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Bombesin analogs containing α-amino-isobutyric acid with potent anticancer activity

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Abstract: Six octapeptide bombesin (BN) analogs were synthesized by substituting α-aminoisobutyric acid (Aib), in place of Ala^9 or Gly^{11}, or both, in the [D-Phe^6, *des*Met^{14}]-BN (6–14) sequence: D-Phe^6-Gln^7-Trp^8-Ala^9-Val^{10}-Gly^{11}-His^{12}-Leu^{13}-NH_2 (**P0**). Additionally, Leu^{13} was replaced with isoleucine in two analogs and one of the analogs was butanoylated at the *N*-terminus. The antiproliferative activity of the analogs was tested *in vitro* on human pancreatic (MiaPaCa-2) and colon cancer (SW620, HT29 and PTC) cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The analogs demonstrated anticancer activity in the above cell lines at concentrations ranging from 0.01 nM to 1 μM. One of the analogs, **P6**, was evaluated for *in vivo* tumor regression in a xenograft model of human primary colon cancer in athymic nude mice and was found to cause significant reduction in tumor volume. NMR and molecular dynamics (MD) simulation studies for this analog revealed the presence of a mixed $3_{10}/\alpha$-helical structure. This study demonstrates that the designed BN analogs retain their anticancer activity after the incorporation of the constrained amino acid, Aib, and are potential molecules for future use in cancer therapy and drug targeting. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bombesin; α-amino-isobutyric acid; anticancer; peptides

INTRODUCTION

Bombesin (BN) is a 14-amino-acid peptide (pGlu^1-Gln^2-Arg^3-Leu^4-Gly^5-Asn^6-Gln^7-Trp^8-Ala^9-Val^{10}-Gly^{11}-His^{12}-Leu^{13}-Met^{14}-NH_2) that was first isolated from the skin of the frog *Bombina bombina* [1]. Its mammalian homolog, the gastrin releasing peptide (GRP), is a 27-residue peptide that has sequence similarity with BN and differs only in one of the ten carboxy-terminal amino acids as in the sequence: H-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2. The normal physiological role of GRP in the human body is to stimulate the secretions of gastrointestinal hormones and the exocrine pancreas and to modulate gastrointestinal motility. Amphibian BN and its mammalian analogs GRP and Neuromedin B (NMB) elicit their effects by interaction with protein receptors located on the cell membranes of target cells. BN receptors belong to the superfamily of membrane bound G-protein coupled receptors and are composed of four subtypes, of which only three receptor subtypes, the GRP-R, the NMB-R and the orphan receptor, BB_3-R, are found in humans [2–4]. GRP and BN show mitogenic effects and stimulate cell proliferation. Rozengurt and Cuttitta reported for the first time that BN-like peptides could function as autocrine growth factors in small cell lung cancer (SCLC) [5,6]. BN and GRP-receptors are expressed in a variety of cancers such as human SCLC and cancers of the prostate, breast, pancreas and colon [7–11]. In the recent years, the BN/GRP-receptors expressed on cancer cells are emerging as attractive targets for cancer treatment [12].

Studies with the anti-BN/GRP antibodies lead to the hypothesis that it may be possible to disrupt the autocrine growth cycle of BN/GRP using designed peptide receptor antagonists. Since then several types of BN antagonists have been reported. These antagonists have been defined by type and position of the substitutions in the natural sequence. Several potent antagonist analogs of BN were designed by modifying the carboxy-terminal (7–14) region of the parent peptide by the incorporation of the D-amino acids, nonpeptide bonds, amide and ester modifications [13–15]. BN (6–14) has been shown to bind to all four BN receptors [16]. A BN/GRP antagonist (RC-3940-II) was found to inhibit the proliferation of SW-1990 human pancreatic adenocarinoma cells *in vivo* and *in vitro* [17]. A similar effect was seen with BN/GRP antagonist RC-3095 on the growth of CFPAC-1 human pancreatic cancer cells transplanted to nude mice or cultured *in vitro* [18]. BN analogs are being extensively explored for targeting cytotoxic drugs to cancer cells [19–22]. Radiolabeled BN/GRP analogs have been explored for their diagnostic and therapeutic potential in GRP-R expressing tumors [23–26].

