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ORIGINAL ARTICLE

Synthesis and in vitro antitumor activity of new octapeptide analogs of somatostatin containing unnatural amino acids

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Abstract Some modified octapeptide analogs of somatostatin with the following structure D-Phe-c(Cys-Phe-D-Trp-Xxx-Yyy-Cys)-Thr-NH_2, where Xxx is Lys or Orn and Yyy is Aib (α-aminoisobutyric acid), Ac5c (1-aminocyclopentanecarboxylic acid) or Ac6c (1-aminocyclohexane carboxylic acid) have been synthesized. The peptides were prepared by standard Fmoc—solid phase peptide chemistry method. The direct disulphide bond formation has been employed on the solid phase by $Tl(CF_3CO_2)_3$. The cytotoxic effects of the compounds were tested in vitro against a panel of tumor cell lines: HT-29 (human colorectal cancer cell line), MDA-MB-23 (human breast cancer cell line), Hep-G2 (human hepatocellular carcinoma cell line), HeLa (cervical cancer cell line) and normal human diploid cell line Lep-3. The new peptides exhibited different concentration-dependent antiproliferative effect against the tumor cell lines after 24 h treatment. The compounds were most effective to the HT-29 tumor cells. The compound **4C** (Orn^5, Aib^6) demonstrated the most pronounced antiproliferative effects on HT-29 cells with the $IC_{50} = 0.0199$ μM.

Keywords **S**omatostatin analog · SPPS · Unnatural amino acids · Cytotoxic

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Introduction

Somatostatin (SST-14 H_2N-Ala-Gly-c(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys)-COOH), is a hormonal cyclopeptide produced by neuroendocrine neurons of the hypothalamus, gastrointestinal, immune cells, as well as by certain tumors. It has a broad range of biological action that includes inhibition of growth hormone (GH), insulin, and glucagon and other hormones secretions, suppression of gastric and pancreatic exocrine secretion, gut motility, cell proliferation and also plays a role as a neurotransmitter (Pollak and Shally 1998; Barrie et al. 1993). Native somatostatin has a very short or transient effect in vivo as it is rapidly inactivated by endo- and exo-peptidases (Haberfeld 2009; Dinnendahl and Fricke 2010). A large number of shortened SST analogs have been synthesized to increase its plasma half-life and have been evaluated for their therapeutic potential.

The SST and its analogs action is mediated through a family of five G-protein-coupled receptors (sstr 1–5, somatostatin receptor subtype 1–5). The distribution of SST receptors ranges widely throughout not only the organs in human body, but also in various tumor cells and proliferating angiogenic vessels (Susini and Buscail 2006; Reubi et al. 2001; Weckbecker et al. 2003). The majority of tumors express sstr 2 followed by sstr 1, sstr 5 and sstr 3, while sstr 4 is expressed in minority tumors. This determines the need of highly selective SST analogs to

SST receptors. Analogs of somatostatin have already been used in clinical practice but many follow-up molecules are still in exploratory phase and under clinical development. Octreotide (Sandostatin, SMS 201-995, D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-ol) and Lanreotide (Somatuline, D-βNal-c(Cys-Tyr-D-Trp-Lys-Val-Cys)-Thr-NH_2) have been used in the management of acromegaly and symptoms caused by neuroendocrine tumors. Vapreotide (RC-160, Sanvar, D-Phe-c(Cys-Tyr-D-Trp-Lys-Val-Cys)-Trp-NH_2) have been used in the treatment of esophageal variceal bleeding in patients with cirrhotic liver disease and AIDS-related diarrhea (Pyronnet et al. 2008; Appetecchia and Baldelli 2010; Pollak 1997; Strosberg and Kvols 2010). In addition, the peptides BIM-23052 (DC-23-99, D-Phe-Phe–Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2), RC-102 (D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-NH_2) and RC-121 (D-Phe-c(Cys-Tyr-D-Trp-Lys-Val-Cys)-Thr-NH_2) have demonstrated antihormone activity (Coy and Murphy 1997; Shimon 2003). The analog RC-102 was known with GH and insulin-inhibitory activities (Cai et al. 1986).

