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Design, synthesis and biological activity of cell-penetrating peptide-modified octreotide analogs

Wenlin Xie,[a,b,c]* Jian Liu,[c] Minghua Qiu,[a] Jiancheng Yuan,[c] and Anlong Xu,[b]

Four novel octreotide analogs with cell-penetrating peptides (CPPs) at the *N*-terminus or *C*-terminus were synthesized by a stepwise Fmoc solid-phase synthesis strategy. The synthesized peptides were analyzed and characterized using reverse phase HPLC and MALDI-TOF mass spectrometry. The antiproliferative activity of the analogs was tested *in vitro* on human gastric (SGC-7901) and hepatocellular cancer (BEL7402) cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Interestingly, these analogs showed a higher anticancer activities than the parent octreotide except CMTPT03 analog. The results demonstrate that the designed octreotide analogs enhance their anticancer activity after linking together the CPPs to octreotide at the *N*-terminus, and are potential molecules for future use in cancer therapy and drug targeting.

Keywords: anticancer activity; cell-penetrating peptide; Octreotide; peptide synthesis

Introduction

The peptide hormone somatostatin is a naturally occurring cyclic tetradecapeptide. It is present in the hypothalamus, the cerebral cortex, the brain stem, the gastrointestinal tract, and the pancreas and exerts an inhibitory effect on several cell functions such as secretion of peptide hormones and growth factors [1–4]. Neuroendocrine tumors have been found to have an increased number of somatostatin receptors. Other common types of neoplasms, such as gliomas [5], lymphomas [6], and breast cancers [7], also contain somatostatin receptors. The medicinal applications of somatostatin *in vivo* are limited by its rapid proteolytic degradation (plasma halflife <3 min) [8]. However, several short synthetic somatostatin analogs with increased metabolic stability have been synthesized, For instance, the octreotide (Figure 1), an octapeptide, is highly resistant to degradation by enzyme attack and more effective than the somatostatin in the suppression of growth hormone secretion [9]. Because of its growth hormone inhibitory effects, octreotide has been used as a clinical therapeutic agent to treat a wide variety of tumors, which contain somatostatin receptors such as carcinoids and tumors of the pituitary and endocrine pancreas [10]. But most peptide- and nucleic acid-based drugs are poorly taken up in cells, and this is considered a major limitation in their development as therapeutic agents [11–13].

Cell-penetrating peptides (CPPs), such as Tat, Penetratin and VP22 are defined as peptides with a maximum of 30 amino acid residues, which are able to traverse the cell membrane by a process called protein transduction and to enter cells in a seemingly energy-independent manner, thus being able to translocate across membranes in a non-endocytotic fashion [14,15]. Short 'protein-transduction domains' are responsible for the cellular uptake of these proteins [16,17]. Indeed, it was discovered that short peptides derived from protein-transduction domains (CPPs) can be internalized in most cell types and, more importantly, allow the cellular delivery of conjugated (or fused) biomolecules [18,19]. A wide range of biomolecules such as antigenic peptides [20], peptide nucleic acids [21], antisense oligonucleotides [22], full-length proteins [23–25], or even nanoparticles [26] and liposomes [27] have been delivered in this way. Therefore, conjugation of therapeutic agents to CPPs could thus become a strategy of choice to improve their pharmacological properties. In this paper, we modified the octreotide at the *N*-terminus or *C*-terminus using the cell-penetrating peptide Tat(RKKRRQRRR) and penetratin(RQIKIWFQNRRMKWKK), Tat derived from HIV-1 Tat protein is a highly basic and hydrophilic peptide, which contains six arginine and two lysine residues in its nine amino acid residues. Penetratin derived from homeodomain which contains 16 amino acid residues. The four novel Octreotide analogs have been synthesized by an Fmoc solid-phase synthesis strategy, in order to improve their bioactivities. We have evaluated the *in vitro* antiproliferative activity of the octreotide analogs on human gastric (SGC-7901) and hepatocellular cancer cell lines (BEL7402) using the MTT assay [28].

