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Substance P analogs containing α,α-dialkylated amino acids with potent anticancer activity

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Abstract: Six analogs (peptides **1–6**) of the potent substance P (SP) derivative known as 'Antagonist D' were synthesized by substituting constrained amino acids Aib or Acp (cycloleucine, 1-amino cyclopentane carboxylic acid) at different positions in the Antagonist D sequence: D-Arg^1-Pro^2-Lys^3-Pro^4-D-Phe^5-Gln^6-D-Trp^7-Phe^8-D-Trp^9-Leu^{10}-Leu^{11}-NH_2. In the preliminary *in vitro* antiproliferative screening of the analogs on different human cancer cell lines by 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT) assay, peptide **1** was found to be the most active. Further, peptide **1** was butanoylated (analog **5**) or octanoylated (analog **6**) at the *N*-terminus. SP analogs **1, 5**, and **6** were evaluated *in vivo* in a xenograft model of human primary colon tumor (PTC) cell line in athymic nude mice and were found to cause tumor regression. This study investigates if the use of the constrained amino acids Aib and Acp in the designed SP analogs can retain the *in vitro* and *in vivo* anticancer activities, which could be useful in cancer therapy and drug targeting. Further, the strategy of incorporation of Aib or Acp in biologically active peptides can be exploited in determining the receptor-bound conformation and in transforming these bioactive peptides into pharmacologically useful drugs. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: substance P; α,α-dialkylated amino acids; anticancer peptides

INTRODUCTION

Substance P (SP) is an 11-amino acid neuropeptide (Arg^1-Pro^2-Lys^3-Pro^4-Gln^5-Gln^6-Phe^7-Phe^8-Gly^9-Leu^{10}-Met^{11}-NH_2) that belongs to the tachykinin family of chemically related peptides and is widely distributed through the central and the peripheral nervous system. It functions as a neurotransmitter and neuromodulator [1], is a potent vasodilator [2], and has been implicated in the regulation of diverse physiological and pathophysiological processes such as pain, smooth muscle contraction, inflammation, emesis, anxiety and depression [3,4]. It exerts its effects by binding to the neurokinin-1 (NK_1) receptor subtype. Using Bolton–Hunter I^{125}-labeled SP, Hennig *et al.* identified SP receptors (of the NK_1 subtype) on a number of tumor types, notably glioblastomas, medullary thyroid carcinomas, and ganglioblastomas. Interestingly, all tumor types including those lacking SP receptors, expressed NK_1 receptors in tumoral and peritumoral vessels [5].

The identification and characterization of substituted derivatives of the neuropeptide SP, which exhibit broad antagonist activity for a variety of neuropeptide receptors expressed on SCLC cells, provides a novel approach for the disruption of multiple neuropeptide autocrine systems [6]. A number of substituted derivatives of SP that exhibit antagonist activity have been synthesized. SP antagonists [D-Arg^1, D-Trp^{5,7,9}, Leu^{11}]-SP (Antagonist A) and [Arg^6, D-Trp^{7,9}, MePhe^8] (6–11)-SP (Antagonist G), and [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-SP (Antagonist D) were found to be potent inhibitors of small-cell lung cancer (SCLC) cells *in vitro* and SCLC xenografts in nude mice *in vivo*; they also inhibited vasopressin, bradykinin, cholecystokinin, gastrin-releasing peptide (GRP), and neurotensin induced Ca^{2+} mobilization and mitogen activated protein kinase (MAPK) activation [7–9]. Guha *et al.* have recently shown that the SP analog, Antagonist A, attenuates tumor cell growth in pancreatic cancer by a dual mechanism involving both antiproliferative and antiangiogenic properties [10].