Previous structure–activity studies of the shortened SST analogs establish that their activities are due to several factors. First one: the sequence required for biological activity consist the β-turn fragment Phe-Trp-Lys-Thr corresponding to the residues 7–10 of the somatostatin. The β-turn conformation is stabilized by intramolecular hydrogen bonds (Veber et al. 1979, 1981; Kazmierski et al. 1995; Prasad et al. 2006). The second one: D-Trp at position 4 is the most important for the activity and its substitution by L-Trp or other amino acid leads to full loss of the activity. The introduction of D-residues increases the plasma half-life and biological activity of the SST analogs (Cai et al. 1986). The presence of aromatic acids Tyr^3 and $D\text{-}Trp^4$ is playing a crucial role in antitumor activity. The Phe^3/Thr^6 and Tyr^3/Val^6 substituted octapeptide analogs have displayed significantly increased antitumor activity (Horvath et al. 1998). On the other hand the replacement of the C-terminal carboxyl group with amide group increases the activity and the N-terminal residues are not involved directly in the receptor recognition process (Murphy et al. 1985).

The α,α-dialkylated amino acids induce conformational constraints in the peptide backbone, thereby stabilizing a desired conformation. The prototypic member of α,α-dialkylated amino acids namely α-aminoisobutyric acid (Aib) has been shown to induce β-turn in small peptides and helical conformation in larger ones (Prasad et al. 2006; Karle and Balaram 1990). The conformational properties of the cyclic side-chain analogs of Aib such as 1-aminocyclopentanecarboxylic acid (Ac5c), 1-aminocyclohexane carboxylic acid (Ac6c) have also been shown to induce folded conformation (Prasad et al. 1995).

On the basis of these data the aim of the present study was the synthesis of new octapeptide analogs of Somatostatin, modified at position 6 by the unnatural amino acids and in vitro examination of their antiproliferative activity against a panel of human tumor cell lines. New modified analogs followed the structure D-Phe-c(Cys-Phe-D-Trp-Xxx-Yyy-Cys)-Thr-NH_2, where Xxx is Lys or Orn and Yyy is Aib (α-aminoisobutyric acid), Ac5c (1-aminocyclopentanecarboxylic acid) or Ac6c (1-aminocyclohexane carboxylic acid).

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 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-fluorenylmethoxycarbonyl) chemistry was employed to synthesize a series of new analogs of SST. Rink amide MBHA resin and TBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3- tetramethyluronium tetrafluoroborate) or DIC (N,N′-Diisopropylcarbodiimide) were used as solid-phase carrier and condensing reagent. Three-functional amino acids were embedded as N^{α}-Fmoc-Thr(tBu)-OH, N^{α}-Fmoc-Cys(Acm)-OH, N^{α}-Fmoc-Lys(Boc)-OH, N^{α}-Fmoc-Orn(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. The Fmoc-group was deprotected by a 20 % piperidine solution in dimethylformamide. The coupling and deprotection reactions were checked by the Kaiser test. For direct disulphide bond formation on the solid phase $Tl(CF_3CO_2)_3$ has been employed, using a mixture of 1.2 mmol $Tl(CF_3CO_2)_3$, 43 mmol anisole and 100 ml DMF at 0 °C for 2.5 h. The oxidized peptidyl-resin was dried. The 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 after evaporation of the TFA precipitated with cold dry ether. The precipitate was filtered, dissolved in water and lyophilized to obtain the crude peptide. The disulphide bridges were proved by reduction of the cyclic peptides with TCEP (tris(2-carboxyethyl)phosphine). The peptide purity was monitored on a RP-HPLC XTera C18 3.5 μm (125 × 2.1 mm) (Waters Co.) column, flow 200 μl/min, using a linear binary gradient of phase B from 10 to