* *Correspondence to: Wenlin Xie, School of Chemistry and Chemical Engineering, Hunan University of Science and Technology, Xiangtan 411201, China. E-mail: xwl2003zsu@yahoo.com.cn*

a *School of Chemistry and Chemical Engineering, Hunan University of Science and Technology, Xiangtan 411201, China*

b *Center of Biopharmaceutical Research, College of Life Science, Zhongshan University, Guangzhou 510275, China*

c *Shenzhen Hanyu Pharmaceutical Co., Ltd., Shenzhen 518057, China*

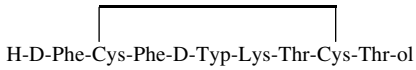

Figure 1. Sequence of the octreotide.

Materials and Methods

Materials

N-α-Fomc-(2*R*,3*R*)-2-amino-3-*t*-butoxy-1-butanol[Fmoc-Thr(*t*Bu)-ol] were prepared as described previously [29,30], 9-Fluorenylmethoxycarbonyl (Fmoc)-α-amino acid derivatives and 2-chlorotrityl chloride resin were purchased from GL Biochem, Ltd. (Shanghai, China). The side chains of amino acids were protected as follows: *t*-butyl (*t*Bu) for threonine and threoninol; *t*-butoxycarbonyl (Boc) for lysines, tryptophan; trityl (trt) for cysteine, asparagine, glutamine; 2,2,4,6,7-pentamethyl-dihydrobenzo-furan-5-sulfonyl (pbf) for arginines. Fmoc-Thr(*t*Bu)-2-chlorotrityl-resin was prepared in-house, as previously described [30]. HOBt, TBTU, DIC, and DIPEA were peptide synthesis grade and purchased from GL Biochem, Ltd. Solvents and other chemicals were all peptide synthesis or analytical grade.

Peptide Synthesis and Purification

Octreotide analogs were synthesized manually on 2-chlorotrityl chloride resin (substitution of 0.6 mmol/g) by standard Fmoc solid-phase synthesis strategy. Attachment of the Fmoc-Thr(*t*Bu)-ol to the resin was performed according to cesium salt procedure [31] and the substitution level was determined by weight gain measurements (Fmoc-Thr(*t*Bu)-Resin, 0.372 mmol/g). Synthesis of desired peptides was achieved by stepwise coupling of Fmoc-amino acids to the growing peptide chain on the resin. All couplings were carried out in DMF, DCM, or a mixture of these solvents. Removal of the N-terminal Fmoc group (deprotection) was performed using a 20%(v/v) solution of piperidine in DMF. Couplings were performed by dissolving an excess (4 mol equiv.) of Fmoc-protected amino acid and HOBt in DMF. The solution was cooled on ice and then TBTU (4 mol equiv.) was added. The reaction mixture was left on ice for 10 min and then at 25 °C for another 10 min. Subsequently, the reaction mixture was added to the resin and allowed to react for 2 h. Coupling efficiency was monitored using the Kaiser ninhydrin test [32]. The coupling step was repeated (double coupling) if Kaiser test was found positive. In all cases where, after second coupling, the test was slightly positive, the remaining free amino groups were acetylated (capping) using a mixture of Ac_2O/DIEA(2/1, v/v, 30 min). In analog CMTPT**04,** the Fmoc group at *N*-terminus was not deprotected after the last amino acid was coupled.

Disulfide bond formation was carried out by treating the peptide resins with iodine in DMF for 2.5 h, Completeness of the formation of the disulfide bond was monitored by the Ellman's test [33]. After peptide synthesis had been completed, the resin was extensively washed (DMF, DCM, and Et_2O,) and then dried *in vacuo* (12 h). The synthesized peptide was cleaved from the resin using a mixture of TFA, thioanisole, ethanedithiol, anisole(90/3/2/1, v/v/v/v) for 3 h. The mixture was filtrated and the filtrate was precipitated with ice-cold dry diethyl ether. The precipitate was filtered, dissolved in water and lyophilized to obtain the crude peptide.