The design of conformationally constrained bioactive peptide derivatives has been one of the widely used approaches for the development of peptide-based therapeutics. Nonstandard amino acids with strong conformational preferences may be used to direct the

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course of polypeptide chain folding by imposing local stereochemical constraints in *de novo* approaches to peptide design. The incorporation of α-aminoisobutyric acid (Aib), an α,α-dialkylated amino acid in peptides, is known to restrict the rotation of ϕ, ψ angles within the molecule, thereby inducing conformational constraint in the peptide backbone and stabilizing a desired peptide conformation. Aib has been shown to induce β-turn in small peptides and a helical conformation in larger ones [27]. Aib has been used to design analogs of different bioactive peptides such as chemotactic peptide [28,29], somatostatin [30], GnRH [31] and parathyroid hormone [32].

Among other strategies to enhance the circulation half-life and bioavailability, lipophilization of bioactive peptides, by attachment of a fatty acyl moiety, is an accepted approach that imparts improved stability, bioavailability, membrane permeability and enhanced receptor selectivity to peptides without loss of their biological activity [33]. *N*-terminal acylation of somatostatin analog RC-160 with long chain fatty acids was found to enhance its stability and antiproliferative activity in human breast carcinoma [34] and oral carcinoma cells [35].

In the present study, we describe the synthesis of six new octapeptide analogs of BN by incorporating Aib and have investigated the structural and functional consequences of this substitution on the anticancer activity of these analogs in human colon and pancreatic cancer. The analogs have been designed by modifying the parent sequence [D-Phe^{6}, *des*Met^{14}]-BN (6–13), labeled **P0**, in the text. In the design of the analogs, Aib residue has been incorporated by replacing either alanine at position 9 or glycine at position 11, or both, in the BN octapeptide sequence, **P0**. In analogs **P3** and **P4**, leucine at position 13 has also been substituted with isoleucine (Ile^{13}). Analog **P6** is the *N*-terminal butanoylated version of analog **P1**. We have evaluated the *in vitro* antiproliferative activity of the BN analogs in human pancreatic and colon cancer cell lines using the MTT assay. One of the analogs, **P6**, was also examined for *in vivo* tumor regression in human primary tumor cells (PTC) colon xenografted in athymic nude mice. NMR and molecular dynamics (MD) simulation studies were carried out to determine the 3D structure of the peptide analog **P6**.

MATERIALS AND METHODS

Peptide Synthesis and Purification

The peptides were synthesized from *C*- to *N*-terminal by standard Fmoc(9-fluorenylmethoxycarbonyl) solid phase peptide chemistry using the peptide synthesizer CS536 (CS Bio, San Carlos, CA, USA). All peptides were synthesized as peptide amides using Rink Amide resin. All the amino acids used were N-Fmoc protected. The side-chains were protected using trityl for glutamine and trityl or Boc protecting groups for histidine. All couplings were carried out in DMF. The Fmoc amino acid, di-isopropyl carbodiimide (DIC) and HOBt (1-hydroxybenzotriazole) were used in threefold molar excess. Successive de-protection and coupling steps were monitored by positive and negative Kaiser (ninhydrin) test, respectively. De-protection of the Fmoc group was carried out in 20% piperidine in DMF (v/v) for 30 min. Usually, no blue/purple color was obtained in the de-protection step following the coupling of Fmoc-Aib-OH in the peptides. Double coupling of the amino acid next to Fmoc-Aib-OH in the synthesis sequence was carried out to prevent deletion. The resin, all Fmoc protected amino acids and reagents were procured from Advanced Chemtech, Louisville, KY, USA. In analog **P6**, butanoic acid was coupled to the *N*-terminus of the peptide on-resin, using DIC and HOBt.

The cleavage of peptides from the resin and simultaneous removal of side-chain protecting groups was performed by treatment with TFA: EDT: water in the ratio 9.5: 0.25: 0.25 (v/v) for 2 h. The peptides were 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 peptides.

