Accepted Manuscript

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PII: S0968-0896(16)30732-5
DOI: http://dx.doi.org/10.1016/j.bmc.2016.09.039
Reference: BMC 13292

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 28 July 2016
Revised Date: 14 September 2016
Accepted Date: 15 September 2016

Please cite this article as: Begum, A.A., Moyle, P.M., Toth, I., Investigation of Bombesin peptide as a targeting ligand for the gastrin releasing peptide (GRP) receptor, *Bioorganic & Medicinal Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.bmc.2016.09.039

Investigation of Bombesin peptide as a targeting ligand for the gastrin releasing peptide (GRP) receptor

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Abstract

Gastrin releasing peptide (GRP) receptor (GRPR), a bombesin family receptor, is overexpressed in many cancers including breast, prostate, pancreatic and lung. The targeting of therapeutics to GRPR can be achieved using the full-length (14 amino acid) GRP analogue Bombesin (BBN) or the truncated BBN(6-14) sequence, both of which bind GRPR with high affinity and specificity. In this study, we have investigated the level of GRPR expression in various cancerous (Caco-2, HeLa, LNCap, MDA-MB-231, and PC-3) and non-cancerous (WPMY-1) cell lines using a western blotting approach. Such information is currently lacking in the literature, and is therefore of importance for the *in vitro* assessment of GRPR targeted therapeutics. Of the cell lines assessed, the PC-3 (prostate cancer) and Caco-2 (colon cancer) cell lines demonstrated the highest and lowest levels of GRPR expression respectively. Using this information, we further investigated the cellular uptake of carboxyfluorescein-labelled BBN and BBN(6-14) peptides by flow cytometry and confocal microscopy using cell lines that express GRPR (Caco-2, HeLa, PC-3). The uptake of each of these peptides was similar, suggesting that the shorter BBN(6-14) peptide is sufficient for GRPR targeting. Further, the uptake of these peptides could be inhibited by competition with unlabelled BBN peptides, suggesting their cellular uptake is GRPR-mediated, while the level of BBN uptake (as measured by flow cytometry) was found to be directly proportional to the level of GRPR expression. Overall, the information obtained from these studies provides useful information for the *in vitro* assessment of GRPR targeted therapeutics.

Keywords:

Bombesin

Cell Uptake

Gastrin releasing peptide receptor

Solid phase peptide synthesis

Targeting

1 Introduction

Cancer cells display a set of heterogeneous characteristic features including uncontrolled cell division, clonal expansion, intravasation and extravasation in the blood and lymphatic vessels, and metastasis to invade different tissues.[1] Growth factor receptors, which are expressed on the cell surface and in the cytoplasm of tumours, are associated with tumour development, progression, angiogenesis and metastasis.[2, 3] Overexpressed growth factor receptors are considered to be tumour biomarkers, which can be used as targets for tumour diagnosis and/or therapeutic targeting. Examples include vascular endothelial growth factor (VEGF),[4] epidermal growth factor (EGF),[5] and fibroblast growth factor (FGF),[6] which have been targeted by anticancer therapies against bladder, prostate, colorectal and breast cancers.

Over the past few decades, research has suggested that bombesin (BBN) family peptides act as growth factors for various cancers including breast, colon, prostate and small cell lung cancer.[2] Bombesin is a tetradecapeptide which was first isolated from the skin of the *Bombina bombina* frog in 1972.[7] Since its initial isolation, extensive investigations have been conducted, which have provided valuable insights into the function of BBN and its corresponding receptors.[8] Three types of mammalian BBN receptors have been identified: gastrin-releasing peptide receptor (GRPR), neuromedin B receptor (NMBR), and bombesin receptor subtype-3 (BRS-3). Each of these receptors belongs to the G-protein coupled receptor super family.[9]

Bombesin and its mammalian counterpart gastrin-releasing peptide (GRP) have structural and functional similarities. The 27-amino acid long peptide, GRP, was first identified from porcine gut in 1979.[10] Both BBN and GRP share a homologous C-terminal seven amino-acid long sequence Trp-Ala-Val-Gly-His-Leu-Met-NH_2,[8] which is important for receptor binding and internalisation.[9] Due to this similarity, BBN and GRP exert similar physiological effects. For example, both peptides function as a gastrointestinal hormone and as a neurotransmitter.[8, 11] In addition, they are both mitogens, and function as autocrine growth factors for tumour cells.[8, 12, 13]