The resulting crude disulfide-bridged peptide was purified on a preparative reverse phase HPLC system (Varian ProStar 320, USA) using Vydac C-18, 5 μm (22 × 250 mm) and eluting with a solvent system of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B), a linear gradient of 20–40% B in 30 min was used, at a flow rate 5 ml/min and detection at 220 nm. The eluted fractions were monitored on analytical HPLC (Agilent 1100, USA) on Vydac C-18, 5 μm (4.6 × 250 mm) reverse phase column. Acetonitrile was evaporated on the rotary evaporator and the fractions were lyophilized to obtain the pure peptide. The purified peptides were characterized by MALDI-TOF mass spectrometry on a Bruker REFLEX III. The calculated mass values for the analogs were in agreement with the protonated molecular ions obtained using MALDI-TOF-MS (Table 1).

In vitro Anticancer Activity

The Octreotide Analogs CMTPT**01**, CMTPT**02**, CMTPT**03**, CMTPT**04** were tested for antiproliferative activity against human gastric (SGC-7901) and hepatocellular cancer (BEL7402) cell lines and compared with the Octreotide using the MTT cytotoxicity assay. The human tumor cell lines representing gastric (SGC-7901) and hepatocellular cancer (BEL7402) were obtained from Shanghai Institutes for Biological Sciences. Briefly, the human tumor cell lines were maintained in a medium (Dulbecco's modified Eagle's medium (DMEM), Gibco, BRL, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, BRL, USA). The cells were plated in 96-well tissue culture plates at a density of 10 000 cells/well and incubated 24 h in a humidified atmosphere of 5% CO_2 at 37 °C to

Table 1. Sequence, mass and HPLC analysis data of the synthesized octreotide analogs

Code	Peptide sequence	Mass[M+H]$^+$		HPLC RT(min.)
		Calculated	Observed	
Octreotide	D-Phe-**c**(CysPhe-D-TrpLysThrCys)Thr-ol	1019.4	1019.8	10.83
CMTPT**01**	**ArgLysLysArgArgGlnArgArgArg**-D-Phe-**c**(CysPhe-D-TrpLysThrCys)Thr-ol	2340.0	2341.4	14.57
CMTPT**02**	**ArgGlnIleLysIleTrpPheGlnAsnArgArgMetLysTrp-LysLys**-D-Phe-c(CysPhe-D-TrpLysThrCys)Thr-ol	3247.0	3247.9	8.27
CMTPT**03**	D-Phe-**c**(CysPhe-D-TrpLysThrCys)Thr-**ArgLysLysArgArgGlnArgArgArg**	2354.7	2354.9	9.87
CMTPT**04**	**Fmoc-ArgLysLysArgArgGlnArgArgArg**-D-Phe-**c**(CysPhe-D-TrpLysThrCys)Thr-ol	2562.3	2563.7	12.74

Mobile phase A: water (0.1%TFA) and mobile phase B: acetonitrile (0.1%TFA); gradient: 20–40% B in 30 min.

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allow complete reattachment of the cells. After 24 h, the medium was removed and new DMEM which did not contain FBS was added in each well, The cells were treated with different concentrations of the ctreotide Analogs (3.91 to 500 μM, final concentration of DMSO <1%). The cells were later incubated for 48 h. The cytotoxicity was measured by adding 5 mg/ml of MTT (Sigma–Aldrich Inc., USA) to each well and incubated for another 3 h. The purple formazan crystals were then dissolved by the addition of dimethylsulfoxide. The absorbance was read at 570 nm in a spectrophotometer. The results were calculated as percent inhibition of cell proliferation according to the formula:

$$\%\ inhibition\ of\ cell\ proliferation = 100 \times (1 - A_t/A_c)$$

where A_t = absorbance of treated sample at 570 nm,

A_c = absorbance of control sample at 570 nm.