Jarpe *et al.* hypothesized that Antagonist D had a unique agonist activity in addition to its antagonist function. According to them, Antagonist D acts as an agonist at GRP receptors, activating the G_{12} family of guanine-nucleotide-binding proteins while blocking signal transduction via Gq, and they proposed a novel pharmacological term, 'biased agonism' to describe this [11]. MacKinnon *et al.* examined the mechanism underlying this activity and showed that Antagonist D binds to the ligand-binding site of the bombesin/GRP receptor and inhibits the bombesin-stimulated increase in Ca^{2+} and growth in SCLC cells within the same concentration range in which it causes sustained activation of c-Jun *N*-terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK) [12]. The SP analog, Antagonist G, completed Phase I clinical trial with minimal toxicity (facial flushing) and

Abbreviations: Acp; cycloleucine or 1-amino cyclopentane carboxylic acid

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successfully blocked SP-induced vasodilatory effects *in vivo*, with no dose-limiting toxicity [13].

The conformational studies of the SP analog, Antagonist D, in solution, by CD, NMR, and MD simulations have shown that this SP analog exhibits flexible conformation in solution – it adopts β-turn conformation in DMSO and water and helical conformation in a hydrophobic environment [14]. In order to restrict the conformation further, we used the cyclic and linear α,α-dialkylated amino acids cycloleucine and Aib, respectively, and designed conformationally constrained analogs of Antagonist D. Both of these amino acids are known to induce either β-turn or helical conformation [15]. α,α-Dialkylated amino acids have been used to design analogs of different bioactive peptides such as chemotactic peptide [16,17], somatostatin [18], GnRH [19], bombesin [20], and parathyroid hormone [21]. Seven C^{α} methylated analogs of the model peptide SP (from positions 5 to 11) were synthesized and conformational analysis revealed that a single C^{α} methylation was not sufficient by itself to drastically stabilize a helical structure except when Gly^9 in the SP sequence was substituted by Aib [22].

In the present study, we describe the design and synthesis of six peptide analogs of SP by incorporating Aib or Acp in the Antagonist D sequence by replacing either leucine at position 11 (peptides **1** and **2**), or both D-Phe at position 5 and leucine at position 10 (peptide **3**). Specific substitutions at the above positions were made so as to keep the polar interactions of the designed peptide analogs with the receptor undisturbed. The heptapeptide (peptide **4**), which is a partial sequence of peptide **1**, has also been synthesized. We also investigate the functional consequences of these substitutions on the *in vitro* anticancer activity in human colon, lung, glioblastoma, and breast cancer cell lines. Further, peptide **1** was acylated at the *N*-terminal by butyric acid (peptide **5**) and octanoic acid (peptide **6**). All the three peptides, **1**, **5**, and **6** were examined for *in vivo* growth inhibition in primary tumor cell line (PTC) (colon) xenograft model in athymic mice.

MATERIALS AND METHODS

Peptide Synthesis and Purification

The peptides were synthesized by standard Fmoc solid phase peptide chemistry using the peptide synthesizer CS536 (CS Bio, San Carlos, CA, USA). All peptides were synthesized as peptide amides using the Rink amide resin. The side-chains were protected using the Trt protecting group for glutamine, Boc for lysine, and Pmc protecting group for arginine. All couplings were carried out in DMF. The Fmoc amino acid, di-isopropyl carbodiimide, and HOBt were used in three-fold molar excess. Successive de-protection and coupling steps were monitored by positive and negative ninhydrin tests, respectively. De-protection of the Fmoc group was carried out in 20% piperidine in DMF (v/v) for 30 min. The *N*-terminal was butanoylated and octanoylated in peptide **1** on solid phase using butyric acid and octanoic acid respectively with DIC and HOBt. The resin, all Fmoc protected amino acids and reagents were procured from Advanced Chemtech, Louisville, KY, USA.