The GRP receptor is overexpressed in a wide range of cancers, including: glioblastomas, small cell lung, gastric, pancreatic, prostate, breast, cervical, and colon cancers, whereas NMBR and BRS-3 are expressed in a limited number of carcinomas.[14-17] Because their

receptors are overexpressed on various tumours, in addition to their ability to internalise GRP-targeted species, BBN and GRP have been investigated as targeting ligands for the diagnostic imaging of tumour cells and for tumour-targeted delivery of therapeutics.[14, 16, 18] For example, assorted *in vivo* and *in vitro* studies have demonstrated high uptake of bombesin peptide analogues by tumour cell lines including the PC-3 and DU145 prostate cancer cell lines;[15, 19] the MDA-MB-231 and T-47-D breast cancer cell lines;[8, 20] and the HeLa cervical cancer cell line.[21] While GRPR agonists have demonstrated the capacity to target GRPR, with subsequent cellular uptake, GRPR antagonists show little to no internalisation following GRPR binding.[22]

In the present study, we have investigated BBN, a native GRPR agonist, as a GRPR targeting ligand that would be useful for the delivery of therapeutics that feature an intracellular site of action, particularly for therapeutics that display poor cellular uptake on their own. It has been reported that the C-terminal seven to nine amino acids of BBN are necessary for high affinity binding to GRPR and for receptor mediated responses.[15, 23] Therefore, we have produced a BBN peptide library that includes both the full length BBN peptide and a truncated BBN peptide [BBN(6-14)] to investigate whether the shorter, more serum stable,[24, 25] and more readily synthesised BBN(6-14) peptide is as effective for GRPR targeting as the full length peptide. Further, the capacity for these peptides to be internalised by receptor mediated cellular internalisation has been studied using 5(6)-carboxyfluorescein fluorescently tagged BBN peptides. Prior to evaluating the cellular uptake of each analogue, the level of GRPR expression in a library of cancerous (Caco-2, HeLa, LNCap, MDA-MB-231, and PC-3) and immortalised primary cell lines (WPMY-1) was evaluated by western blotting. Quantitative (flow cytometry) and qualitative (confocal microscopy) cell uptake studies were then conducted to evaluate the level of cellular internalisation and the intracellular localisation of each BBN peptide. To evaluate the mechanism of internalisation competitive, and temperature dependent cell uptake studies were conducted. A major aim of this study was to compare the capacity to target GRPR expressing cancer cell lines using BBN peptides as a means to improve the targeted delivery of anticancer therapeutics. Toward this goal, a comparison of cellular uptake in breast (MDA-MB-231), colon (Caco-2), prostate (PC-3) and cervical cancer (HeLa) cell lines was performed using both BBN peptide analogues.