Table 1 Structures and characteristics of the synthesized analogs

No.	Structure	MF	MM_{exact}	$[MH]^+$ observed	T_R min	$*\alpha_{546}^{20}$
1C	D-Phe-c(Cys-Phe-D-TrpL-ys-Aib-Cys)-Thr-NH_2	$C_{49}H_{65}N_{11}O_9S_2$	1,015.4408	1,016.4504	11.00	−45.16
2C	D-Phe-c(Cys-Phe-D-Trp-Lys-Ac5c-Cys)-Thr-NH_2	$C_{51}H_{67}N_{11}O_9S_2$	1,041.4565	1,042.4659	11.41	−36.84
3C	D-Phe-c(Cys-Phe-D-Trp-Lys-Ac6c-Cys)-Thr-NH_2	$C_{52}H_{69}N_{11}O_9S_2$	1,055.4721	1,056.4778	11.04	−69.23
4C	D-Phe-c(Cys-Phe-D-Trp-Orn-Aib-Cys)-Thr-NH_2	$C_{48}H_{63}N_{11}O_9S_2$	1,001.4252	1,002.4330	11.6	−43.75
5C	D-Phe-c(Cys-Phe-D-Trp-Orn-Ac5c-Cys)-Thr-NH_2	$C_{50}H_{65}N_{11}O_9S_2$	1,027.4408	1,028.4464	10.52	−60.87
6C	D-Phe-c(Cys-Phe-D-Trp-Orn-Ac6c-Cys)-Thr-NH_2	$C_{51}H_{67}N_{11}O_9S_2$	1,041.4565	1,042.4633	10.85	−48.00
$7C_{rf}$	D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-NH_2	$C_{49}H_{67}N_{11}O_{10}S_2$	1,031.4357	1,032.4401	8.7	−55.32

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

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 was evaluated by the MTS-dye reduction assay for cell viability (CellTiter 96 Non-Radioactive Cell proliferation assay, Technical Bulletin 1999) 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), Lep-3 (normal human diploid cell line) as a control. Cells were cultivated with different amounts of the substances applying concentrations in the range from 4.10^{-3} to 4.10^{-8} M.

MTS test

Cells were seeded in 96-well flat-bottomed microplates (Orange Scientific) (100 µl aliquots/well) at a concentration of 2×10^4 cells/well. After the 24th hour, cells were covered with DMEM medium containing different concentrations of the tested compound. Each concentration was applied in three wells. Samples of cells grown in non-modified medium served as a control. After 24 h incubation, the MTS colorimetric assay of cell survival was performed as described in the protocol of ''Promega''. This consisted of 24 h incubation with MTS solution at 37 °C under 5 % carbon dioxide and 95 % air. 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 curve was obtained for every one experiment. Each data point represents an average of three independent assays. All cells were incubated with tenfold dilution (in the interval from 4.10^{-3} to 4.10^{-8} M).

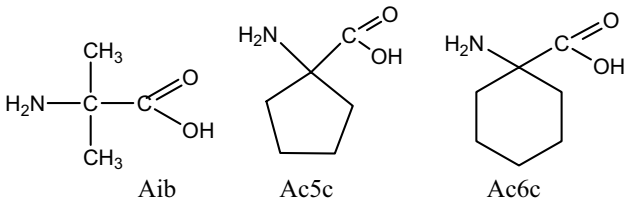

Fig. 1 Structure of Aib (α-aminoisobutyric acid), Ac5c (1-aminocyclopentane carboxylic acid) and Ac6c (1-aminocyclohexane carboxylic acid)

Statistical analysis

Statistical deviations for the viability were calculated automatically by Excel 2007 software program (SE ≤ 5 %) and for the IC_{50}—by the ''Origin Pro 7.5'' and ''Origin 6.1'' (for $7C_{rf}$) PC-program.

Results and discussion

On the base of RC-102 (D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-NH_2) new analogs of Somatostatin were synthesized. The Thr at position 6 was replaced by the steric-restricted amino acids Aib, Ac5c and Ac6c to stabilize the desired conformation necessary for biological activity (Fig. 1).

In compounds **4C–6C** the Lys at position 5 was substituted by its analog Orn. All analogs were synthesized as C-terminal amides to increase plasma half-life. We synthesized the SST analog RC-102 (compound $\mathbf{7C_{rf}}$) to compare its in vitro cytotoxic activity to the other compounds.