The crude peptides were purified on preparative HPLC system (Shimadzu Corporation, Japan) using LichroCART C-18 (250 mm × 10 mm) reverse phase column (Merck, Darmstadt, Germany) using a gradient of 0.1% TFA in acetonitrile and water. The eluted fractions were monitored on analytical HPLC (Shimadzu Corporation, Japan) on C18 reverse phase column (Lichrosper, WP300 (250 mm × 4 mm)). Acetonitrile was evaporated on the rotary evaporator and the fractions were lyophilized to obtain the pure peptide. The purified peptides were characterized 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 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 20–40% B in 20 min; System 2, using a solvent system of 0.1 M triethylamine phosphate (TEAP) buffer (A) and acetonitrile (B) and a linear gradient of 20–40% B in 25 min. The peptide sequence and analytical data of the synthesized analogs is summarized in Table 1.

In Vitro Anticancer Activity

The BN analogs **P1, P2, P3, P4, P5** and **P6** were tested for antiproliferative activity against four human tumor cell lines representing pancreatic (MiaPaCa-2) and colon cancers (SW620, HT29 and PTC) and compared with the octapeptide **P0** using the MTT cytotoxicity assay, as described previously [36]. The human tumor cell lines representing pancreas (MiaPaCa-2) and colon cancers (SW620, HT29) were obtained from National Centre for Cell Science, Pune, India while the primary tumor cell line, PTC, was derived from primary human colon adenocarcinoma biopsies [37]. Briefly, these cell lines were cultured to 70% confluence in 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 0.2mM EDTA solution. The cells were re-plated in 96-well tissue culture plates at a density of 5000–10 000

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Table 1 Sequence, mass and HPLC retention times of the synthesized BN analogs

Code	Peptide sequence	Mass		HPLC RT (min.)	
		Calculated	Observed $[M + H]^+$	System 1[a]	System 2[b]
P0	D-Phe^6-Gln^7-Trp^8-Ala^9-Val^{10}-Gly^{11}-His^{12}-Leu^{13}-NH_2	955.2	956.8	10.48	5.76
P1	D-Phe-Gln-Trp-Ala-Val-**Aib**-His-Leu-NH_2	983.1	984.6	14.65	9.79
P2	D-Phe-Gln-Trp-**Aib**-Val-Gly-His-Leu-NH_2	969.0	970.5	12.14	6.89
P3	D-Phe-Gln-Trp-**Aib**-Val-Gly-His-Ile-NH_2	969.1	970.5	11.42	6.35
P4	D-Phe-Gln-Trp-Ala-Val-**Aib**-His-Ile-NH_2	983.1	984.6	13.98	9.06
P5	D-Phe-Gln-Trp-**Aib**-Val-**Aib**-His-Leu-NH_2	997.1	998.6	15.85	11.02
P6	Butanoyl-D-Phe-Gln-Trp-Ala-Val-**Aib**-His-Leu-NH_2	1053.2	1054.6	20.01[c]	21.87[c]

[a] System 1: mobile phase A: water (0.1%TFA) and mobile phase B: acetonitrile (0.1%TFA); gradient: 20–40% B in 20 min.
[b] System 2: mobile phase A: 0.1M TEAP buffer and mobile phase B: acetonitrile; gradient: 20–40% B in 25 min.
[c] Gradient: 20–80% B in 40 min.

cells/well and incubated overnight in a humidified atmosphere of 5% CO_2 at 37 °C to allow complete reattachment of the cells. The BN analogs were added in tenfold incremental concentrations (ranging from 0.01nM to 1 μM, in triplicate wells, once every 24 h, for a period of 72 h) to get a dose dependent response. After 72 h, the assay was terminated and results were calculated as percentage inhibition of cell proliferation according to the formula:

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

Where X = absorbance of treated sample at 540 nm,

R_1 = absorbance of control sample at 540 nm.

For each of the cell lines, MiaPaCa-2 (pancreatic cancer) and SW620, HT29 and PTC (human colon cancers), the values for percentage inhibition of cell proliferation (± standard error) were plotted on the y-axis (mean values from three independent experiments) at different concentrations (ranging from 0.01 nM to 1 μM on the x-axis) as shown in Figure 1(a)–(d).