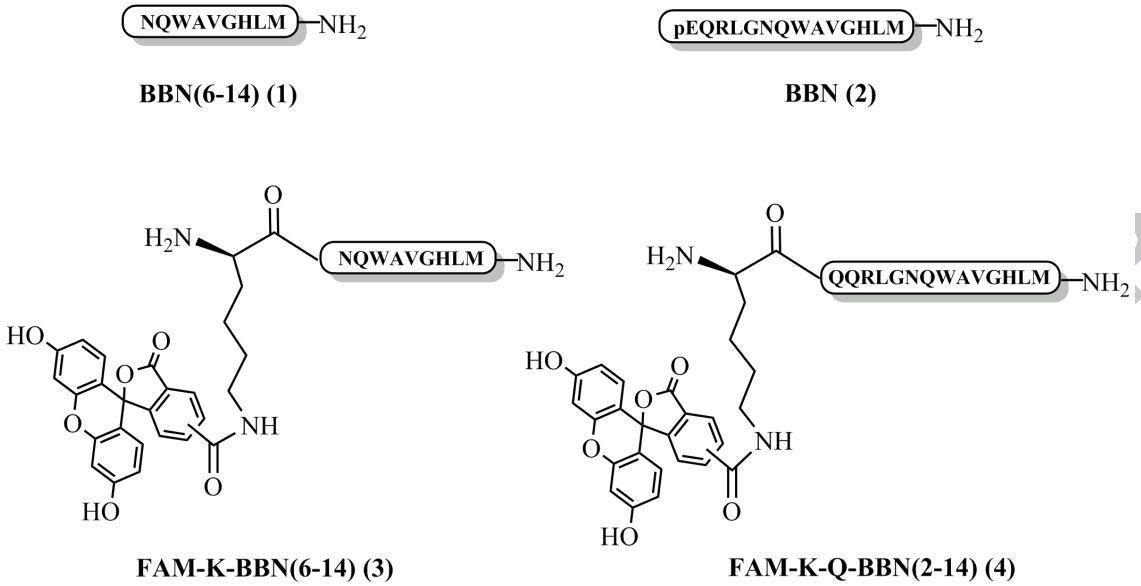

Figure 1. Library of BBN peptide analogues. (BBN: Bombesin peptide, FAM: 5(6)-carboxyfluorescein)

2 Results and discussion

2.1 BBN Peptide synthesis and characterisation

Each peptide (**1-4**) was successfully synthesised using manual solid phase peptide synthesis. The fluoresecent tag 5(6)-carboxyfluorescein (FAM), was coupled to the N-terminus of peptides **1** and **2** through a lysine spacer to produce compounds **3** and **4** using 1-hydroxybenzotriazole (HOBt) and *N,N'*-diisopropylcarbodiimide (DIC) as coupling reagents. Coupling was performed in the dark to minimise photo bleaching, followed by the removal of any ester linked FAM by repeateted 20% piperidine washing (5 min each cycle) until no further color was observed when washed. The peptides were cleaved from the resin using trifluoroacetic acid (TFA) in the presence of 2.5 % 1,2-ethanedithiol (EDT) to minimize methionine oxidation to methionine sulfoxide.[26] The crude peptides were purified by reversed phase-high performance liquid chromatography (RP-HPLC) to > 99% purity using water/acetonitrile gradients (Table 1). The purified fractions were lyophilised to yield the final pure compounds **1-4**, which were characterised by RP-HPLC and electrospray ionization-mass spectroscopy (ESI-MS) (Supplementary Fig. 1). Purification and characterisation data (RP-HPLC and MS) are presented in Table 1.

Table 1

Summary of purification yields and characterisation data (RP-HPLC and ESI-MS) for the peptide library

Compound no.	MW (Da)	Purity	RP-HPLC retention time [a] (gradient)[b]	ESI-MS		
				Protonation state	Calculated	Found
1	1054.24	>99%	17.3 min (0-80% solvent B)	$[M+H]^{+}$	1055.2	1055.1
			24.1 min(15-35% solvent B)	$[M+2H]^{2+}$	528.1	528.6
2	1619.87	>99%	18.2min(0-80% solvent B)	$[M+H]^{+}$	1620.8	1620.0
			26.8min(15-35% solvent B)	$[M+2H]^{2+}$	810.9	811.1
3	1540.72	>99%	20.0 min (0-80% solvent B)	$[M+H]^{+}$	1541.7	1541.9
			25.1min(20-45% Solvent B)	$[M+2H]^{2+}$	771.3	771.4
				$[M+3H]^{3+}$	514.5	514.9
4	2123.38	>99%	19.6min (0-80% solvent B)	$[M+2H]^{2+}$	1062.6	1062.0
			21.4min(25-40% solvent B)	$[M+3H]^{3+}$	708.7	709.0
				$[M+4H]^{4+}$	531.8	532.0

[a] HPLC conditions: 1 mL/min flow rate, 214 nm detection, Vydac C18 column (218TP54, 4.6 mm × 250 mm, 5 μm)

[b] All the gradients were run over 30 min using solvent A: 0.1% (v/v) TFA-H_2O; solvent B: 90% (v/v) MeCN-0.1% (v/v) TFA-H_2O.

