Journal of the University of Chemical Technology and Metallurgy, 47, 3, 2012, 297-302

SYNTHESIS AND *IN VITRO* ANTITUMOR ACTIVITY OF NEW LINEAR SOMATOSTATIN ANALOGS

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Received 29 May 2012
Accepted 25 June 2012

ABSTRACT

Some linear analogs of somatostatin were synthesized and tested for in vitro *cytotoxic activity against a panel of human tumor cell lines: HT-29 (human colorectal cancer cell line), MDA-MB-23 (human breast cancer cell line), HepG2 (human hepatocellular carcinoma cell line) and HeLa (cervical cancer cell line). The new compounds are analogues of BIM-23052 (DC-23-99 D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2) containing fragment responsible for the activity and sequence composed of hydrophobic amino acids that support easier penetration of the cell membrane. The compounds exhibited moderated antiproliferative effects against the human tumor cell lines after 24 h treatment. The most active compounds are* ***2L*** *(Pro-Phe-D-Val-Tyr-Leu-Ile-Trp-Lys-Tle-Thr-NH_2) and* ***3L*** *(where D-Val was replaced by Val and Trp by D-Trp) against HeLa and HepG-2 cells at greatest concentrations where the vitality was about 80 %.*

Keywords: somatostatin analogs, unnatural amino acids, cytotoxic, SPPS.

INTRODUCTION

Somatostatin (SST, SRIF, somatotropin release–inhibiting factor) is an inhibitory tetradecapeptide hormone, which plays an important regulatory role in several cell functions, including inhibition of endocrine secretion and cell proliferation and functions as a neuromodulator in the central nervous system (CNS) [1, 2]. Most of the effects of SST and of its currently available analogs are mediated via five different G protein-coupled receptor, codenamed SSTR1–5 (somatostatin receptors subtype 1-5) [3, 4]. The different SSTR subtypes are expressed in different tissues and in some tumor cells [5]. Based on the findings that various tumor cells express SSTR2 and/or SSTR5 [6-10] peptide analogs with high affinity to these receptors were designed for targeting to such tumors and their metastases [11].

Native somatostatin has a very short or transient effect *in vivo* as it is rapidly inactivated by endo- and exo-peptidases [12, 13]. The introduction of D-residues like D-Trp increased the plasma half-life and biological activity of the SST analogs. The key residues of native SST essential for binding and biologic activity were identified. These residues include Phe^6-Phe^7-Trp^8-Lys^9-Thr^{10}-Phe^{11} (SST-14: H-Ala-Gly-c(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys)-OH). The linear and cyclic SSTR2- and SSTR5-selective octapeptide analogs contained the smaller pharmacophore Phe/Tyr-D-Trp-Lys-Thr/Val/ necessary for biologic activity and were modified at the N-terminus to increase their length of action [14]. Extensive NMR and molecular dynamics studies demonstrated that a β-turn structure about Trp-Lys (or Orn) assure preferred stable conformation of the octapeptide analogs [15, 16]. The activity of previously

described analogs of SST is dependent on the presence of a disulfide bridge between cysteine residues located at or near the ends of the peptide. The disulfide linkage results in a cyclic conformation necessary for activity. Some authors were synthesized linear SSTAs, like BIM-23052 (DC-23-99 D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2) and BIM-23053 (DC-25-4 D-Phe-Ala-Tyr-D-Trp-Lys-Val-Ala-β-D-Nal-NH_2) with high binding affinity to SSTR5 and low affinity to SSTR2 in pancreas, SCLC (small cell lung carcinoma) and brain cells and with GH inhibitor activity in cultured rat pituitary cells. The linear octapeptides utilize non-covalent interactions between the side chains of critically positioned constituent amino acid residues to confer a hairpin or quasi-cyclic conformation on the peptides [17, 18].

Dasgupta at al. synthesized lipophilized derivatives of the SST analog RC-160 with long chain acids [19]. These analogues show higher receptor affinity, GH-inhibitor and antiproliferative activity. These effects vary as a function of the hydrophobicity of the peptides [20]. Hence, optimizing the peptide hydrophobicity should be a key consideration in the rational design and synthesis of potent and selective lipopeptides [19]. Recently Futaki and other authors reported a small hydrophobic sequence PFVYLI that successfully translocated cargos across the cell membrane [21, 22].