Upon completion of synthesis, the *N*-terminal Fmoc group was deprotected and the peptide was cleaved from the resin in a cleavage mixture consisting of phenol, 1,2-ethanedithiol, thioanisole, distilled water, and TFA. The cleavage time was 2–3 h. The peptides were obtained as a filtrate in TFA and were precipitated with cold dry ether. The precipitate was filtered, dissolved in water or water–acetonitrile mixture, and lyophilized to obtain the crude peptide. The crude lyophilized peptide was analyzed by analytical HPLC and purified by preparative HPLC (Shimadzu Corporation, Japan) on a C-18 reverse phase column using a gradient of 0.1% TFA in acetonitrile and water. The correct molecular mass was obtained for the purified peptides by LC-MS (Micromass, Quattro, triple quadrupole mass spectrometer, Micromass, UK). The calculated mass values for the analogs were in agreement with the protonated molecular ions obtained using LC-MS. The HPLC retention time (RT) for each of the analogs was recorded in two different solvent systems: System 1, using a solvent system of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) and a linear gradient of 35–80% (B) in 30 min;

Table 1 Sequence, Mass and HPLC RTs of the synthesized SP analogs in two different solvent systems

PeptideCode	Peptide sequence	Mass		HPLC RT (min.)	
		Calculated Mass	Observed $[M+H^+]$	System 1[a]	System 2[b]
1	**D**-Arg-Pro-Lys-Pro-**D**-Phe-Gln-**D**-Trp-Phe-**D**-Trp-Leu-**Acp**^{11}-NH_2	1513.83	1515.6	12.16	7.42
2	**D**-Arg-Pro-Lys-Pro-**D**-Phe-Gln-**D**-Trp-Phe-**D**-Trp-Leu-**Aib**^{11}-NH_2	1487.75	1488.8	10.58	5.92
3	**D**-Arg-Pro-Lys-Pro-**Aib**^{5}-Gln-**D**-Trp-Phe-**D**-Trp-**Aib**^{10}-Leu-NH_2	1425.67	1425.8	8.04	4.20
4	D-Phe^5-Gln^6-D-Trp^7-Phe^8-D-Trp^9-Leu^{10}-**Acp**^{11}-NH_2	1035.23	1036.6	12.43	10.08
5	Butanoyl-**D**-Arg-Pro-Lys-Pro-**D**-Phe-Gln-**D**-Trp-Phe-**D**-Trp-Leu-**Acp**^{11}-NH_2	1583.93	1584.8	13.04	9.30
6	Octanoyl-**D**-Arg-Pro-Lys-Pro-**D**-Phe-Gln-**D**-Trp-Phe-**D**-Trp-Leu-**Acp**^{11}-NH_2	1639.83	1640.8	16.76	12.42

[a] System1: Water (0.1% TFA) (A) and acetonitrile (0.1% TFA) (B); 35–80% (B) in 30 min. [b] System2: 0.1 M TEAP (A) and acetonitrile (B); 35–80% (B) in 30 min.

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Table 2 Percentage inhibition of cell proliferation by the SP analog, peptide **1**, at different concentrations

	% inhibition of cell proliferation at different concentrations				
Cell line	1 nM	10 nM	100 nM	1 μM	10 μM
MCF-7 (Breast)	2.4 ± 1.2	7.5 ± 1.5	8.6 ± 1.6	12.4 ± 2.6	18.4 ± 6.4
U373 (Glioblastoma)	3.1 ± 0.5	5.0 ± 0.7	4.5 ± 0.5	17.1 ± 1.9	37.2 ± 5.3
PTC (Colon)	3.1 ± 0.5	3.1 ± 1.9	6.7 ± 1.3	25.5 ± 4.8	35.5 ± 4.8
L132 (Lung)	15.0 ± 5.8	19.0 ± 3.3	21.0 ± 1.2	20.0 ± 2.3	23.0 ± 2.3

System 2, using a solvent system of 0.1 M TEAP (triethylamine phosphate) buffer (A) and acetonitrile (B) and a linear gradient of 35–80% (B) in 30 min. The peptide sequence, mass, and HPLC RTs of the synthesized SP analogs in two different solvent systems are summarized in Table 1.