2.2 Analysis of GRPR expression by western blotting

Western blotting was performed to investigate the level of GRPR expression in various human tumour-derived (HeLa, PC-3, MDA-MB-231, LNCaP, and Caco-2) and immortalized non-tumour-derived (WPMY-1) cell lines. Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer, and the protein content for each group quantified using a bicinchoninic acid (BCA) assay. Equivalent amounts (15 μg/lane) of total protein from each cell line were separated by SDS-PAGE on a 10% gel, electrotransferred onto a PVDF membrane, and probed using a polyclonal rabbit α-GRPR antibody (Abcam ab39963). GRPR bands were then detected using enhanced chemiluminescence (ECL) after probing with a goat α-rabbit IgG-peroxidase conjugate (Sigma A6154) secondary antibody. Caco-2 cells were used as a positive control for GRPR expression, as recommended by the antibody manufacturer, with glyceraldehyde-3-phosphate dehydrogenase (GADPH) staining used as a loading control.[27]

Each of the cell lines tested was observed to express GRPR by western blotting (Fig. 2A). The highest level of GRPR expression was observed in the PC-3 prostate cancer epithelial cell line, with the Caco-2 colon cancer epithelial cell line demonstrating the lowest level of GRPR expression (Fig. 2A). The level of GRPR expression in PC-3 prostate cancer cells was also compared to other prostate-derived cell lines including the androgen-sensitive human prostate adenocarcinoma cell line LNCaP and the transformed human non-tumour-derived prostate myofibroblast cell line WPMY-1. This comparison demonstrated the GRPR expression level in PC-3 cells was significantly higher (4.6-fold higher than Caco-2 cells) than observed in LNCaP (1.7-fold higher than Caco-2 cells) or WPMY-1 cell lines (2.2-fold higher than Caco-2 cells) (Fig. 2B). In addition, the GRPR expression level in HeLa human cervical adenocarcinoma and MDA-MB-231 human breast invasive ductal carcinoma cell lines were also assessed. These cell lines exhibited GRPR expression levels in between PC-3 and LNCaP cells (3- and 2.6-fold higher than Caco-2 cells respectively). Overall, this data provides important information relating to the expression levels of GRPR in cultured human cell lines, which is lacking in the literature, and will prove useful for the selection of appropriate cell lines for *in vitro* assessment of GRPR targeted therapeutics.

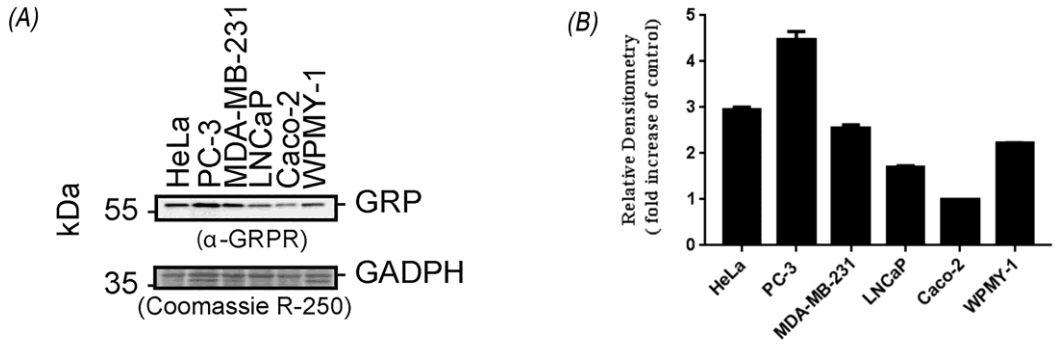

Figure 2. (A)Western blot analysis of GRPR expression in six different cell lines. Data are representative of at least two independent experiments. GADPH is used as a loading control.(B) The band density was quantified by densitometry. Data is presented as the fold increase in GRP band density in comparison to the Caco-2 cell group.

2.3. Analysis of the cellular uptake of BBN peptides by flow cytometry

In order to evaluate how effectively BBN peptides are internalised by GRPR expressing cells, the cellular uptake of fluorophore-labelled BBN peptides **3** and **4** was evaluated by flow

cytometry in PC-3 cells at three different concentrations (1, 5, and 10 μM). Trypan blue was used to quench any fluorescence associated with surface bound peptides prior to analysis by flow cytometry (with the results reported as intracellular fluorescence in Fig. 3) in order to differentiate between peptides that are associated with the surface of PC-3 cells, and peptides that were internalised.[28] This experiment demonstrated that the percentage of PC-3 cells containing internalised BBN peptides **3** or **4** increased as the concentration of each peptide was increased (from 1.76% to 29.8% and from 1.86% to 29.7% at 1 and 10 μM for both compound 3 and 4, respectively), with comparible results observed at each tested concentration for peptides **3** and **4** ($p > 0.05$) (Fig. 3A). This data indicated that the truncated BBN(6-14) peptide **3**, which is easier and less expensive to synthesize in comparison to the full-length BBN peptide **4**, would be preferable for incorporation into GRPR-targeted delivery systems.

