pollak MN, Shally AV (1998) Mechanisms of antineoplastic action of somatostatin analogs. Proc Soc Exp Biol Med 217:143–152

Pyronnet S, Bousquet C, Najib S, Azar R, Laklai H, Susini C (2008) Antitumor effect of somatostatin. Mol Cell Endocrinol 286:230–237. https://doi.org/10.1016/j.mce.2008.02.002

Reisine T, Bell GI (1995) Molecular biology of somatostatin receptors. Endocr Rev 16:427–442. https://doi.org/10.1210/edrv-16-4-427

Reubi J-C, Waser B, Schaer JC, Markwalder R (1995) Somatostatin receptors in human prostate and prostate cancer. J Clin Endocrinol Metab 80:2806–2814. https://doi.org/10.1210/jcem.80.9.7673428

Reubi JC, Waster B, Schaer JC, Laissue JA (2001) Somatostatin receptor sst1–sst5 expression in normal and neoplastic human tissues using receptor autoradiography with subtype-selective ligands. Eur J Nucl Med 28:836–846

Rose DP, Gottardis M, Noonan JJ (1983) Rat mammary carcinoma regressions during suppression of serum growth hormone and prolactin. Anticancer Res 3:323–325

Sali A, Blundell TL (1993) Comparative protein modeling by satisfaction of spatial restraints. J Mol Biol 234:779–815

Saltz L, Trochanowski B, Buckley M, Heffernan B, Niedzwiecki D, Tao Y, Kelsen D (1993) Octreotide as an antineoplastic agent in the treatment of functional and nonfunctional neuroendocrine tumors. Cancer 72(1):244–248

Schally AV, Redding TW (1987) Somatostatin analogs as adjuncts to agonists of luteinizing hormone-releasing hormone in the treatment of experimental prostate cancer. Proc Natl Acad Sci USA 84:7275–7279

Schally AV, Comaru-Schally AM, Redding TW (1984) Antitumor effects of analogs of hypothalamic hormones in endocrine-dependent cancers. Proc Soc Exp Biol Med 175:259–281

Schally AV, Redding TW, Comaru-Schally AM (1984) Potential use of analogs of luteinizing hormone-releasing hormones in the treatment of hormone-sensitive neoplasms. Cancer Treat Rep 68:281–289

Shimon I (2003) Somatostatin receptors in pituitary and development of somatostatin receptor subtype-selective analogs. Endocrine 20:265–269. https://doi.org/10.1385/ENDO:20:3:265

Staykova STS, Mihaylova BD, Goshev IG, Wesselinova DW, Vezenkov LT, Naydenova ED (2012a) Antioxidant capacity of new analogs of Octreotide. Bulg Chem Commun 44:233–237

Staykova S, Naydenova E, Wesselinova D, Kalistratova A, Vezenkov L (2012b) Synthesis and in vitro study of the anticancer activity of new analogs of Octreotide. Prot Pept Lett 19:1257–1262. https://doi.org/10.2174/092986612803521611

Staykova S, Naydenova E, Wesselinova D, Vezenkov L (2012c) Synthesis and in vitro antitumor activity of new linear somatostatin analogs. JUCTM 47:297–302

Staykova STS, Wesselinova DW, Vezenkov LT, Naydenova ED (2015) Synthesis and in vitro antitumor activity of new octapeptide analogs of somatostatin containing unnatural amino acids. Amino Acids 47:1007–1013. https://doi.org/10.1007/s00726-015-1929-x

Strowski MZ, Blake AD (2008) Function and expression of somatostatin receptors of the endocrine pancreas. Mol Cell Endocrinol 286(1–2):169–179

Susini C, Buscail L (2006) Rationale for the use of somatostatin analogs as antitumor agents. Ann Onc 17:1733–1742. https://doi.org/10.1093/annonc/mdl105

Thayer M (2011) Improving peptides. Chem Eng News 89:13–20

Torre N, Wass J, Turner H (2002) Antiangiogenic effects of somatostatin analogs. Clin Endocrinol 57(4):425–441

Weckbecker G, Lewis I, Albert R, Schmid HA, Hoyer D, Bruns C (2003) Opportunities in somatostatin research: biological, chemical and therapeutic aspects. Nat Rev Drug Discov 2:999–1017. https://doi.org/10.1038/nrd1255

Zalatnai A, Paz-Bouza JI, Redding TW, Schally AV (1988) Histologic changes in the rat prostate cancer model after treatment with somatostatin analogs and D-Trp-6-LH-RH. Prostate (NY) 12:85–98