

# Delivering multiple anticancer peptides as a single prodrug using lysyl-lysine as a facile linker

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**Abstract:** A large 40-residue precursor peptide (propeptide 5) was synthesized by linking together four designed anticancer peptide analogs to the neuropeptides: vasoactive intestinal peptide, somatostatin, bombesin and substance P, using enzyme cleavable lysyl-lysine linkers. On incubation with the enzyme trypsin, propeptide 5 was cleaved in a sequence-specific manner at the lysyl-lysine residues in the linker to release the individual peptide fragments which were identified by LC-MS. Another precursor peptide (propeptide 5a), consisting of two of the peptide analogs linked through lysyl-lysine linker, was also preferentially cleaved at the Lys-Lys site on incubation with the enzyme trypsin. Propeptide 5 showed potent anticancer activity, both *in vitro* and *in vivo*, which was greater than that of the individual component peptides. The enhanced activity suggests that the propeptide is possibly cleaved in the biological system at the lysyl-lysine site to yield the individual peptide analogs, which together show a synergistic effect. On the basis of these experimental findings, it can be concluded that pairs of basic amino acids such as Lys-Lys can be used as facile linkers for delivering multiple biologically active peptides. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** neuropeptide; dibasic amino acid linker; anticancer peptides; propeptide

## INTRODUCTION

Peptides and peptide receptors are expressed in large quantities in certain tumors and have become attractive targets for cancer diagnosis and therapy. Studies have shown that neuropeptides such as vasoactive intestinal peptide (VIP), somatostatin, bombesin, substance P and several others act by binding to specific high-affinity receptors on cancer cells and play a key role in tumor cell proliferation [1]. Specially designed agonists of growth-inhibiting peptides such as somatostatin or antagonists of growth stimulating peptides such as VIP, bombesin and substance P can abrogate the proliferative effects of these peptides by binding to their receptors [2–6]. We have designed and synthesized several novel and conformationally constrained analogs of the VIP receptor binding inhibitor [7,8], somatostatin [9], bombesin [10] and substance P [11].

Malignant tumor cells are often characterized by the deregulation of multiple signaling pathways that eventually promote proliferation, inhibit apoptosis and enable metastasis and angiogenesis. A single drug may have a modest to striking impact on the tumor behavior initially, but the regressing cancer cells frequently acquire additional mutations that make them resistant to the original therapy leading to tumor recurrence. It is

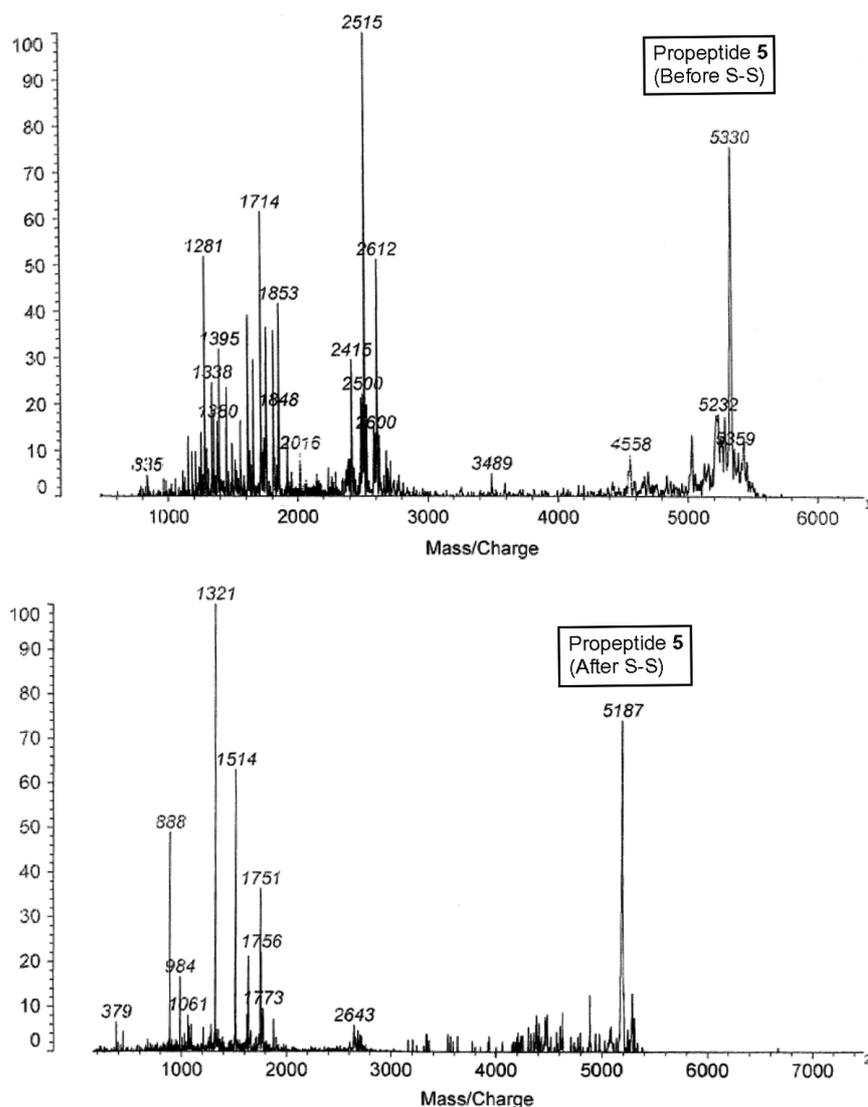
believed that the most useful and long-term therapeutic benefits are likely to be achieved with combination regimens that address several targets [12]. We selected peptide analogs of VIP, somatostatin, bombesin and substance P with potent anticancer activities and reasoned that it was possible to use these analogs either simultaneously or sequentially in combination and modulate multiple signaling pathways. We found that when these peptides analogs were used in combination, they exerted a synergistic effect, which was greater than the anticancer activity when used individually [13,14].