The peptides were synthesized by standard solid phase peptide chemistry methods—Fmoc (fluorenylmethoxycarbonyl)-strategy, using TBTU (2-(1H-benzo-triazole-

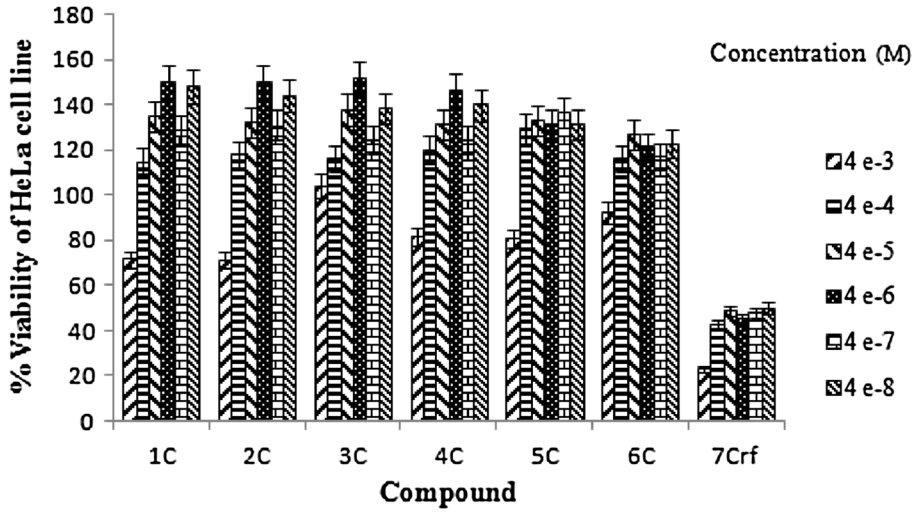

Fig. 2 Antiproliferative activity of compounds **1C–7C** in a human cervical cancer cell line HeLa after 24 h treatment (SE ≤ 5 %; e^{-n} is a negative exponentiation with base $e = 10$ and exponent n)

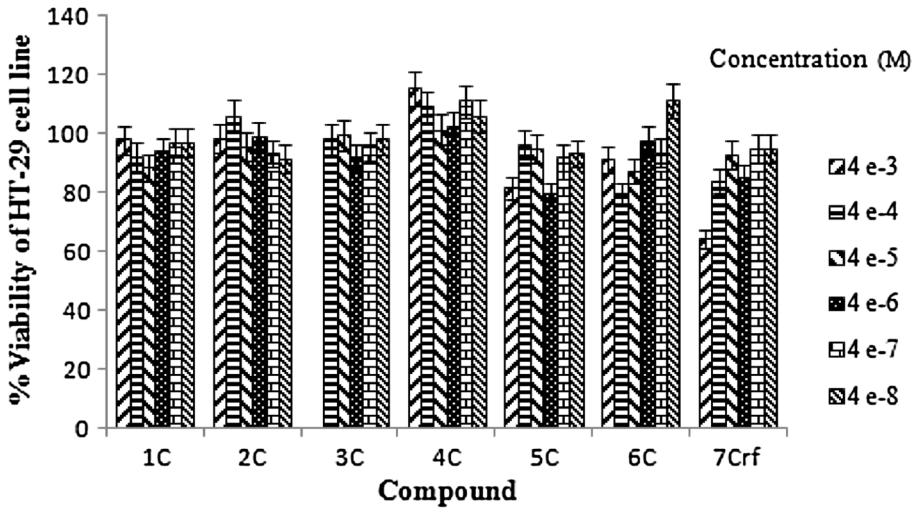

Fig. 3 Antiproliferative activity of compounds **1C–7C** in a human liver carcinoma cell line Hep G-2 after 24 h treatment (SE ≤ 5 %; e^{-n} is a negative exponentiation with base $e = 10$ and exponent n)

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 direct disulphide bond formation has been employed on the solid phase with $Tl(CF_3CO_2)_3$ in DMF. The disulphide bridges were proved by reduction of the cyclic peptides with TCEP (tris-(2-carboxyethyl)-phosphine). The cleavage of the synthesized peptides from the resin was done, using TFA. The compounds were checked by LC-electrospray ionization mass spectrometry and the optical rotation was measured in water. The data are summarized in Table 1.

The investigated compounds **1C-7C$_{rf}$** were evaluated for cytotoxicity effect against a panel of four tumor cell lines of human origin (Hep G-2, HT-29, HeLa, MDA-MB-231) and normal human diploid cells Lep-3 by using the MTS-dye reduction assay. 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). As expected the cytotoxicity of each compound was different to the different cell kinds. The results are presented at Figs. 2, 3, 4, 5 and 6.

For the HeLa cells all substances had cytotoxic effect only at the high concentrations (4.10^{-3} M) and this can be seen by the calculations of their EC_{50} (estimated by the computer program "Origin Pro7.5"). The data are summarized in Table 2.