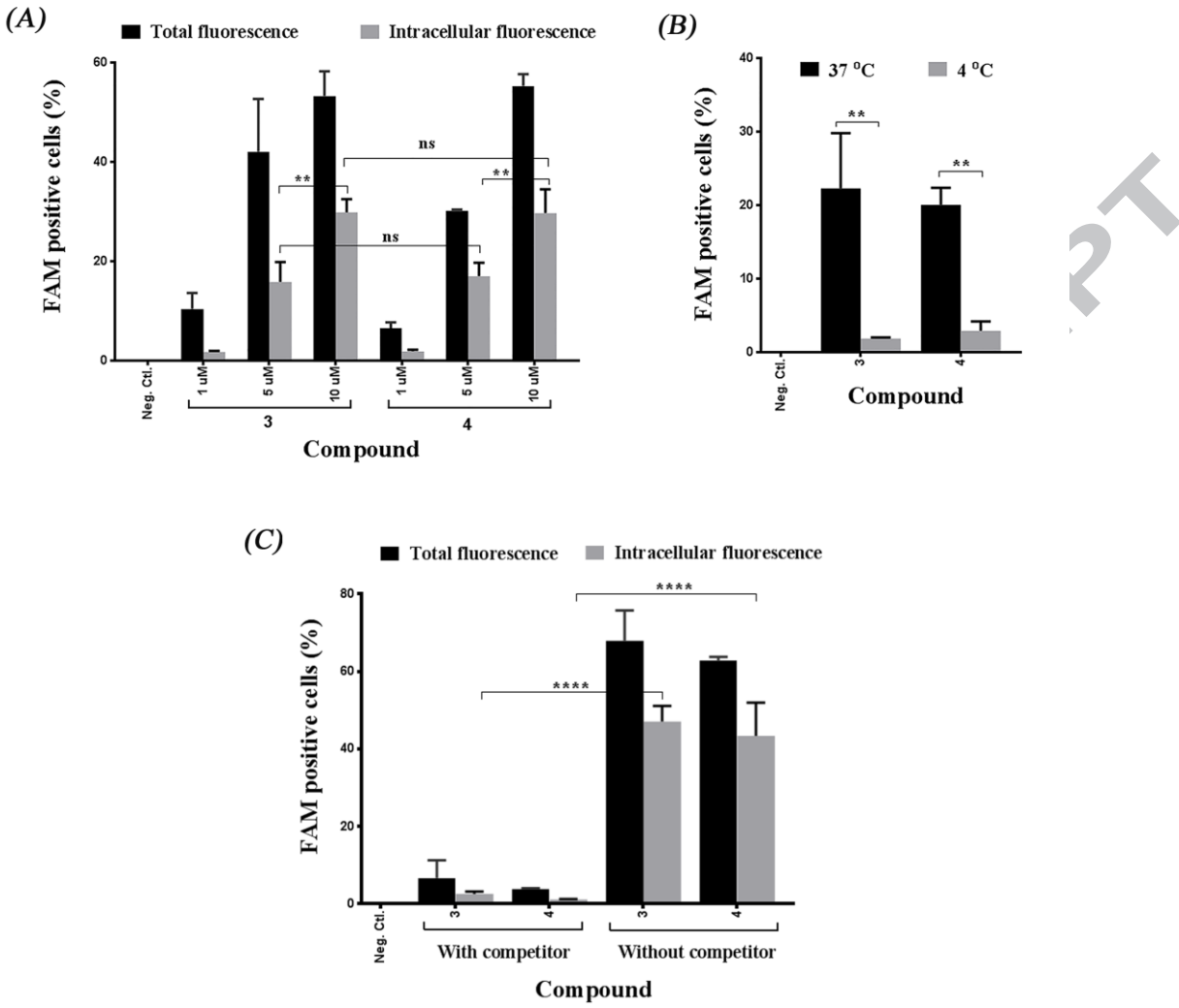

Figure 3. Cellular uptake of FAM-labelled BBN peptides **3** and **4** in PC-3 cells as measured by flow cytometry. The effect of: (A) BBN peptide concentration (1, 5, and 10 μM); (B) temperature (4 °C vs. 37 °C; 5 μM BBN peptides); and (C) competition with a 10-fold excess of peptides **1** or **2** over peptides **3** or **4 (**5 μM) respectively. Untreated PC-3 cells were used as a negative control (Neg. Ctl.). Data is presented as the mean (± SD) percent FAM-positive PC-3 cells based on total cellular or intracellular fluorescence after 4 h incubation for triplicate samples.

To gain additional insight into the mechanism of cellular uptake, the cell uptake experiment was repeated at 4 °C, a temperature at which energy-dependent endocytosis is inhibited.[29] Comparing the percentage FAM-positive cells after incubating PC-3 cells with peptides **3** or **4** at 4 °C and 37 °C revealed that the cellular uptake of both peptides was significantly reduced at 4 °C ($p < 0.01$; Fig. 3B). The percent FAM-positive cells ranged from 20-22% for

each peptide at 37 °C, and were reduced to 2-4% at 4 °C, suggesting that BBN cellular uptake is predominantly associated with energy dependent pathways.

2.4 Competative cell uptake study

Because fluorescein analogs have been demonstrated to be internalised by cultured cells,[30] including conjugates with high molecular weight polymers,[31, 32] we wanted to demonstrate that the observed internalisation of peptides **3** and **4** was due to BBN and not the FAM-label. This was achieved by conducting the uptake assay in PC-3 cells with peptides **3** or **4** (5 μM) in the presence of a 10× excess of the unlabelled BBN peptides **1** or **2**. If the BBN peptides were responsible for cellular uptake, rather than the FAM-label, the excess of peptides **1** or **2** would compete for uptake, and a large reduction in the uptake of **3** or **4** would be observed by flow cytometry. This outcome was observed, with the incubation of PC-3 cells with FAM-labelled peptides **3** and **4** exhibiting only 4 and 2% FAM-positive cells respectively in the presence of a 10× excess of peptides **1** or **2** (Fig. 3C). In comparison, where PC-3 