In the present work, four designed analogs of VIP, bombesin, substance P and somatostatin are linked together using a linker consisting of pairs of basic amino acids such as Lys-Lys to form a propeptide. It is assumed that the designed individual analogs will be released from the polypeptide by specific enzymatic cleavage at the linker site *in vivo*. Such cleavage at pairs of basic residues is also known in biological systems. In fact, most peptides in the human body are synthesized as large precursors or propeptides and undergo endoproteolytic cleavage at pairs of basic residues (Lys-Arg, Arg-Arg, Lys-Lys or Arg-Lys) by enzymes called prohormone convertases (PCs), to give rise to the active molecule [15–17].

The sequences of the four designed peptide analogs used in the present study are: Leu-Met-Tyr-Pro-Thr-Tyr-Leu-Lys-OH (**1**), the VIP receptor binding inhibitor [8]; D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH<sub>2</sub>

Abbreviations: CyLeu, cycloleucine or 1-aminocyclopentane-1-carboxylic acid; NMP, *N*-methylpyrrolidinone; TPCK, *L*-1-tosylamide-2-phenylethyl chloromethyl ketone.

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**Figure 1** MALDI-MS spectra of propeptide **5** before and after the disulfide bond formation.

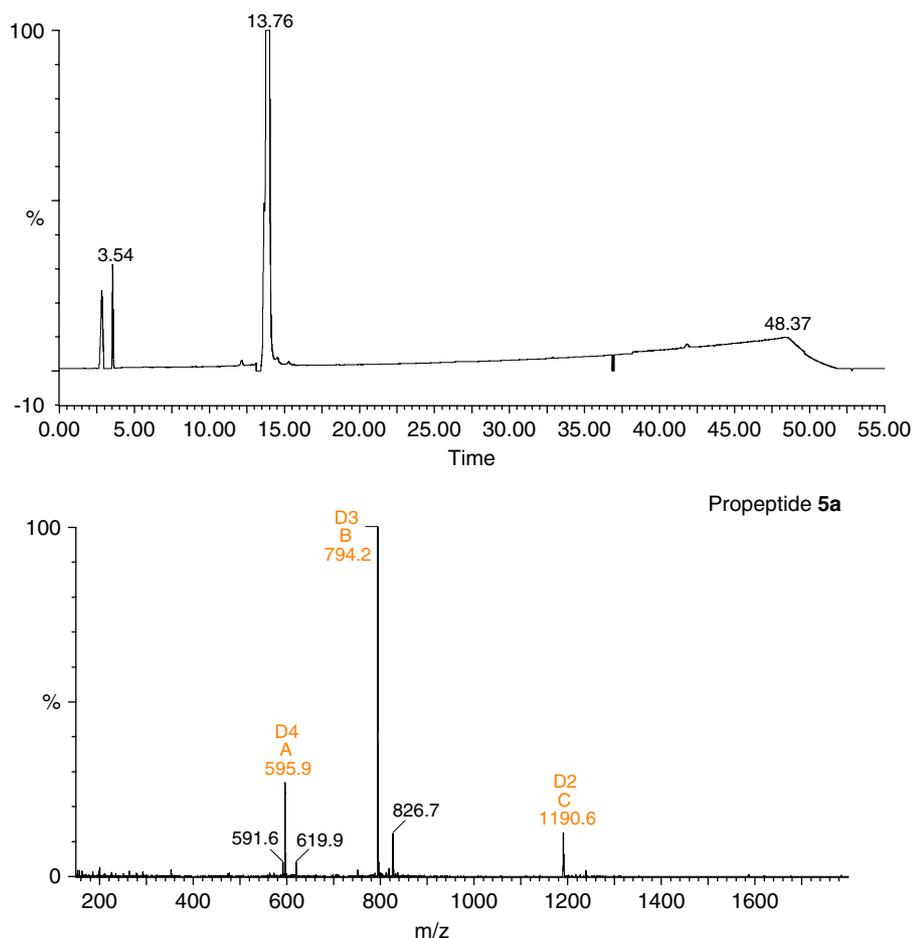
(**2**), the bombesin antagonist [10]; D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-CyLeu-NH<sub>2</sub> (**3**), the substance P antagonist [11] and D-Phe-Cys-Tyr-D-Trp-Orn-CyLeu-Pen-Thr-NH<sub>2</sub>, with a disulfide bond between Cys and Pen amino acids (**4**), the somatostatin analog [9]. In order to co-administer all the four peptides analogs as a single moiety, the peptides are linked using the Lys-Lys linker to form a precursor peptide of 40 amino acids. The sequence of the propeptide synthesized is: D-Phe<sup>1</sup>-Cys-Tyr-D-Trp-Orn<sup>5</sup>-CyLeu-Pen-Thr-Lys-Lys<sup>10</sup>-D-Arg-Pro-Lys-Pro-D-Phe<sup>15</sup>-Gln-D-Trp-Phe-D-Trp-Leu<sup>20</sup>-CyLeu-Lys-Lys-Leu-Met<sup>25</sup>-Tyr-Pro-Thr-Tyr-Leu<sup>30</sup>-Lys-Lys-D-Phe-Gln-Trp<sup>35</sup>-Ala-Val-Aib-His-Leu<sup>40</sup>-NH<sub>2</sub>, with a disulfide bond between Cys and Pen amino acids (**5**). Additionally, propeptide **5a**, having the sequence: Lys<sup>22</sup>-Lys-Leu-Met<sup>25</sup>-Tyr-Pro-Thr-Tyr-Leu<sup>30</sup>-Lys-Lys-D-Phe-Gln-Trp<sup>35</sup>-Ala-Val-Aib-His-Leu<sup>40</sup>-NH<sub>2</sub>, was also synthesized. The propeptides **5** and **5a** were incubated with the enzyme trypsin, and the site of