Compounds **1C** (Lys^5, Aib^6) and **2C** (Lys^5, $Ac5c^6$) inhibit the vitality of HeLa cell line to 71 % followed by the compounds **4C** (Orn^5, Aib^6) and **5C** (Orn^5, $Ac5c^6$) (81 % viability) at the concentration 4.10^{-3} M. The referent compound **7C$_{rf}$** is most cytotoxic (23 % viability at 4.10^{-3} M and 50 % at 4.10^{-8} M) with the $IC_{50} = 420$ μM (Fig. 2).

The Hep G-2 cells were sensitive to almost all substances (vitality up to 100 % and only few above this). The compounds **1C** (Lys^5, Aib^6), **3C** (Lys^5, $Ac6c^6$) and **6C** (Orn^5, $Ac6c^6$) inhibit the growth of Hep G-2 at the highest

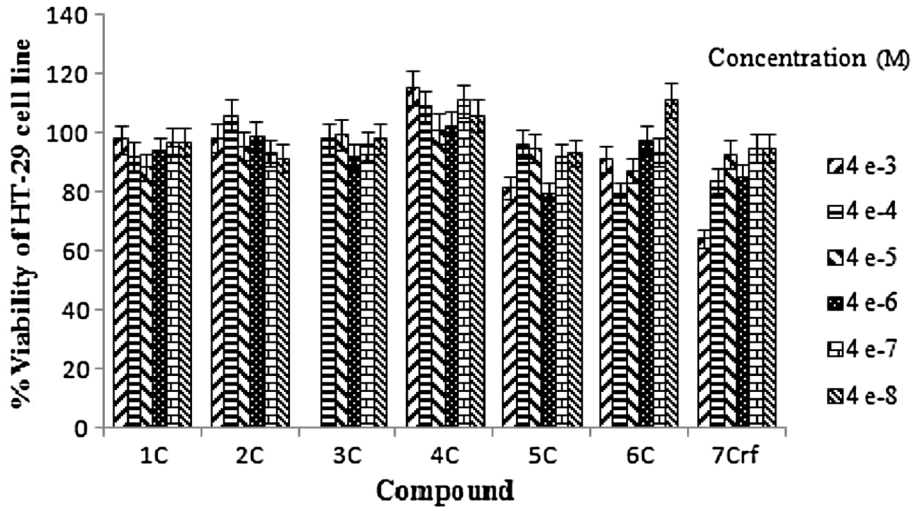

Fig. 4 Antiproliferative activity of compounds **1C–7C** in a human colorectal carcinoma cell line HT-29 after 24 h treatment (SE $\leq$5 %; e^{-n} is a negative exponentiation with base $e = 10$ and exponent n)

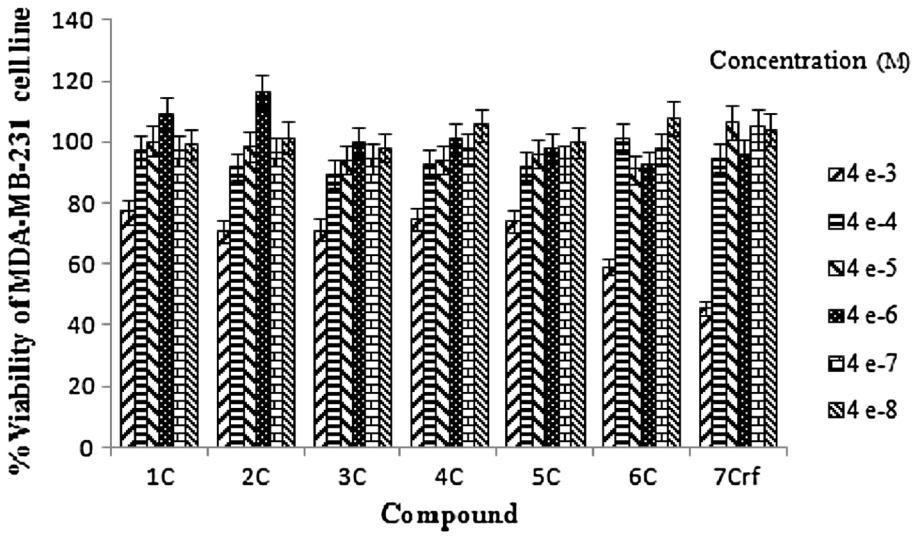

Fig. 5 Antiproliferative activity of compounds **1C–7C** in a human breast cancer cell line MDA-MB-231 after 24 h treatment (SE $\leq$5 %; e^{-n} is a negative exponentiation with base $e = 10$ and exponent n)

