Bulgarian Chemical Communications, Volume 44, Number 3 (pp. 233 – 237) 2012

Antioxidant capacity of new analogs of octreotide

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Received June 10, 2012; accepted August 10, 2012

New C-amide analogs of octreotide (SMS 201–995) modified at positions 5 with Orn, Dab (diaminobutanoic acid) and Dap (diaminopropanoic acid) and at positions 6 with the unnatural amino acids Tle (t-leucine) were synthesized. The antioxidant capacity of the compounds was tested by ORAC (Oxygen Radical Antioxidant Capacity) and HORAC (Hydroxyl Radical Averting Capacity) methods. All substances express significantly higher antioxidant capacity by comparison with gallic acid. Compound **1** (D-Phe-c(Cys-Phe-D-Trp-Dab-Tle-Cys)-Thr-NH_2) showed the highest antioxidant effect.

Kew words: antioxidant, antiproliferative, somatostatin analogs, SPPS, unnatural amino acids

INTRODUCTION

Oxygen free radicals or, more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems. Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defense against infectious agents and in the function of a number of cellular signaling systems. The harmful effect of free radicals (oxidative stress) is observed by the excess of ROS which can damage cellular lipids, proteins, or DNA inhibiting their normal function [1].

Naturally occurring somatostatins have diverse biological effects in many cells and organs throughout the body. They are produced by normal endocrine, gastrointestinal, immune and neuronal cells, as well as by certain tumors. The effects of somatostatins are broadly inhibitory on the secretion of hormones (e. g. growth hormone), as well as on the proliferation and survival of both normal and tumor cells [2]. Native somatostatin (SST) has a very short or transient effect *in vivo* as it is rapidly inactivated by endo- and exo-peptidases [3, 4]. In recent years, increasing data have supported the hypothesis that different somatostatin analogs with longer half-life can also function as antineoplastic agents capable of inhibiting tumor growth and tumor angiogenesis [5–9].

Octreotide (Sandostatin, SMS 201-995: D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-ol) is the somatostatin analog used in clinical practice that mimics natural hormone somatostatin (SST-14: Ala-Gly-c(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys) pharmacologically, but it is more potent inhibitor of growth hormone, glucagon, and insulin than SST [10]. It is used for the treatment of acromegaly, diarrhea and flushing episodes associated with carcinoid syndrome, in nuclear medicine imaging and peptide receptor radionuclide therapy [11–13].

Previous structure-function studies indicate that the sequence required for biological activity of the shortened SSAs consists of the β-turn fragment Phe-Trp-Lys-Thr corresponding to the residues 7–10 of the somatostatin. A β-turn structure about Trp-Lys (or Orn) in the analogs was a preferred stable conformation [14–17]. The introduction of D-residues increased the half-life and biological activity of the SST analogs. The substitutions of D-Trp decreased significantly or lead to full loss of antitumor activity [18]. The replacement of the C-terminal carboxyl group with amide group led to

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molecules with high biological activity [14–19]. RC-102 is the SST analog with GH and insulin-inhibitory activity [18].

Recently new modified C-amide analogs of Octreotide were synthesized substituting ornithine (Orn), diaminobutanoic (Dab) and diaminopropanoic (Dap) acids for lysine (Lys) and with Tle (t-leucine) for the threonine (Thr) based on templates D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-ol (Octreotide, Sandostatin, SMS 201-995). The *in vitro* antiproliferative activity were evaluated against 4 human tumor cell lines (HeLa, Hep G-2, MDA-MB-231, HT-29) and in the non-tumor Lep-3 cell line. The compounds showed different activity depending on the cell line and amount applied. The compound **2** (D-Phe-c(Cys-Phe-D-Trp-Dap-Tle-Cys)-Thr-NH_2) had antiproliferative effects on MDA-MB-231 cells with the IC_{50} 0.03 mM. The most antiproliferative effect against the HeLa and HepG-2 cells exert the peptides **3** (D-Phe-c(Cys-Phe-D-Trp-Lys-Tle-Cys)-Thr-NH_2), **4** (D-Phe-c(Cys-Phe-D-Trp-Orn-Tle-Cys)-Thr-NH_2) and **5** (RC-102). The peptides were not cytotoxic to the normal Lep-3 cells [20].