preferential enzymatic cleavage was confirmed by analyzing the peptide fragments obtained by LC-MS/MS. The enhanced *in vitro* and *in vivo* anticancer activity also suggests that propeptide **5** is possibly cleaved at the Lys-Lys site to release the individual peptides that act synergistically.

## MATERIALS AND METHODS

### Peptide Synthesis and Purification

The propeptide **5** was synthesized on the peptide synthesizer CS536 (CS Bio, San Carlos, CA, USA) by standard Fmoc solid-phase peptide chemistry. The peptide was assembled from C- to N-terminal on the Rink Amide resin [4-(2', 4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy-methyl-derivatized polystyrene 1% divinylbenzene resin, 100–200 mesh, substitution of 0.6 mm/gm] as peptide amide. All the amino acids used were N-Fmoc protected. The side chains were protected using the trityl protecting group for glutamine,



**Figure 2** LC chromatogram and LC-MS spectra of propeptide **5a**.

trityl or Boc protecting groups for histidine, acetamidomethyl (Acm) for cysteine and penicillamine, Boc group for lysine and ornithine, *t*-butyl (tBu) protecting group for tyrosine and threonine and Pmc (pentamethyl chroman-6-sulphonyl) protecting group for arginine. All couplings were carried out in DMF, DCM or NMP or a mixture of these solvents. Coupling reactions were carried out using HBTU/HOBt and DIEA in three- to fourfold molar excess. Deprotection of the Fmoc group was carried out in 20% piperidine in DMF (v/v) for 30 min. Successive deprotection and coupling steps were monitored by positive and negative Kaiser (ninhydrin) test [18], respectively. The resin, all Fmoc protected amino acids and reagents were procured from Advanced Chemtech, Louisville, KY, USA.

After completion of synthesis and deprotection of the *N*-terminal amino acid, the cleavage of peptide from the resin and simultaneous removal of side-chain protecting groups were performed by treatment with a cleavage mixture consisting of crystalline phenol, 1,2-ethanedithiol, thioanisole, distilled water and trifluoroacetic acid for 3 h. The peptide was filtered from the resin and precipitated with cold dry ether. The precipitate was filtered, dissolved in water and lyophilized to obtain the crude peptide. Disulfide bond formation was carried out by conventional methods using iodine or thallium trifluoroacetate and monitored by MALDI-MS. The resulting crude disulfide-bridged peptide was purified on a preparative HPLC system (Shimadzu Corporation, Japan) on a C-18 reverse phase column using a gradient of 0.1%

TFA in acetonitrile and water. The purified propeptide **5** was characterized by MALDI-MS (Kompag SEQ MALDI-TOF mass spectrometer, Kratos Analytical, Manchester, UK).

In between the synthesis, an aliquot of the peptide-resin was taken to obtain propeptide **5a**, having the sequence: Lys<sup>22</sup>-Lys-Leu-Met<sup>25</sup>-Tyr-Pro-Thr-Tyr-Leu<sup>30</sup>-Lys-Lys-D-Phe-Gln-Trp<sup>35</sup>-Ala-Val-Aib-His-Leu<sup>40</sup>-NH<sub>2</sub>. This peptide was similarly cleaved from the resin and purified. The mass spectrum of the purified peptide was recorded by LC-MS (Micromass, Quattro, triple-quadrupole mass spectrometer, Micromass, UK).

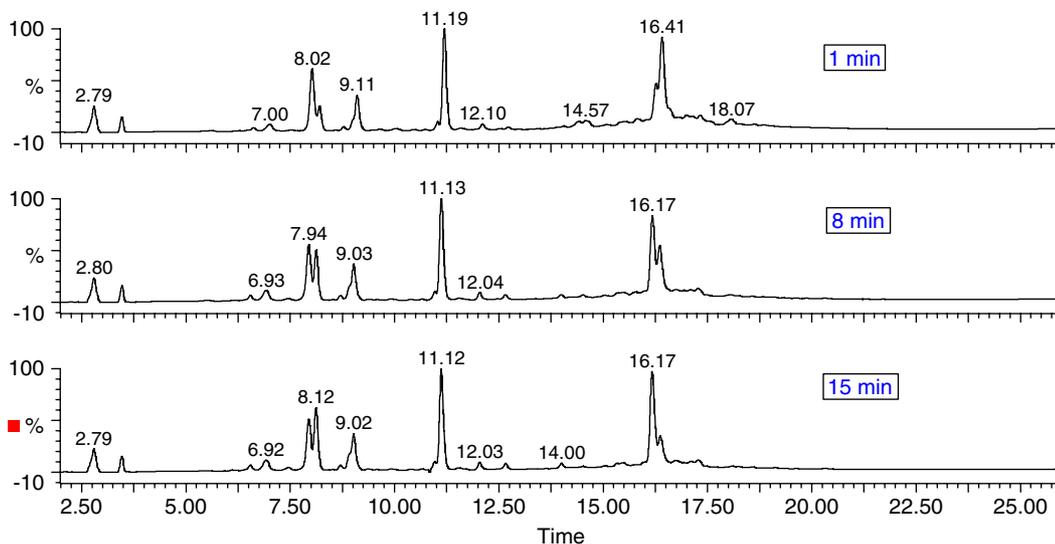
### Trypsin Digestion of Propeptides **5** and **5a**