Biological Activity

In vitro anticancer activity using MTT assay. Preliminary screening of the SP analogs **1**, **2**, **3**, and **4** for antiproliferative activity against different human tumor cell lines using the MTT cytotoxicity assay [23] revealed that the SP analog **1** was the most active. The values for the percentage inhibition of cell proliferation for the analog **1** in human tumor cell lines representing breast (MCF-7), glioblastoma (U373), lung (L132), and PTC colon cancer cell lines were determined at different concentrations ranging from 1 nM to 10 μM, in triplicates. While the cell lines MCF-7, U373, and L132 were obtained either from the ATCC or National Centre for Cell Science, Pune, India the PTC was derived from primary human colon adenocarcinoma biopsies [24]. Results were calculated as percent inhibition of cell proliferation according to the formula:

$$\% \text{ inhibition of cell proliferation} = 100 \times [1 - (X/R_1)]$$

Where $X = [(\text{absorbance of treated sample at 540 nm}) - (\text{absorbance of control at 540 nm})]$ and $R_1 = \text{absorbance of control sample at 540 nm}$.

In vivo anti-tumor activity in primary tumor (colon) xenografts in athymic mice. Human primary colon adenocarcinoma (PTC) xenografts were grown in Balb/c athymic nude mice by the subcutaneous inoculation of a single suspension of PTC cells (15×10^6 cells/100 μl). The tumor-bearing animals were divided into four groups (control, and treated with peptides **1**, **5**, and **6**) of three animals each. Treatment with peptides was initiated when the average tumor volume, as measured using a Vernier calipers, was between 250 and 800 mm^3. Peptides were prepared at a concentration of 42.5μg/ml and administered intravenously to the tumor-bearing animals at a dose of 4.25 μg/100 μl, twice a day, so that a total dose of 8.5 μg per day was administered to each animal. The tumor volumes were monitored till day 22 of tumor initiation. The antitumor activity of the compound was measured using the formula W × W × L × 0.4 (W = smaller diameter, L = larger diameter). The percentage inhibition of tumor growth was calculated using the formula: [1 – tumor volume (treated)/tumour volume (control)] ×100.

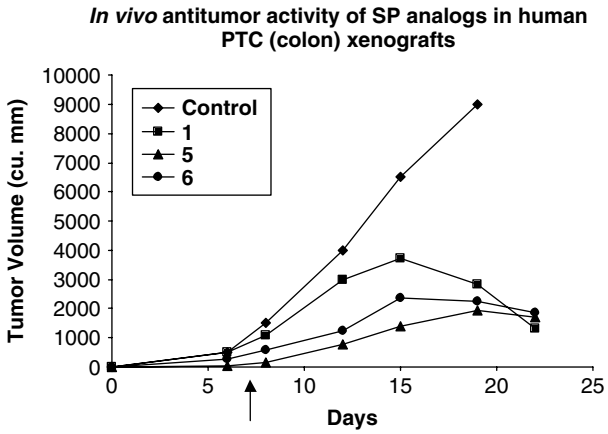

Figure 1 *In vivo* antitumor activity of SP analogs (**1**, **5**, and **6**) treated *versus* control (untreated) animals in human primary colon tumor (PTC) xenografts in athymic nude mice. The arrow represents the day of initiation of treatment.

RESULTS

The peptide sequence, mass and HPLC RTs of the synthesized SP analogs in two different solvent systems are summarized in Table 1.

In vitro Anticancer Activity

The values obtained for the *in vitro* antiproliferative activity (% inhibition of cell proliferation ± standard error) of the SP analog **1** as obtained by the MTT assay for each of the human tumor cell lines representing breast (MCF-7), glioblastoma (U373), lung (L132), and colon (PTC) at ten-fold incremental concentrations ranging from 1 nM to 10 μM, are shown in Table 2.