The compound concentration causing a 50% cell growth inhibition (IC_{50}) was determined by interpolation from dose–response curves.

Results and Discussion

Synthesis and Characterization of Peptides

The four new analogs of octreotide, modified with CPPs Tat and Penetratin at the *N*-terminus or *C*-terminus, were synthesized by stepwise coupling of Fmoc-amino acids to the growing peptide chain on 2-chlorotrityl chloride resin. The couplings were mediated by the TBTU/HOBt method. On completion of the coupling, the crude linear precursors were cyclized by treating the peptide resins with iodine in DMF, The progression of cyclization reaction was monitored by HPLC. To further confirm the disulfide bond formation, an Ellman test for free thiols was carried out and the result showed that the free thiols were completely consumed if Ellman test was found negative. The synthesized peptide was cleaved from the resin using a mixture of TFA, thioanisole, ethanedithiol, anisole(90/3/2/1, v/v/v/v).The crude peptides were purified by preparative reversed-phase HPLC. The homogeneity of the purified cyclic peptides was checked by analytical HPLC (Figure 2) and their structures were confirmed by MALDI-TOF-MS (Figure 3). MS analyses were correspondence to the expected molecular weights. The HPLC results of the prepared compounds displayed that they had high purity in a range of 95.8–98.5%. Yields of the products were in the range of 25–38% based on the Fmoc-Thr(tBu)-2-chlorotrityl-resin loadings. The peptide sequence and analytical data of the synthesized octreotide analogs are summarized in Table 1.

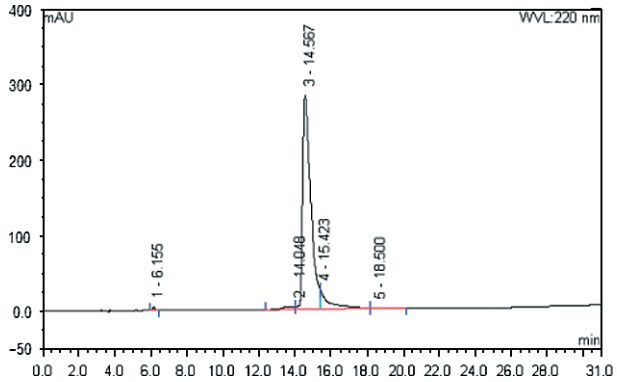

Figure 2. HPLC profiles for the CMTPT**01** at 220 nm.

Biological Assays

The *in vitro* antiproliferative activity of the synthesized octreotide analogs against human gastric (SGC7901) and hepatocellular cancer(BEL7402) cell lines were evaluated by the standard MTT assay using octreotide as reference compounds. Antitumor potency of the compounds was indicated by IC_{50} values [that is the concentration (μM) of a compound able to cause 50% of cell death with respect to the control culture] that were calculated by linear regression analysis of the concentration–response curves obtained for each compound. Data are represented in Table 2.