The propeptides **5** and **5a** were weighed and dissolved in 1 : 1 (v/v) of water: ammonium bicarbonate buffer (pH 8.0) and mixed with the freshly made enzyme trypsin (Sigma, TPCK treated; 1mg/ml in water) in a ratio of 1 : 25 (w/w) of enzyme: substrate, according to published protocol [19]. The mixture was incubated at 37 °C. Aliquots were taken at times 1, 8 and 15 min and analyzed by LC-MS.

### *In vitro* Anticancer Activity of Propeptide **5** Using MTT Assay

The propeptide **5** was tested for anti-proliferative activity against five human tumor cell lines. While the cell lines

## Propeptide 5-Trypsin Digest



**Figure 3** LC chromatograms of trypsin digest samples of propeptide **5** at 1, 8 and 15 min.

MOLT-4 (leukemia), MCF-7 (breast), MiaPaCa.2 (pancreas) and KB (oral) were obtained either from ATCC or the National Centre for Cell Science, Pune, India, the primary tumor cell line, primary colon adenocarcinoma (PTC), was derived from primary human colon adenocarcinoma biopsies [20]. The MTT assay [21] is based on the principle of uptake of MTT [(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide), a tetrazolium salt, by metabolically active cells where it is metabolized by active mitochondria into a blue colored formazan product which can be read spectrophotometrically. Briefly, the human tumor cell lines were cultured to 70% confluence in a medium (Dulbecco's modified Eagle's medium (DMEM), Gibco, BRL, USA) supplemented with 10% fetal calf serum (Gibco, BRL, USA) and subsequently harvested using 0.25% trypsin containing 0.2 mM EDTA solution. The cells were replated in 96-well tissue culture plates at a density of 5000–10 000 cells/well and incubated overnight in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C to allow complete reattachment of the cells. Propeptide **5** was added in 10-fold incremental concentrations (ranging from 1 nM to 10 μM, in triplicate wells, once every 24 h, for a period of 72 h) to get a dose response. After 72 h, the assay was terminated and 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  $X = [(\text{absorbance of treated sample at } 540 \text{ nm}) - (\text{absorbance of control at } 540 \text{ nm})]$  and  $R_1 = \text{absorbance of control sample at } 540 \text{ nm}$

The percentage inhibition of cell proliferation was determined at different concentrations (ranging from 1 nM to 10 μM) and ED<sub>50</sub> values were calculated for propeptide **5** in each of the cell lines tested.

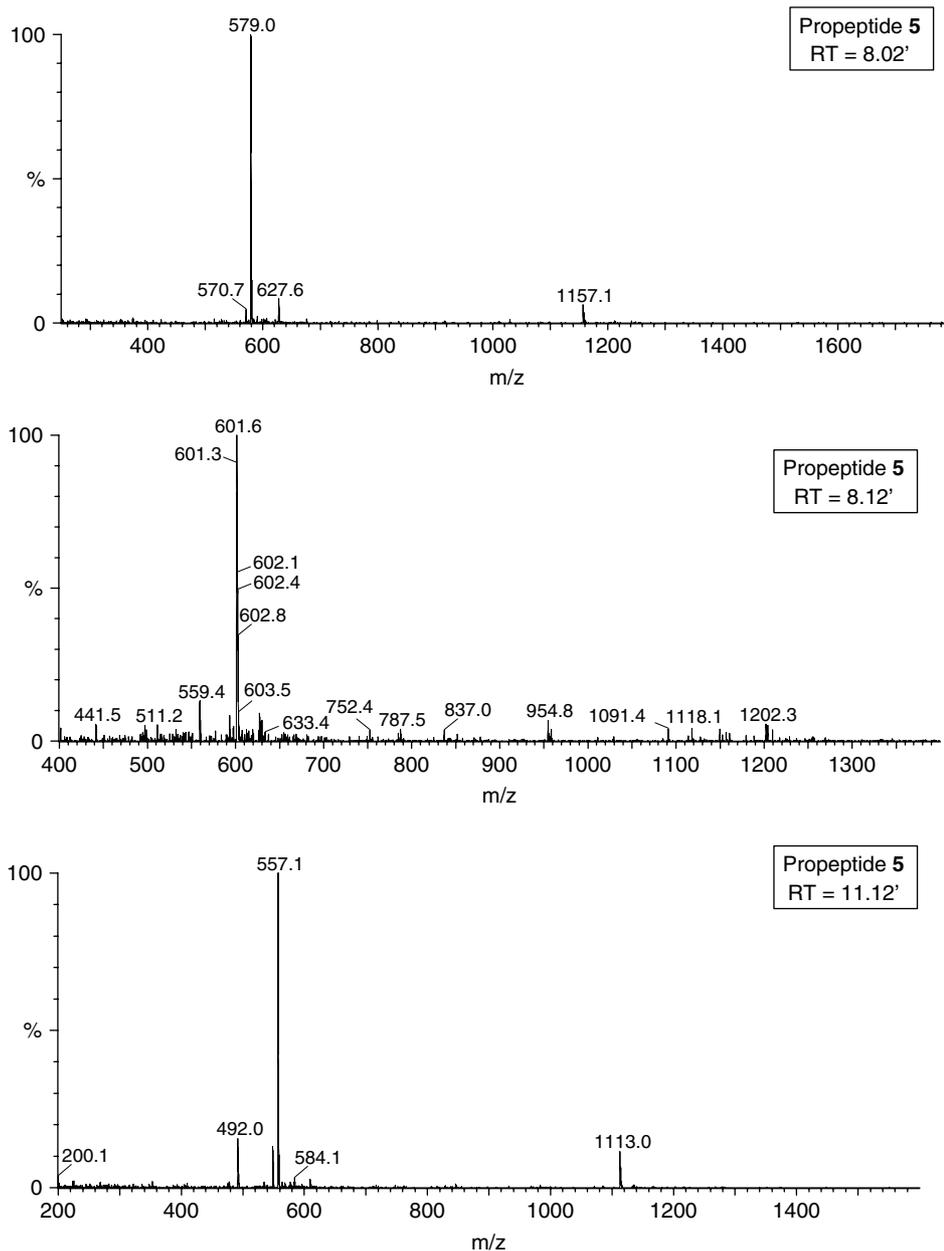
### ***In vivo* Antitumour Activity of Propeptide 5 in Tumor Xenografted Nude Mice**