The aim of the present study was the synthesis and the biological screening of new linear somatostatin analogs which contain hydrophobic amino acids that support easier penetration of the analogues through the cell membrane.

EXPERIMENTAL

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 bought from Merck (Germany). The LC/MC spectra were recorded on a LTQ XL Orbitrap Discovery instrument, Thermo Corporation, USA. The optical rotation was measured on automatic standard polarimeter Polamat A, Carl Zeis, Jena.

The conventional solid-phase peptide synthesis based on Fmoc (9-fluorenylmethyloxycarbonyl) chemistry was employed to synthesize a series of new analogues of SST. Rink-amide MBHA resin and TBTU (2-(1-O-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) were used as a solid-phase carrier and condensing reagent. Three-functional amino acids were embedded as 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. The Fmoc-group was deprotected by 20 % piperidine solution in dimetilformamide. The coupling and deprotection reactions were checked by the Kaiser test. The cleavage of the synthesized peptide from the resin was done, using a mixture of 95 % trifluoroacetic acid (TFA), 2.5 % triisopropylsilan (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 on a RP-HPLC XTera C_{18} 3.5 μm (125x2.1mm) (Waters Co.) column, flow 200 μl/min, using a linear binary gradient of phase B from 10 % to 90 % for 15 min (phase A: 0.1 % HCOOH/H_2O; phase B: 0.1 % HCOOH/AcCN). The compounds were checked by electrospray ionization mass spectrometry and the optical rotation was measured in water. The analytical data for the synthesized peptides are shown in Table 1.

Cytotoxic effect

Cytotoxicity of the substances was measured *in vitro*, using cultivated human tumor cell lines (American Type Culture Collection ATCC, Rockville, MD, USA). The cytotoxic activity of the tested somatostatin analogs (**1L-4L**) was evaluated by the MTS-dye reduction assay for cell viability against the Hep G-2 (human hepatocellular carcinoma cell line), MDA-MB-231 (human breast cancer cell line), HT-29 (human colorectal cancer cell line), HeLa (cervical cancer cell line). Cells were cultivated with different amounts of the substances at concentration from 4.10^{-3} - 4.10^{-8} M.

MTS test

The test (CellTiter 96 Non-Radioactive Cell proliferation assay, Promega Corporation USA) was performed according to protocol of "Promega" and the details are previously described [23 - 25]. The absorbance of each well at 490 nm was read by an automatic microplate reader (Absorbance Reader "Tecan"/Austria). Relative cell viability, expressed as a percentage of the untreated control (100 % viability), was calculated for each concentration. Concentration-response curves were constructed

manually for each experiment. All data points represent an average of three independent assays.

Statistical analysis

Statistical deviations were calculated automatically by Excel 2007 software program and the IC_{50}-by the “Origin 6.1” PC-program.

RESULTS AND DISCUSSION

The peptides shown in Table 1 were prepared with good yield by solid phase synthesis using TBTU, an efficient peptide coupling reagent. On the base of the sequence D-Trp-Lys-Thr necessary for the biological activity, the new analogs contain the fragments: D-Trp/Trp-Lys-Tle-Thr [25]. In order to stabilize the desired conformation Thr was replaced by the steric restricted amino acid Tle (t-leucine). To support easier penetration of the analogues through the cell membrane the new analogs include the sequence Pro-Phe/D-Phe-Val/D-Val-Tyr-Leu-Ile consisting of hydrophobic amino acids. This strategy was used in other examinations and gave hopeful results [21, 22, 26, 27]. To increase plasma half-life and biological activity of the new peptides they were synthesized as C-terminal amides and all of them included at least one D-amino acids.

The compounds were proved by electrospray ionization masspectrometry and the optical rotation was measured in water. The data are summarized in Table 1.