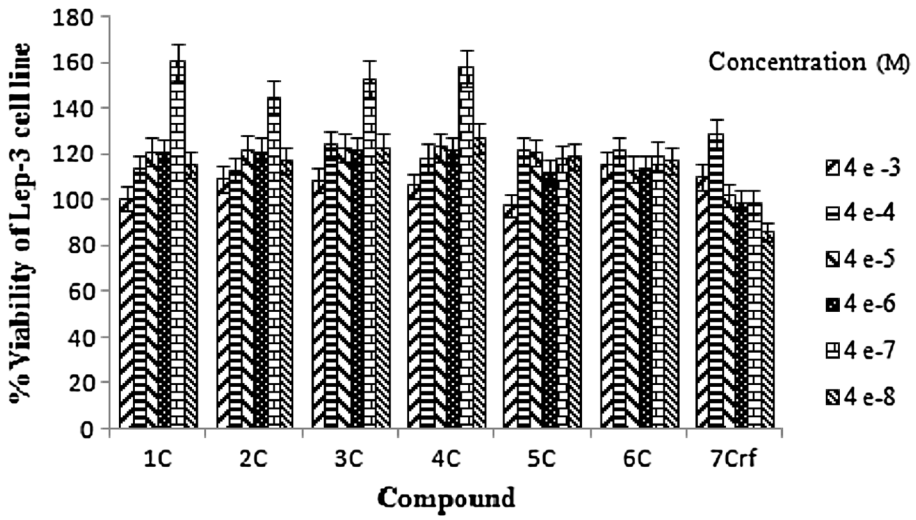

Fig. 6 Antiproliferative activity of compounds **1C–7C** in a normal human diploid cell line Lep-3 after 24 h treatment (SE $\leq$5 %; e^{-n} is a negative exponentiation with base $e = 10$ and exponent n)

Table 2 In vitro cytotoxicity of compounds **1C–7C$_{rf}$** after 24 h treatment

Comp.	HeLa		Hep G-2		HT-29		MDA-MB-231		Lep-3
	$EC_{50} \pm SE$ (mM)	$IC_{50} \pm SE$ (mM)	$EC_{50} \pm SE$ (mM)	$IC_{50} \pm SE$ (mM)	$EC_{50} \pm SE$ (mM)	$IC_{50} \pm SE$ (mM)	$EC_{50} \pm SE$ (mM)	$IC_{50} \pm SE$ (mM)	$EC_{50} \pm SE$ (mM)
1C	1.18	–	nd	–	–	0.98 ± 0.05	2.11 ± 0.06	–	0.21 ± 0.10
2C	0.16 ± 0.11	–	$38.9 \times 10^{-5} \pm 0.09$	–	–	$1.17 \times 10^{-3} \pm 0.066$	0.97 ± 0.078	–	0.08 ± 0.07
3C	0.16 ± 0.06	–	4.12 ± 0.24	–	–	250.82 ± 0.08	22.46 ± 0.06	–	$3.99 \times 10^{5} \pm 0.08$
4C	0.17 ± 0.08	–	6.21 ± 0.15	–	–	$1.99 \times 10^{-5} \pm 0.07$	$2.54 \times 10^{6} \pm 0.07$	–	0.05 ± 0.09
5C	30.9 ± 0.08	–	9.37×10^{48}	–	19.99 ± 0.09	–	2.24 ± 0.06	–	1.6 ± 0.05
6C	0.47 ± 0.05	–	$1,401.92 \pm 0.29$	–	$6.22 \times 10^{-11} \pm 0.14$	–	$1.13 \times 10^{27} \pm 0.10$	–	$0.78 \times 10^{-3} \pm 0.02$
$7C_{rf}$	–	0.42 ± 0.07	–	0.83 ± 7.48	–	15.9 ± 0.09	–	0.54 ± 0.18	nd

concentrations (4.10^{-3} M), respectively the measured viability were 56, 58 and 38 % (Fig. 3).