Human PTC tumor xenografts were initiated in Balb/c athymic nude mice by subcutaneous inoculation of a single cell suspension of PTC cells (15 × 10<sup>6</sup> cells/100 μl). The tumor-bearing animals were divided into two groups of three animals each of treated and untreated (control) animals. Treatment with peptide **5** was initiated when the average tumor volumes, as measured using a Vernier callipers, were ~300–800 mm<sup>3</sup>. Peptide **5** was prepared at a concentration of 250 μg/ml so as to deliver a dose of 50 μg/200 μl. The injections were given twice a day at 12-h intervals so as to deliver a total dose of 100 μg. The treatment was continued daily for a period of 15 days. The tumor volumes were measured using the formula  $W \times W \times L \times 0.4$  ( $W$  = smaller diameter,  $L$  = larger diameter). The percentage inhibition of tumor growth was calculated using the formula:  $[1 - \text{tumor volume (treated)}/\text{tumor volume (control)}] \times 100$ .

## **RESULTS**

### **Peptide Synthesis**

The presence of several unnatural amino acids such as Aib, CyLeu and Pen makes the synthesis of the peptide difficult. Usually, no blue/purple color was obtained in the deprotection step following the coupling of the unnatural amino acids, Fmoc-Aib-OH and Fmoc-CyLeu-OH, in propeptide **5**. Double coupling of the amino acid next to these unnatural amino acids in the synthesis sequence was carried out to prevent deletion. The presence of 'close-by' peaks made it difficult to purify propeptide **5** beyond 90% purity by HPLC. Correct molecular mass was



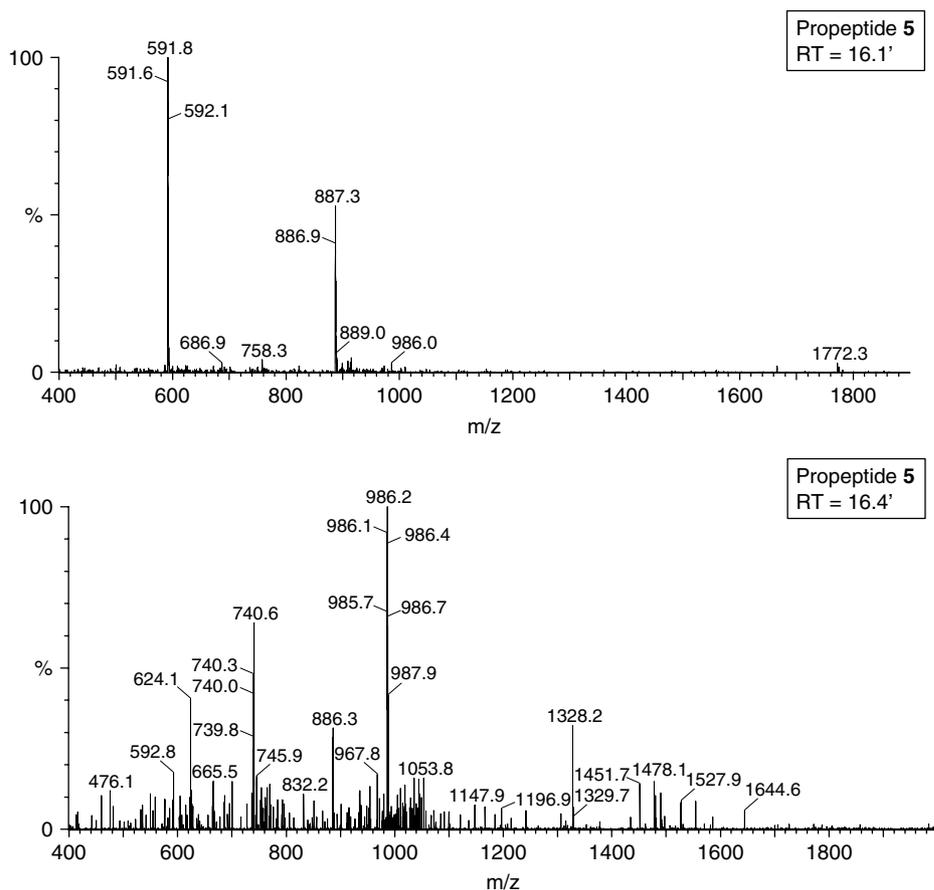
**Figure 4** (a) and (b) LC-MS spectra corresponding to fragments of propeptide **5**, according to retention time, as obtained in the LC chromatograms of trypsin digest samples of propeptide **5**.

obtained for peptide **5** by MALDI-MS before (calculated mass = 5331.4 Da, observed mass = 5330 Da) and after disulfide bond formation (calculated mass = 5188.4 Da, observed mass = 5187 Da) as shown in Figure 1. Propeptide **5a** could, however, be purified to >97% purity and its correct molecular mass was obtained by LC-MS (calculated mass = 2379 Da, observed  $M/2 = 1190.6$ ,  $M/3 = 794.2$ ,  $M/4 = 595.9$ ) as shown in Figure 2.