The investigated compounds **1L-4L** were evaluated for their antiproliferative effect against a panel of four tumor cell lines of human origin: HT-29, MDA-MB-231, HepG-2 and HeLa cell lines. The results are shown in Figs. 1-4. Relative cell viability, expressed as a percentage of the untreated control (100 % viability),

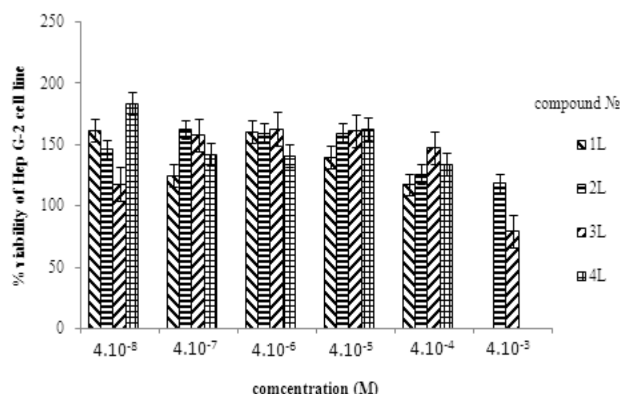

Fig. 1. Vitality of tested compounds **1L−4L** in a human liver carcinoma cell line HepG-2 after 24 h treatment.

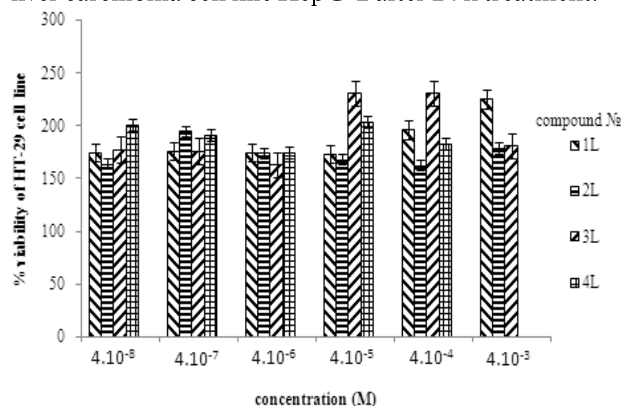

Fig. 2. Vitality of tested compounds **1L–4L** in a human colorectal carcinoma cell line HT-29 after 24 h treatment.

was calculated for each concentration. The treated with different substances tumor cells gave specific answers to this intervention.

The HepG-2 cells showed comparatively stable condition and their vitality did not change significantly after 24 h incubation with the compounds. The compound **3L** is cytotoxic only at higher tested concentration where the vitality was 79 % (Fig. 1).

Table 1. Structures and characteristics of the synthesized analogs:

№	STRUCTURE	GF	Exact Mass	$[MH]^+$ observed	RT [min]	$^{20}_{546}$ *
1L	Pro-D-Phe-Val-Tyr-Leu-Ile-Trp-Lys-Tle-Thr-NH_2	$C_{67}H_{99}N_{13}O_{12}$	1277.7536	1278.7612	18.80	-63.2
2L	Pro-Phe-D-Val-Tyr-Leu-Ile-Trp-Lys-Tle-Thr-NH_2	$C_{67}H_{99}N_{13}O_{12}$	1277.7536	1278.7613	18.22	-35.3
3L	Pro-Phe-Val-Tyr-Leu-Ile-D-Trp-Lys-Tle-Thr-NH_2	$C_{67}H_{99}N_{13}O_{12}$	1277.7536	1278.7617	14.05	-15.4
4L	Pro-D-Phe-Val-Tyr-Leu-Ile-D-Trp-Lys-Tle-Thr-NH_2	$C_{67}H_{99}N_{13}O_{12}$	1277.7536	1278.7609	14.87	-45.3

*Optical rotation in H_2O (c 0.25) at 20°C.

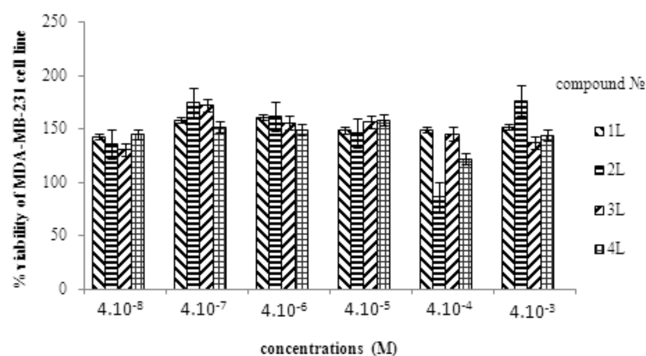

Fig. 3. Vitality of tested compounds **1L–4L** in a breast cancer cells MDA-MB-231 after 24 h treatment.