In Vivo Anticancer Activity on Primary Tumor (Colon) Xenografted Mice

Human primary colon adenocarcinoma (PTC) xenografts were grown in Balb/c athymic nude mice by a subcutaneous inoculation of a single suspension of PTC cells (15×10^6 cells/100 μl). The tumor-bearing animals were divided into two groups (control, treated with peptide **P6**) of six animals. Treatment with peptides was initiated when the average tumor volume, as measured using vernier calipers, was between 400 and 800 mm^3. Peptide solutions were prepared at a concentration of 100 μg/ml and administered intravenously to a treated group of tumor-bearing animals at a dose of 0.5 mg/kg body weight, twice a day, so that the total dose of 1 mg/kg body weight per day was administered to each animal. The treatment was continued for a period of 25 days. The tumor volume was 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:

$$\text{Inhibition of tumor growth (\%)} = 1 - [\text{tumor volume}_{(\text{treated})}/\text{tumor volume}_{(\text{control})}] \times 100.$$

NMR Studies of Butanoyl-D-Phe^6-Gln^7-Trp^8-Ala^9-Val^{10}-Aib^{11}-His^{12}-Leu^{13}-NH_2(P6)

^{1}H NMR studies of peptide **P6** were carried out in approximately 5–7 mM solution in dimethyl sulfoxide (DMSO)-$d6$ on a Bruker NMR spectrometer operating at a proton frequency of 500 MHz. The chemical shifts were measured with respect to the residual protons in DMSO-$d6$ as an internal reference (2.49 ppm). Total correlation spectroscopy (TOCSY) and rotating frame nuclear Overhauser effect spectroscopy (ROESY) were used for the resonance assignment using standard pulse sequences. ROESY experiments, along with temperature coefficients of amide proton chemical shifts ($d\delta/dT$) and vicinal couplings between CαH and NH ($^3J_{NH-C\alpha H}$) were used to obtain the structural information.

Molecular Dynamics Studies of Peptide P6

Molecular mechanic/dynamics calculations were carried out using Sybyl 6.8 program on a silicon graphics O2 workstation. The tripos force field with default parameters was used throughout the simulation. A dielectric constant of 47 D was used in all minimizations as well as simulated annealing (SA) runs. For peptide **P6**, 28 inter residue and 8 intra residue constraints were used in the minimization as well as in the SA runs. The rOe derived distance restraints, classified empirically as 'strong', 'medium' and 'weak', were applied as biharmonic restraints with lower and upper bounds of 1.8–2.8, 1.8–3.3 and 1.8–4.2 A° respectively, and a force constant of 15 kcal/A° was employed. An additional H-bonding constraint of 2.2 A° with a force constant of 30 kcal/A° was initially used to derive the starting energy-minimized structures of these molecules. Minimizations were done first with steepest descent, followed by conjugate gradient methods for a maximum of 1000 iterations each or root mean square (RMS) deviation of 0.05 kcal/mol, whichever was earlier. The energy-minimized structures were then subjected to 30 ps (10 cycles, each of 3 ps period) of SA run. The atomic velocities were applied following Boltzmann distribution about the center of mass to obtain a starting temperature of 700 K. After simulating for 1 ps at this high temperature, the system temperature was reduced step wise over a 2-ps period to reach a final temperature of 300 K. Resulting structures were sampled after every 3 ps (one cycle), leading to an ensemble