The compounds were most effective to the HT-29 tumor cells, which vitality decreased below 100 %. This effect was denoted for all concentrations of the studied compounds (Fig. 4). The IC_{50} were in accordance with the lowered vitality. The best result against HT-29 cells was obtained for the compound **4C** (D-Phe-c(Cys-Phe-D-Trp-Orn-Aib-Cys)-Thr-NH_2), $IC_{50} = 0.0199$ μM (Table 2).

The MDA-MB-231 cells have shown vitality, which values depends also on the compounds' concentrations. As it is well visible in the Fig. 5, in the most cases they did not reach 100 %. The substance **3C** was the most cytotoxic. At the highest concentration (4.10^{-3} M) only, the vitality of MDA-MB-231 cells was depressed to 59 and 46 %, respectively by compound **6C** (Orn^5, $Ac6c^6$) and **7C$_{rf}$**. The other peptides suppress the viability up to 77 % at the same concentration (Fig. 5).

The vitality of the normal (Lep-3) cells was found above 100 % in all studied concentrations (even at the highest ones) of the compounds (Fig. 6). This result is very important, because it means that the normal Lep-3 cells are not affected by the targeted substances.

Recently we have reported the synthesis of newly modified analogs of Octreotide, RC-160 and RC-121 substituting Thr^6 (respectively Val^6) by Tle (t-leucine) as well as substituting Lys^5 by Orn, Dab (diaminobutanoic acid) or Dap (diaminopropanoic acid) so to elucidate the influence of the modification to the in vitro antitumor activity. The activities were evaluated against the same four tumor cell line (HeLa, Hep G-2, MDA-MB-231, HT-29) and in the non-tumor cell line (Lep-3). (Staykova et al. 2012a). The results obtained revealed that the amino acid Tle at position 6 did not increase the cytotoxicity and the length of the amino acid side chain at position 5 doesn't significantly affect the activity. It was found that the Phe^3/Tle^6 octapeptides demonstrated higher cytotoxic activities than Tyr^3/Tle^6 substituted peptides (Staykova et al. 2012b). The examined compounds influence only the tumor cells of each kind and did not influence the normal Lep-3 cell line.

Because of the low activity of the peptides analogs containing Tle^6, new modifications at position 6 were designed. In the current study the replacement of Thr^6 by α,α-dialkylated amino acids (Aib), 1-aminocyclopentane carboxylic acid (Ac5c) or 1-aminocyclohexane carboxylic acid (Ac6c) is presented. The new octapeptide SST analogs demonstrated exceptional selectivity. They have shown high cytotoxic effect only against HT-29 cell line (Table 2). The best results was obtained for compound **4C** (Orn^5, Aib^6) with $IC_{50} = 1.99x10^{-2}$ μM followed by compound **2C** (Lys^5, $Ac5c^6$) with $IC_{50} = 1.17$ μM. The substitution of Lys^5 by Orn in compound **2C** led to complete loss of the activity that was confirmed by compound **5C**

(Orn^5, Ac5c^6). The same tendency was observed comparing the activity of both compounds: **3C** (Lys^5, Ac6c^6) and **6C** (Orn^5, Ac6c^6), indicating the significance of the Lys at position 5. At lower concentrations compound **6C** displayed proliferative activities. Reverse tendency has been observed at the analogs containing Aib at position 6. By the shortened of the side chain's length of the located at position 5 amino acid, the activity considerably increases. It's interesting to mention that the substitution of Lys^5 by Orn led to noticeably increasing of the activity of compound **4C** in comparison with compound **1C**. Compound **4C** has shown $IC_{50} = 1.99 \times 10^{-2}$ μM, while the IC_{50} found for compound **1C** (Lys^5, Aib^6) was 0.98×10^3 μM. The referent compound **7C$_{rf}$** (Lys^5, Thr^6) has shown cytotoxic activity against all tumor cell lines.

We can conclude that the incorporated α,α-dialkylated amino acids influence the in vitro antitumor activity of the modified analogs. The activity is directly related also to the type of the amino acid in position 5 (Lys or Orn) and the tumor cells' kind. The normal Lep-3 cells are not affected by the examined substances.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard This article does not contain any studies with human participants or animals performed by any of the authors.

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