Our results demonstrated that these new somatostatin analogs exhibit cytotoxic effect against some tumor cell lines (HeLa, Hep G2, MDA-MB-231 and HT-29) but their antioxidant effect is still unknown.

The aim of present investigation was to study the antioxidant capacity of the previously synthesized modified C-amide analogs of Octreotide: D-Phe-c(Cys-Phe-D-Trp-Dab-Tle-Cys)-Thr-NH_2 (**1**), D-Phe-c(Cys-Phe-D-Trp-Dap-Tle-Cys)-Thr-NH_2 (**2**), D-Phe-c(Cys-Phe-D-Trp-Lys-Tle-Cys)-Thr-NH_2 (**3**), D-Phe-c(Cys-Phe-D-Trp-Orn-Tle-Cys)-Thr-NH_2 (**4**) and D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-NH_2 (**5**) (RC-102) by ORAC (Oxygen Radical Antioxidant Capacity) and HORAC (Hydroxyl Radical Averting Capacity) methods.

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-fluorenylmethoxycarbonyl) chemistry was employed to synthesize a series of new analogues of SSAs. Rink-amide MBHA resin and TBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) 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, s N^{α}-Fmoc-Lys(Boc)-OH, N^{α}-Fmoc-Orn(Boc)-OH, N^{α}-Fmoc-Dab(Boc)-OH, N^{α}-Fmoc-Dap(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 a 20% piperidine solution in dimetilformamide. 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 anisol and 100 ml DMF at 0ºC for 2.5 h. The oxidized peptidyl-resin was dryed and next cleaved from the resin. 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 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 (125×2.1mm) (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 masspectrometry and the optical rotation was measured in water.

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 (**1**–**5**) 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), Lep-3 (normal human diploid cell line, delivered from 3-month embryo) as a control. Cells were cultivated with different amounts of the substances at concentration from 4.10^{-3} – 4.10^{-8} M.

The test (Cell Titer 96 Non-Radioactive Cell proliferation assay, Promega Corporation USA) was performed according to protocol of "Promega" and the details are previously described [20–22].

ANTIOXYDANT ACTIVITY

Preparation of the samples

Approximately 2 mg of each sample were dissolved in 2 mL acetone: dist. water: conc. acetic acid = 70:29.5:0.5 at room temperature for 1 hour. This solvent system is widely applied for enhanced extraction of phenolic substances from plant materials, foods etc. [23]. The solutions obtained were applied for determination of the antioxidant capacity immediately.

Determination of antioxidant capacity

AAPH, (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride), a water-soluble free radical (peroxyl radical, ROO$^{\bullet}$) generator was delivered from Cayman Chemical Company. Fluorescein-disodium salt, gallic acid and TROLOX® (6–Hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid) (a water-soluble analog of Vitamin E) were delivered from Sigma Aldrich. All other chemicals were of reagent grade.

HORAC (Hydroxyl Radical Averting Capacity)

The applied procedure is based on a scheme given by Ou et al. with some modifications [24]. Fluorescein-disodium salt (λex 493 nm, λem 518 nm) was used as indicator for monitoring the generation of hydroxyl radical according to Fenton reaction mechanism and the effect of the tested substance.

ORAC (Oxygen Radical Antioxidant Capacity)

The procedure used is based on the thermal destruction of AAPH, which is accompanied by generation of peroxyl radicals (ROO$^{\bullet}$) as it is described by Yilmaz and Toledo [25], Wada and Ou [26] and Gheldof and Engeseth [27].

The experiments in both cases (HORAC & ORAC) were performed on a Perkin Elmer LS 5 spectrofluorimeter, equipped with thermostated cell holder (working temperature 37^{o}C) in a 10 mm quartz cell. Data were collected for 30 min at a constant step of 0.5 sec.