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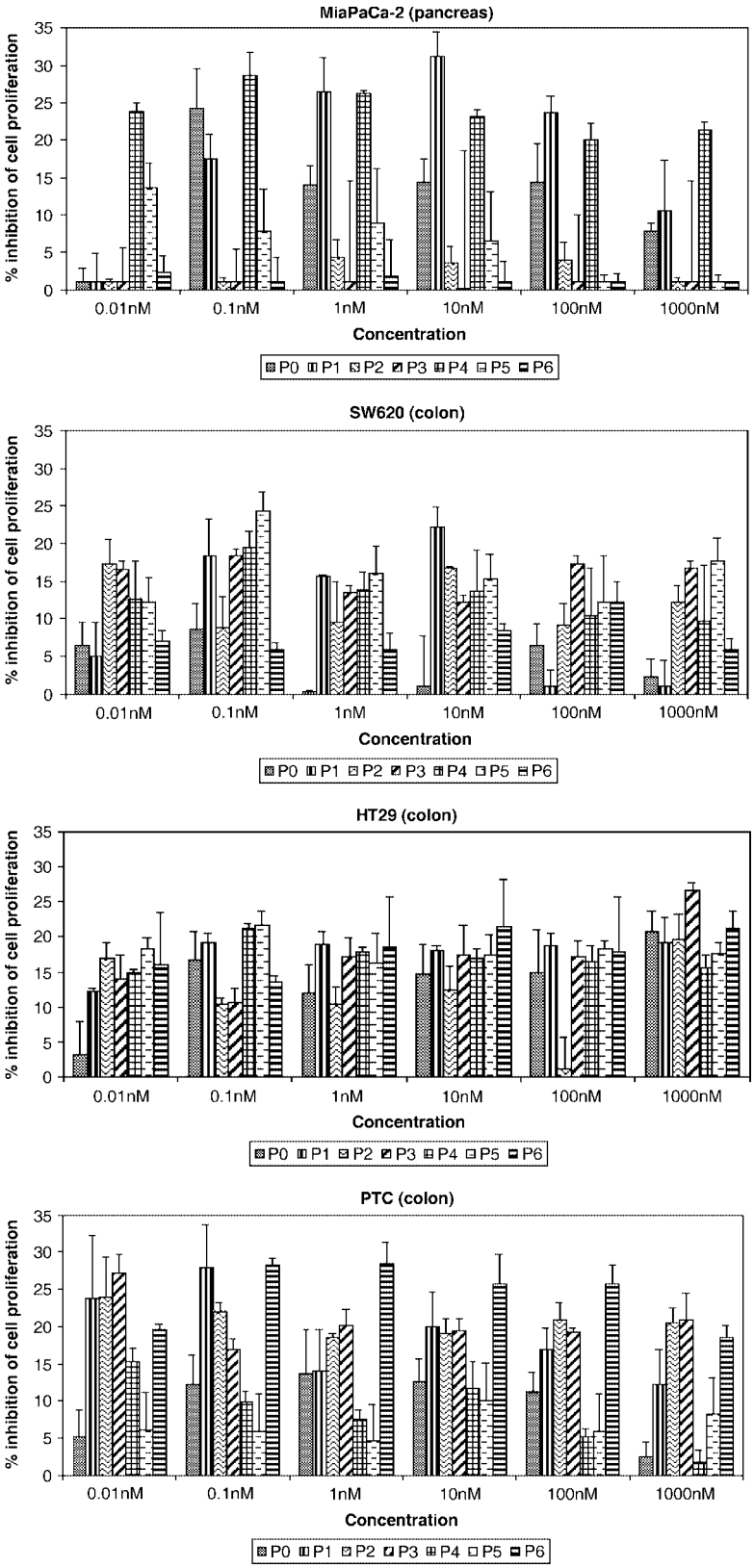

Figure 1 (a) *In vitro* percentage inhibition of cell proliferation (± standard error) at different concentrations ranging from 0.01 nM to 1 μM in human pancreatic cancer cell line MiaPaCa-2. (b) *In vitro* percentage inhibition of cell proliferation (± standard error) at different concentrations ranging from 0.01 nM to 1 μM in human colon cancer cell line SW620. (c) *In vitro* percentage inhibition of cell proliferation (± standard error) at different concentrations ranging from 0.01 nM to 1 μM in human colon cancer cell line HT29. (d) *In vitro* percentage inhibition of cell proliferation (± standard error) at different concentrations ranging from 0.01 nM to 1 μM in human colon cancer cell line PTC.

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of total ten structures. The samples were minimized using the above-mentioned protocol, compared and superimposed as shown in Figure 2.