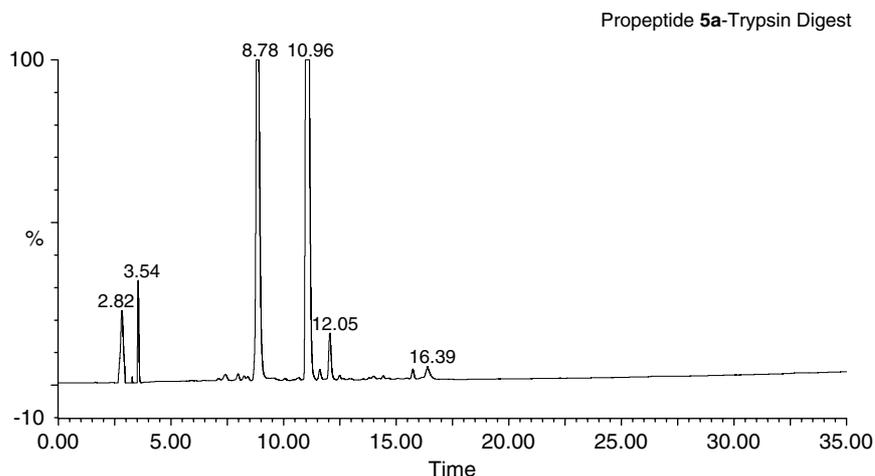
#### LC-MS Data of Trypsin Digest of Propeptide 5

Aliquots of the trypsin digest samples of propeptide **5**, taken at 1, 8 and 15 min, were diluted 1:1 (v/v)

with acetonitrile and analyzed as shown in Figure 3. The individual mass values for peptides **1**, **2**, **3** and **4** are 1027.5, 984.6, 1514.9 and 1071.6, respectively. Mass data for the peaks at the following retention time (RT) could be assigned: molecular mass of 1157.1 ( $M/2 = 579.0$ ) corresponding to the RT of 8.02 min could be assigned to peptide **1** + Lys; the peak at 8.12 min increased with time and had the molecular mass of 1202.0 ( $M/2 = 601.6$ ), which corresponds to the mass of (Peptide **4** (SH-SH) + Lys). The peak at the RT of 11.12 min corresponds to (Lys + Peptide **2**) at a molecular mass of 1113.1 ( $M/2 = 557.0$ ). The peak at 16.41 min corresponds to (peptide **4** + Lys + Lys + Peptide **3** + Lys) with a molecular mass of