Processing of data. The data obtained for each sample, the corresponding standard and the blank probe are normalized to the initial value (I_0) and the measuring interval (0.5 sec). Then the Area Under Curve (AUC) value is calculated according to the formula:

$$AUC = I_2/I_0 + I_3/I_0 + \ldots + I_{n-1}/I_0$$

The calculation of the antioxidant capacity proceeds according to the equation:

$$\text{Antioxidant capacity} = \frac{AUC_{Sample} - AUC_{Blank}}{AUC_{Std} - AUC_{Blank}} * \frac{C_{eff}^{Std}}{C_{eff}^{Sample}}$$

[μM Std Equivalents/g sample]

if the effective concentration of the sample is in g/L.

The effective concentrations of the standard and the sample are their final concentrations in the cuvette. If we introduce in the formula the molecular mass of the tested substance, the result is obtained as μM Std Equivalents/M sample. The data processing can be achieved using the standard Excel software.

RESULTS AND DISCUSSION

In our previously investigation we synthesized some new modified C-amide analogs of Octreotide with the following sequences:

D-Phe-c(Cys-Phe-D-Trp-**Xxx**-**Yyy**-Cys)-Thr-NH_2

where:

Xxx: Dab (compound **1**), Dap (compound **2**), Lys (compounds **3, 5**), Orn (compound **4**)

Yyy: Tle (compounds **1- 4**), Thr (compound **5**)

The synthesis and cytotoxic assays were described [20].

All tested substances express significant antioxidant capacity. In Fig.1 and Fig. 2 are shown the fluorescence decay curves in the HORAC - procedure for tested substances.

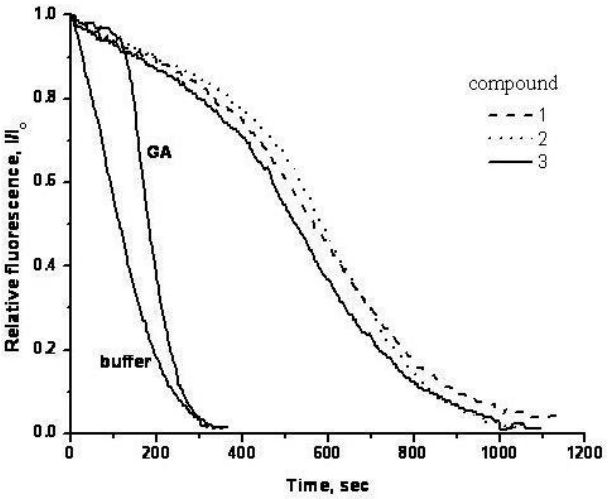

Fig.1. Fluorescence decay curves of compounds **1**, **2** and **3** in the determination of HORAC

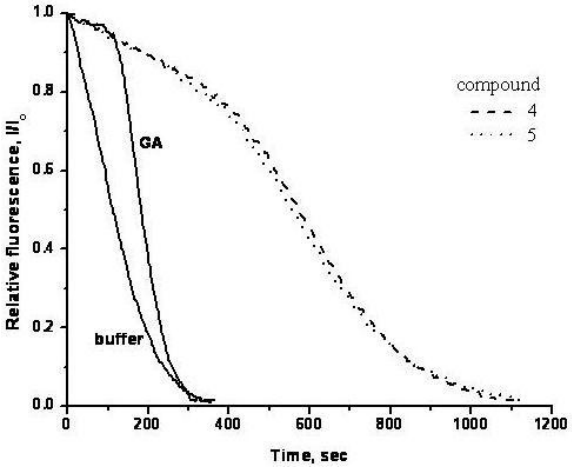

Fig. 2. Fluorescence decay curves of compounds **4** and **5** in the determination of HORAC

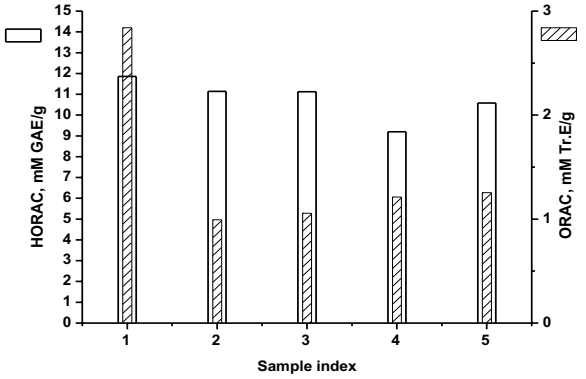

Fig. 3. Comparison of HORAC and ORAC values of the tested substances. GAE – gallic acid equivalents; Tr.E – TROLOX – equivalents.