RESULTS AND DISCUSSION

In view of the limited efficacy and considerable toxicity of conventional chemotherapy, the development of novel cytotoxic agents and innovative noncytotoxic approaches to cancer treatment are becoming increasingly important. Neuropeptides like BN are emerging as important targets for anticancer drug development. The carboxyl terminal heptapeptide region of BN/GRP was found to be the minimal fragment important for receptor interaction and biological activity [38,39]. The aim of the present study was to attempt to discover novel BN analogs with potent anticancer activity. Six octapeptide BN analogs were designed, synthesized and characterized using the noncoded amino acid Aib, in place of Ala^{9} or Gly^{11}, or both, and also substituting isoleucine (Ile^{13}) in place of leucine (Leu^{13}) in the parent sequence [D-Phe^{6}, *des*Met^{14}]-BN (6–13) or **P0**. All positions are with respect to the amino acid positions in the native BN sequence. The peptide sequence and analytical data of the synthesized BN analogs is summarized in Table 1.

Aib or α-aminoisobutyric acid is a naturally occurring amino acid that belongs to the class of α,α-disubstituted amino acids. It is not one of the 20 amino acids found in the proteins and peptides derived from animals and plants but occurs extensively in microbial peptide antibiotics, several of which have been reported to form transmembrane ion channels. The conformation of peptides containing Aib has been extensively studied and it has been established that the presence of two methyl groups on the C^{α}-carbon imposes a marked restriction on the available conformational space for the peptide backbone. The incorporation of Aib restricts the ϕ (N_{α}–C), ψ (C_{α}–C) angles in the peptide backbone and induces either β-turn in small peptides or helical conformation [27]. Since it has been reported that BN adopts a helical structure in the region from Asn^{6} to Met^{14} [40], we used the strategy of substituting the conformationally restricted amino acid, Aib, in the octapeptide BN (6–13) sequence, with the hope that it would stabilize the conformation and either retain or possibly enhance the antiproliferative activity.

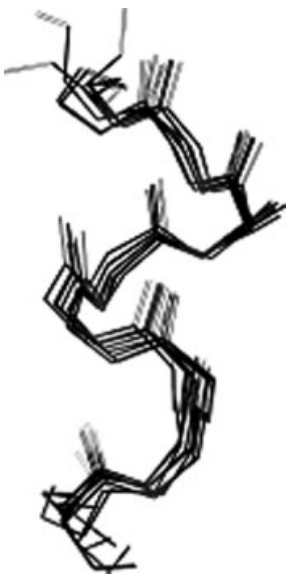

Figure 2 Energy-minimized structure of peptide **P6** ($3_{10}/\alpha$-helix mixture).

We tried to investigate the effect of the substitutions on the antiproliferative activity in human pancreatic (MiaPaCa-2) and colon cancer cell lines (SW620, HT 29 and PTC) *in vitro*. The *in vitro* antiproliferative activity (percentage inhibition of cell proliferation) of **P0** and the Aib containing BN analogs as obtained by the MTT assay is represented as bar graphs as shown in Figure 1(a)–(d). Most of the peptides tested showed a characteristic dose response when tested on human colon and pancreatic cancer cell lines.

In the pancreatic cancer cell line, MiaPaCa-2, the parent analog, **P0**, showed 24% cytotoxicity at 0.1 nM concentration, whereas the peptide **P1**, incorporating Aib residue instead of glycine at position 11, showed 31.1% inhibition of cell proliferation at 10 nM concentration and the peptide **P4**, with Aib^{11} and substitution of isoleucine for leucine at position 13 also showed growth inhibition of a maximum of 28.6% at 0.1 nM concentration. There was a complete loss of activity when Aib was substituted for alanine at position 9 as in the analogs **P2** and **P3** in the pancreatic cell line MiaPaCa-2. Thus, while a single Aib substitution in place of Gly^{11} in analogs **P1** and **P4** caused appreciable growth inhibition in the pancreatic cancer cell line MiaPaCa-2, the single Aib substitution at Ala^{9} (**P2** and **P3**), or, two Aib substitutions at Ala^{9} and Gly^{11}, as in analog **P5** were not as effective in inhibiting the growth of pancreatic cancer cells *in vitro*.