**Figure 4** (Continued).

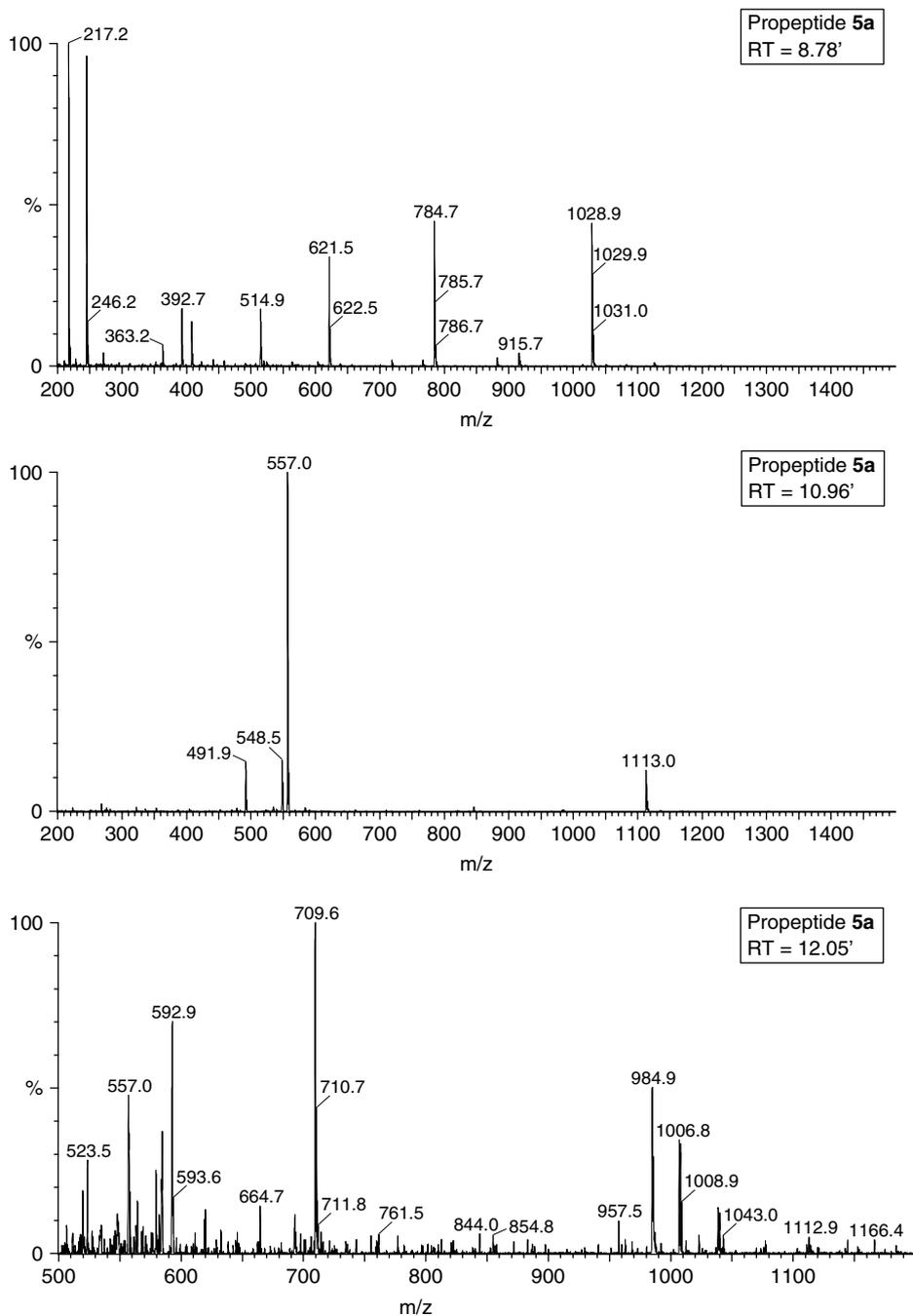


**Figure 5** Representative LC chromatogram of trypsin digest sample of propeptide 5a.

2956.5 Da ( $M/2 = 1478.1$ ,  $M/3 = 986.2$ ,  $M/4 = 740.6$ ,  $M/5 = 592.3$ ). This peak decreases with time, while the peak at 16.17 min increases with time and corresponds to the peptide fragment (Lys + Peptide 3 + Lys) at a molecular mass of 1772.3 ( $M/2 = 887.3$ ,  $M/3 = 591.8$ ). The recorded mass data for the assigned peptide fragments of propeptide 5 according to the RTs is depicted in Figure 4(a) and (b).

#### LC-MS Data of Trypsin Digest of Propeptide 5a

Propeptide 5a was similarly incubated with the enzyme trypsin at 37 °C and pH 8.0 and data analyzed for the time points 1, 8 and 15 min by LC-MS. A similar peak pattern was observed at the different time points. A representative chromatogram depicting the trypsin digest of peptide 5a is shown in Figure 5. Mass



**Figure 6** LC-MS spectra corresponding to the assigned trypsin digest fragments of propeptide **5a**, according to retention time, as obtained in the LC chromatogram (Figure 5) of propeptide **5a**.

data for the peaks at the following RTs could be assigned: the molecular mass of 1028.9 at the RT of 8.78 min could be assigned to Peptide **1**; the molecular mass of 1113.0 ( $M/2 = 557.0$ ) at the RT of 10.96 min corresponds to [Lys + Peptide **2**]; the mass of 984.9 Da ( $M + Na^+ = 1006.8$ ) at the RT of 12.05 min could be assigned to peptide **2**. The recorded mass data for the assigned peptide fragments of propeptide **5a** according to the RTs is depicted in Figure 6.

### ***In vitro* Inhibition of Cell Proliferation of Peptide 5 on Tumor Cell Lines**

Table 1 shows the dose response of peptide **5** over a concentration range of 1 nM to 10  $\mu$ M along with the respective  $ED_{50}$  values for different tumor cell lines.

### ***In vivo* Antitumor Activity of Peptide 5 on PTC (colon) Tumor Xenografts**

The tumor volumes for the propeptide **5**-treated and the untreated (control) animals were plotted on the

**Table 1** Dose response and ED<sub>50</sub> values of peptide **5** on human tumor cell lines: KB (oral), Mia Pa Ca.2 (pancreas), PTC (colon), MCF-7 (breast) and MOLT-4 (leukemia)

Cell line	Percentage inhibition of cell proliferation					ED <sub>50</sub> (μM)
	1 nM	10 nM	100 nM	1 μM	10 μM	
KB (Oral)	10.6 ± 1.2	18.2 ± 1.9	30.7 ± 3.9	43.1 ± 5.3	98.3 ± 5.7	2.1
MiaPaCa.2 (pancreas)	24.2 ± 2.7	37.8 ± 4.6	48.2 ± 4.8	59.6 ± 4.9	84.5 ± 6.3	0.210
PTC (colon)	16.5 ± 1.6	14.2 ± 2.2	16.8 ± 1.0	21.7 ± 2.9	27.9 ± 2.8	>10
MCF-7 (breast)	29.7 ± 3.8	28.2 ± 4.3	35.3 ± 4.8	59.4 ± 4.8	85.4 ± 5.0	0.34
MOLT-4 (leukemia)	10.4 ± 0.7	18.7 ± 1.9	34.7 ± 4.7	54.3 ± 5.2	94.5 ± 6.1	0.29

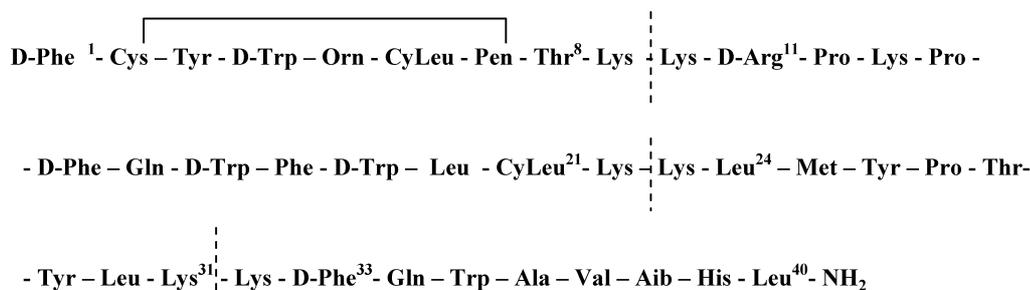
Y-axis and the number of days (post-treatment) was plotted on the X-axis as shown in Figure 7. A tumor regression of 73.7% was seen in propeptide **5**-treated tumor-bearing mice as compared to untreated mice.