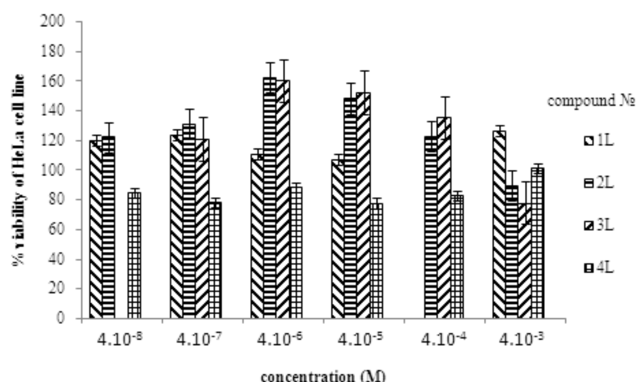

Fig. 4. Vitality of tested compounds **1–4** in a cervical cancer cell line HeLa after 24 h treatment.

The investigated substances did not influence the vitality of the HT 29 cells, even at the greatest concentrations the cultivated cells continue to proliferate and the vitality did not fall below 100 %. Substance **1L** acted quite stimulating on the cellular response (Fig. 2). This is evident from the calculated EC_{50} shown in Table 2. It could be explained with the type of cellular line and the lack of sensitivity to these compounds.

The MDA-MB-231 cells proliferate after incubation with the investigated compounds but not as much as the HT 29 cells (Fig. 3).

The HeLa cells were more sensitive than the other experimental cell lines which is in coincidence with our previously studies [25]. Compounds **3L** and **4L** did not allow the cells to proliferate sufficiently. The 4L did not allow them to reach the 100 % level even at the highest dilution. The new analog **3L** is cytotoxic at higher tested concentration where the vitality was 78 % (Fig. 4).

The *in vitro* investigation of cytotoxic activity demonstrated that the compound **2L** (Pro-Phe-D-Val-Tyr-Leu-Ile-Trp-Lys-Tle-Thr-NH_2) and **3L** (Pro-Phe-Val-Tyr-Leu-Ile-D-Trp-Lys-Tle-Thr-NH_2) slightly inhibit the growth of HeLa (cervical cancer cell line). Compound **3L** inhibit the growth of Hep G2 (human hepatocellular carcinoma cell line) cells at higher tested concentrations where the vitality was about 80 %.

The IC_{50} values for all compounds and cell lines are presented in Table **2**. It is important to mention that IC_{50} calculations of all substances revealed best inhibitory effect towards the HeLa and Hep G-2. Good result was obtained with compound **1** against Hep G-2 cells (IC_{50} 0.11 mM).

In comparison Kalfin and Alexandrova [28] showed that SST-14 did not affect significantly the viability of Hep G-2 cell line at concentrations 10^{-9} M to 10^{-5} M. The lipophilized analogs of the cyclic RC-160 have antiproliferative activity in pancreatic and prostate cell lines *in vitro* at concentration rang of 0.1-1.0 ng/ml [20].

We would suggest that the absence of disulfide bridge of the tested analogues leads to low antiproliferative effect (or no effect) or this could be due to different expressions of SSTRs (for example HT-29 cell line) (not examined in our investigation).

Table 2. *In vitro* cytotoxicity after 24 h treatment.

Comp.	MDA-MB-231		HT-29		HeLa		HepG-2	
	IC_{50} ± SE (mM)	EC_{50} ± SE (mM)	IC_{50} ± SE (mM)	EC_{50} ± SE (mM)	IC_{50} ± SE (mM)	EC_{50} ± SE (mM)	IC_{50} ± SE (mM)	EC_{50} ± SE (mM)
1L	-	5.10^{-5} ± 0.03	-	0.42 ± 0.11	-	3.07 ± 0.04	0.11 ± 0.12	-
2L	-	3.78 ± 0.17	-	nd	0.44±0.13		0.39 ± 0.13	-
3L	-	4.10^{-5}± 0.08	-	0.01 ± 0.13	0.77±0.19		0.89± 0.17	-
4L	-	nd	-	3.48 ± 0.07	-	0.7 ± 0.11	nd	-

CONCLUSIONS

Comparing the results between all cell kinds one can observe, that the compounds had moderated antiproliferative effects against the HeLa and Hep G-2 cell lines after 24 h treatment. This finding is important because each of these compounds may find its application on some tumor cells.

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