In the colon cancer cell line SW620, in general, all analogs show better inhibition of cell proliferation than the parent peptide **P0**. While **P0** showed growth inhibition of $<10\%$ over the entire concentration range, analog **P5**, with Aib substitutions at both positions 9 and 11, showed maximum inhibition of cell proliferation of 24.2% at 0.1 nM concentration and analog **P1** showed 22.2% growth inhibition at 10 nM concentration.

In the colon cancer cell line HT29, the maximum inhibition of proliferation of 26.5% (analog **P3**) was achieved at a higher concentration (1 μM) in comparison to the other cell lines. The growth inhibition value of **P0** was 16.6% at 0.1 nM concentration while the percentage growth inhibition values for analogs **P1**, **P4** and **P5** also at 0.1 nM concentration were closely similar at ~20%.

In the primary colon tumor cell line PTC, while the analogs **P1** and **P3** were closely similar with growth

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inhibition of 27.8% at 0.1 nM concentration (analog **P1**) and 27.3% at 0.01 nM concentration (analog **P3**), analog **P6** showed the maximum inhibition of proliferation (28.3% at 0.1 nM and 28.5% at 1 nM concentrations).

In the absence of clear-cut data in favor of any particular analog from the *in vitro* data, we decided to assess the *in vivo* tumor regression potential of one of the potent analogs in human PTC (colon) xenograft model in athymic nude mice. The colon cancer cell lines derived from primary tumor biopsies expresses BN receptors [37,41] and hence human PTC (colon) xenografts were chosen for the *in vivo* study. Analog **P6** had shown maximum (28.3% at 0.1 nM and 28.5% at 1 nM) *in vitro* growth inhibition in PTC colon cancer cell line. Also the *N*-terminal protection with the butanoyl group may improve the *in vivo* metabolic stability and bioavailability of the peptide; hence peptide **P6** was selected for the *in vivo* tumor regression assay in the PTC (colon) tumor xenograft mouse model. Figure 3 shows tumor kinetics of colon (PTC) tumor xenografts till day 29 in the **P6** treated and control (untreated) animals. The peptide **P6** significantly inhibited the growth of tumor xenografts by day 29 as compared to control ($p < 0.05$). The percentage inhibition of tumor growth, calculated as percentage of tumor volume of **P6** treated group as compared to control, on day 29, was 44.3%.

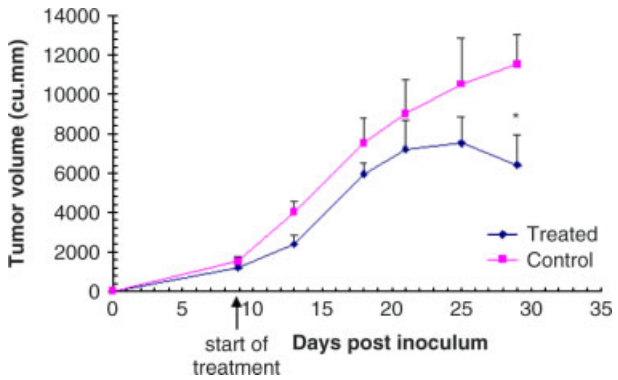

Figure 3 *In vivo* antitumor activity of BN analog **P6** treated *versus* control (untreated) animals in human primary colon tumor (PTC) xenograft in athymic nude mice. (Data shown are mean ± SD, $n = 6$. *represents $p < 0.05$ compared with control. Groups were compared using multiple comparison procedure, SAS 9.1.3).

To better comprehend the anticancer activity of BN analog **P6** from the structural point of view, we decided to determine the three-dimensional structure of peptide **P6** by 2D NMR and MD simulations. The chemical shifts, coupling constant and temperature coefficients of peptide **P6** have been summarized in Table 2 and Table 3. Peptide **P6** shows several signatures of helical structure. The observation of moderate intensity nOe cross peaks between sequentially connected amide protons ($NH_{(i)}$–$NH_{(i+1)}$) throughout the length of the molecule, as well as presence of several $^3J_{NH-C\alpha H}$ in the vicinity of 6 Hz are the characteristics of helical structure. Small values of ($d\delta/dT$) for (Trp NH, −3.1; Ala NH, −2.6; His NH, −2.7; and Leu NH, −2.9 ppb/K) imply their participation in intramolecular hydrogen bonding. The detection of $d_{\alpha N(i,i+2)}$, characteristics of 3_{10} helices, and $d_{\alpha N(i,i+4)}$, characteristics of α-helices, denote the presence of both type of structures in solution. On the basis of these results we show that large fraction of peptide **P6** takes a helical structure. Residues in the *N*-terminal take a 3_{10} helical structure in which the ones at the *C*-terminal end have a tendency to adopt α-helical structure.