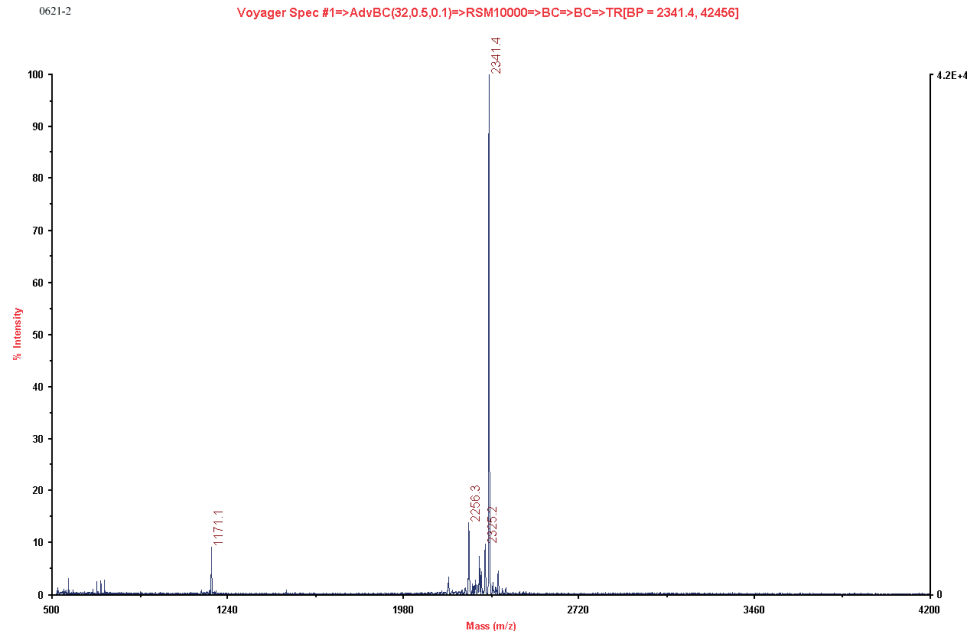

Figure 3. MS profiles for the CMTPT**01,** MS analysis of peak at14.57 min. $[M+H]^+$ calculated for CMTPT**01** is 2341.4.

Table 2. Cytotoxicity of the prepared compounds against several human cancer cell lines

Code	IC_{50}/μM				
	Oct	CMTPT**01**	CMTPT**02**	CMTPT**03**	CMTPT**04**
BEL-402	34.68	<3	9.8	73.82	9.3
SGC-7901	43.9	6.6	19.7	54.4	5.9

* Significant at $P < 0.05$.

As shown in Table 2, most of the peptides tested showed potent inhibitory effects on human gastric(SGC7901) and hepatocellular cancer(BEL7402) cell lines, Except for CMTPT**03** analog. The potencies of CMTPT**01**, CMTPT**02**, and CMTPT**04** were better than the parent analog octreotide. The results indicated that the CPPs can enhance the antiproliferative activity of octreotide. Compared with the positive control octreotide (IC_{50} of 34.68 and 43.9 μM, respectively), peptide CMTPT**01** (with IC_{50} of <3 and 6.6 μM, respectively) among the new analogs tested was found to show the most potent *in vitro* inhibitory activity against BEL7402 and SGC7901.

The potencies of the resulting octreotide analogs appeared to be related to the position of CPPs in peptide sequence. It has been observed from Table 2 that the octreotide analogs with cell-penetrating peptide at the *N*-terminus have higher inhibitory effects than octreotide. Conversely, the peptide CMTPT**03,** which contains the CPP TAT at the *C*-terminus of octreotide, exhibits a lower inhibitory potency than parent peptide. Indeed the IC50 of CMTPT**03** against BEL7402 and SGC7901 cell lines is 73.82 and 54.4 M, respectively, while the IC50 of octreotide is 34.68 and 43.9 M, respectively. This lower activity is due to the change of the *C*-terminal L-threoninol to L-threonine as from structure–function studies it is known that the C-terminal L-threoninol is required to stabilize the active conformation. It was found that the β-turn/β-sheet conformation is stabilized by intramolecular hydrogen bonds between the *N*-terminal residue and the *C*-terminal L-threoninol [34,35].

Conclusions

In conclusion, we have designed and synthesized four novel octreotide analogs with CPPs at the *N*-terminus or *C*-terminus using the CPPs Tat and Penetratin, and evaluated their anti-tumor activity *in vitro*. The results indicated that these analogs showed higher antiproliferative activity against human gastric (SGC-7901) and hepatocellular cancer (BEL7402) cell lines than the parent octreotide except CMTPT03 analog. It may be concluded that the cell-penetrating peptide can enhance the antiproliferative activity of octreotide. This study may provide valuable information for further designing more potent anticancer agents.

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