In vivo Anticancer Activity in PTC (colon) Tumor Xenograft Mouse Model

Figure 1 shows the tumor kinetics of primary colon tumor (PTC) xenografts till day 22 in the treated (**1, 5** and **6**) and control (untreated) animals. All untreated animals died by day 19 of tumor initiation. All SP

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analogs caused regression of tumor xenografts as compared to control. The percentage inhibition of tumor growth on day 19 was 68.35% for analog **1**, 78.54% for **5** and 75.17% for peptide **6** treated mice.

DISCUSSION

Peptides and peptide receptors are emerging as promising targets for cancer diagnosis and therapy. Peptide receptors are expressed in large quantities in many tumors. Most peptide receptors belong to the family of G-protein coupled receptors (GPCR), whose agonists function as autocrine/paracrine growth factors in cancer; hence, broad-spectrum antagonists could potentially arrest the growth of cancer cells by binding to these receptors [25,26].

The present study is a part of our studies aimed at developing a neuropeptide-based drug for the treatment of cancer [27–29]. Agonists to the neuropeptide somatostatin and antagonists to the neuropeptides bombesin, vasoactive intestinal peptide receptor binding inhibitor, and several others, inhibit cancer cell proliferation *in vitro* and *in vivo* [18,20,30]. Multiple neuropeptide analogs have also been linked together as a propeptide to deliver the component peptides and exert growth inhibition *in vitro* and *in vivo* [29]. SP and its receptor, the NK1 receptor, are also involved in a number of processes related to oncogenesis such as mitogenesis, angiogenesis, cell migration, and metastasis [31]. We designed and synthesized six Aib or cycloleucine-containing analogs of the potent SP analog, Antagonist D. Site-specific modifications were made at positions of hydrophobic amino acids without disturbing any polar interactions. In a preliminary *in vitro* MTT assay on human cancer cell lines, we tried to investigate the effect of the substitutions on antiproliferative activity, where peptide **1** showed 25.5 and 35.5% inhibition of cell proliferation in the human primary colon cancer cell line PTC at 1 μM and 10 μM concentrations, respectively. It also showed a maximum inhibition of cell proliferation of 37.2% at 10 μM concentration in U373 glioblastoma cell line as depicted in Table 2. We also tried to improve the biological half-life of peptide **1** by blocking the *N*-terminus with butanoyl (peptide **5**) and octanoyl (peptide **6**) groups. *N*-terminal acylation of the somatostain analog RC-160 has been shown to improve its stability, biological availability, and anticancer activity in human breast adenocarcinoma cells [32]. We decided to assess the *in vivo* tumor regression potential of analog **1** and the *N*-terminal acylated analogs **5** and **6** in the human PTC colon tumor xenograft model in athymic nude mice. We presumed that colon cancer cell lines derived from primary tumor biopsies would closely represent the clinical situation, and hence human PTC (colon) xenografts were chosen for the *in vivo* study. All the three SP analogs **1**, **5**, and **6** caused appreciable reduction in tumor volumes in human PTC xenografts in athymic nude mice by day 19 (68.35% for analog **1**, 78.54% for **5** and 75.17% for **6**). Although the tumor regression values for the three analogs are closely similar, the butanoylated analog **5** and the octanoylated analog **6** were found to be more toxic (unpublished data) and were not studied further. Also, there was further reduction in tumor volume with analog **1** by day 22 when compared with analogs **5** and **6**.

CONCLUSIONS

This study demonstrates that the constrained amino acids Aib and Acp can be used to design antagonists of SP with potent *in vitro* and *in vivo* anticancer activities. *N*-terminal butanoylation or octanoylation may retain or enhance the *in vivo* anticancer activities, but also makes the molecule more toxic in this case (unpublished data). Thus, SP analogs containing site-specific substitutions with α,α-dialkylated amino acids could prove to be promising candidate molecules for future use of these analogs in cancer diagnosis, therapy, or drug targeting.

Further, the strategy of incorporation of Aib or Acp in biologically active peptides can be exploited in determining the receptor-bound conformation and in transforming these bioactive peptides into pharmacologically useful drugs.

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