It is evident that there is no significant difference in the hydroxyl radical averting capacity of the individual samples, especially in the case of peptide **4** and **5**. The capacity of compound **1** is slightly higher. The lag-phase is not well defined. All tested substances express significantly higher capacity by comparison with gallic acid, i.e. they are good helators of metal ions thus preventing the generation of hydroxyl radicals.

The scavenging effect of the tested substances towards peroxyl radical (ORAC) follows the same logics (data not shown). The substance **1** is a stronger free radical scavenger by comparison with the other tested substances (Table 1 and Fig. 3).

Table 1. Summarized data on the antioxidant properties of the tested substances

№	STRUCTURE	MM_{exact}	HORAC M GAE/M	ORAC M Tr.E/M
1	D-Phe-c(Cys-Phe-D-Trp-Dab-Tle-Cys)-Thr-NH_2	1015.4408	12.030	2.883
2	D-Phe-c(Cys-Phe-D-Trp-Dap-Tle-Cys)-Thr-NH_2	1001.4252	11.150	0.996
3	D-Phe-c(Cys-Phe-D-Trp-Lys-Tle-Cys)-Thr-NH_2	1043.4721	10.560	1.104
4	D-Phe-c(Cys-Phe-D-Trp-Orn-Tle-Cys)-Thr-NH_2	1029.4565	9.470	1.249
5	D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-NH_2	1031.4357	10.910	1.294

The antioxidant activity of proteins is thought to encompass both free radicals scavenging by amino acid residues and chelation of prooxidative transition metals; however the precise mechanism by which this occurs, remains unclear [28]. In beta-lactoglobulin particularly, Cys was oxidized more easily than Trp. Further, the extent of exposure of a given amino acid residue greatly affects its oxidation kinetics. In our case theoretically the Lys-residue and its analogs (Dap, Dab, Orn) are possible target points. The pKa-value of the epsilon-NH_2-group of Lys is about 10.0 and theoretically it should decrease following the order Dap<Dab<Orn [29]. This means that these amino acid residues are relatively reactive and modifiable under physiological conditions. In other words, the observed antioxidative capacity is a result of the corresponding modifications of lysine residues in the peptide structure. The elucidation of the detailed mechanism requires further investigations.

CONCLUSION

In conclusion we can summarize that the new previously synthesized somatostatin analogs with established *in vitro* anticancer activity express considerable antioxidant activity. Compound **1** has the greatest anti-ROS capacity and moderate anti-proliferative response. To determine the relationship between these activities further investigations are needed.

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АНТИОКСИДАНТЕН КАПАЦИТЕТ НА НОВИ АНАЛОЗИ НА ОКТРЕОТИД

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Получена на 10 юни, 2012 г.; приета на 10 август, 2012 г.

(Резюме)

Ние синтезирахме нови аналози на октреотид (SMS 201-995) под формата на С-крайни амиди. Аналозите модифицирахме с некодираните аминокиселини Orn (орнитин), Dab (диаминобутанова киселина) и Dap (диаминопропанова киселина) в позиция 5, както и с аминокиселината Tle (третичен левцин) в позиция 6. Антиоксидантната активност на новосинтезираните пептиди изследвахме с помощта на методите ORAC (Oxygen Radical Antioxidant Capacity) и HORAC (Hydroxyl Radical Averting Capacity). Всички съединения проявяват значително по-висока антиоксидантна активност в сравнение с използвания стандарт галова киселина. Пептид **1** **(**D-Phe-c(Cys-Phe-D-Trp-Dab-Tle-Cys)-Thr-NH_2) показва най-висок антиоксидантен капацитет.