It is evident from literature that the *C*-terminal region of BN and BN analogs has the tendency to adopt a helical or a partially helical structure in aqueous solvent, DMSO and trifluoroethanol (TFE) [40,42–44] also indicates that the *C*-terminal region of BN has the tendency toward a helical or a partially helical structure. In our studies we have tried to stabilize the helical structure by incorporating Aib in designing the BN analogs. Further, we carried out the conformational study of only the bioactive peptide, **P6**. A membrane-mimicking solvent such as TFE would have been ideal for carrying out the NMR experiments. However, we observed a helical structure for peptide **P6** in DMSO-*d*6, and it is likely that **P6** will adopt a helical conformation in a helix-promoting solvent such as TFE.

In the molecular dynamics studies of peptide **P6**, the energy minimizations were carried out based on NMR derived distance restraints. A stereo view of the ten superimposed, energy-minimized, mixed $3_{10}/\alpha$-helical

Table 2 ^{1}H NMR chemical shift values (δ in ppm) of peptide **P6** in DMSO-d_6 at 500 MHz

Protons	Butanoyl	D-Phe	Gln	Trp	Ala	Val	Aib	His	Leu
NH	—	8.57	7.96	7.70	7.54	8.04	7.60	7.70	—
CαH	—	4.36	3.92	4.35	4.21	3.87	—	4.41	4.14
CβH	—	2.91, 2.85	1.83, 1.53	3.14, 3.09	1.24	2.01	1.29, 1.27	3.18, 3.04	1.59, 1.49
CγH	—	—	1.74	—	—	0.89, 0.85	—	—	1.59
CδH	—	—	—	—	—	—	—	—	0.85, 0.80

Others: 6.72–7.50 (aromatic protons and NH_2 s).

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Table 3 NOEs, coupling constant values (J in Hz) and temperature coefficients ($d\delta/dT$ in ppb/K) of peptide **P6** in DMSO-d_6 at 500 MHz

Sequence	Butanoyl-	D-Phe	Gln	Trp	Ala	Val	Aib	His	Leu
$d_{NN(i, i+1)}$									
$d_{\alpha N(i, i+1)}$									
$d_{\alpha N(i, i+2)}$									
$d_{\alpha N(i, i+3)}$									
$d_{\alpha N(i, i+4)}$									
$d_{\beta N(i, i+1)}$									
$d_{\alpha\beta(i, i+3)}$									
$J_{NH\text{-}\alpha H}$ (Hz)		5.9	6.3	7.2	6.6	6.6	—	7.6	8.0
$-d/\delta dT$ (ppb/^{0}K)		6.5	4.4	3.1	2.6	4.5	6.0	2.7	2.9

Strong nOe(▬), medium nOe(▬), weak nOe(▬).

structures of peptide **P6** using MD simulations is shown in Figure 2.

CONCLUSIONS

The experimental results obtained from the present study show that the incorporation of Aib in one of the BN analogs induces helical conformation. Further, we can infer from the *in vitro* growth inhibition data in human pancreatic and colon cancer cell lines that all the Aib incorporating BN analogs, in general, show either retention or enhancement of anticancer activity when compared with the [D-Phe^6, *des*Met^{14}]-BN (6–14) peptide, **P0**. The BN analog **P6**, which shows stable helical conformation because of incorporation of Aib, reduces tumor volume by ~44% in PTC xenografted mice. Thus, BN analogs containing site-specific Aib substitutions could prove to be promising candidate molecules for future use in cancer diagnosis, therapy or drug targeting. Further, the strategy of incorporation of Aib in biologically active peptides can be exploited in determining their receptor bound conformation and in transforming these bioactive peptides into pharmacologically useful drugs.

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