## DISCUSSION

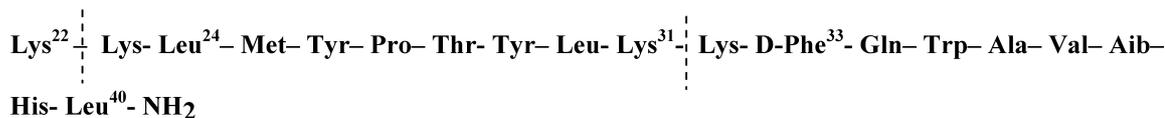
Most peptides in the human body are synthesized as larger precursors or propeptides. The generation of active forms of the peptides often requires enzymatic processing of the precursor peptides at sites containing two or more basic amino acids such as Lys-Lys, Lys-Arg, Arg-Arg or Arg-Lys. Processing at these basic amino acids involves endopeptidase action by enzymes such as PC 1 or 2 followed by the removal of the basic

through pairs of lysine residues. We checked for preferential enzymatic cleavage at the lysyl-lysine site by incubating propeptides **5** and **5a** with the enzyme trypsin (TPCK treated) as a model enzyme, and analyzed the peptide fragments obtained by LC-MS. It is known that at pH 8.0 and 37°C, the conditions required for its optimal activity, the enzyme trypsin also undergoes autolysis to produce among other products, ψ-trypsin, which has chymotrypsin-like activity; the treatment with TPCK specifically inhibits chymotrypsin, which may contaminate trypsin preparations [21].

It was possible to identify all the four peptides (linked to amino acid lysine) by LC-MS after incubation of propeptide **5** with enzyme trypsin. Similarly, the two constituent peptides were identified in the trypsin digest of propeptide **5a**. The following enzymatic cleavage pattern emerged:



Enzymatic cleavage sites in propeptide **5** sequence.



Enzymatic cleavage sites in propeptide **5a** sequence.

residue(s) primarily by the enzyme carboxypeptidase E [15,22]. Using a similar strategy, we synthesized a large 40-residue propeptide **5**, by linking together four neuropeptide analogs of known anticancer activities

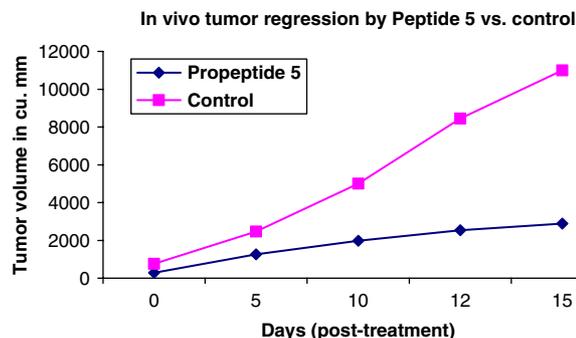
We could not determine the enzymatic degradation pattern *in vivo* in mouse or rat model because the half-life of the four peptides was short and it was difficult to

quantify them by HPLC or LC-MS/MS even when they were injected individually in rat or mice.

The four neuropeptide analogs individually exert anticancer activities by targeting different receptors and signaling pathways. It is therefore possible for the precursor peptide to release the peptide analogs, target several pathways simultaneously and even achieve synergistic activity. We have reported in our previous studies that the *in vitro* inhibition of cell proliferation for individual peptide analogs was 10–40% over a concentration range and ED<sub>50</sub> values were seldom achieved [8–11]; however, we observed ED<sub>50</sub> values in the micro molar range for propeptide **5** in most of the human tumor cell lines. Also, while we report an *in vivo* tumor regression of 73% for propeptide **5** in this study, we have earlier reported tumor regression only in the range of 30–68% for the individual analogs [8–11]. This enhanced *in vitro* and *in vivo* anticancer activity found for the designed propeptide **5** can be explained on this basis of the possible cleavage of the peptide **5** at the lysyl–lysine site in the biological system and the release of individual peptide analogs to exert a synergistic effect.

## CONCLUSIONS

On the basis of these experimental findings, it can be concluded that pairs of basic amino acids can be used as facile linkers to deliver multiple biologically active peptides simultaneously for possible additive or synergistic effect. The strategy of using a multipronged approach to treat cancer by targeting multiple pathways is gaining momentum [12]. We propose the concept of multiple-receptor targeting in cancer and the use of easily cleavable linkers to deliver multiple peptide ligands. This approach can have important applications in cancer therapy and diagnosis in future.



**Figure 7** *In vivo* tumor regression (of 73.7%) of propeptide **5**-treated versus untreated (control) animals in human primary colon (PTC) tumor xenografts in athymic nude mice. Tumor volumes (in mm<sup>3</sup>) are plotted on the Y-axis and the days (post-treatment) on the X-axis.

## Acknowledgements

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