cells were not exposed to peptides **1** or **2**, 47 and 42% FAM-positive cells respectively were observed (Fig. 3C). This experiment strongly supported the hypothesis that cellular uptake of peptides **3** and **4** was due to BBN, and not the FAM-label. In addition, since the uptake process was energy dependent, and could be competed with BBN peptides, this data suggested that the uptake of peptides **3** and **4** was associated with BBN receptors. The data also demonstrated that N-terminal modification of BBN peptides is compatible with BBN receptor targeting and cellular uptake, and thus may serve as a modification site for targeting therapeutics to cells that overexpress GRPR.

2.5 Comparing the uptake of bombesin peptides in different GRPR-expressing cell lines

In this report western blotting was used to assess the level of GRPR expression in a library of different cultured cell lines. Such information is currently lacking in the literature, and provides an important resource for designing *in vitro* experiments to evaluate GRPR targeted delivery systems. However, it is curently unknown if GRPR expression levels alone directly correlate with BBN peptide uptake levels in different cell lines. For this reason, we wanted to compare the level of FAM-labelled BBN peptide uptake to the level of GRPR expression observed by western blotting. For this purpose we compared the uptake of peptides **3** and **4** (at 5 μM concentration) in three different human tumour cell lines: Caco-2 (colon epithelial), HeLa (cervical epithelial), and PC-3 (prostate epithelial), by flow cytometry. Of these cell lines, PC-3 cells demonstrated the highest uptake, with 47 and 50% of PC-3 cells

demonstrating uptake of peptides **3** and **4** respectively (Fig. 4). In comparison, Caco-2 cells demonstrated the lowest uptake (5 and 10%), and HeLa cells showed intermediate uptake (16 and 25%) (Fig. 4). This data was in direct agreement with the GRPR expresion levels detected by western blotting of lysates for each of the assessed cell lines (Fig. 2A), and thus suggests that the level of BBN peptide uptake is proportional to the level of GRPR expression. As such, BBN peptides may prove useful for targeted prostate cancer therapies.

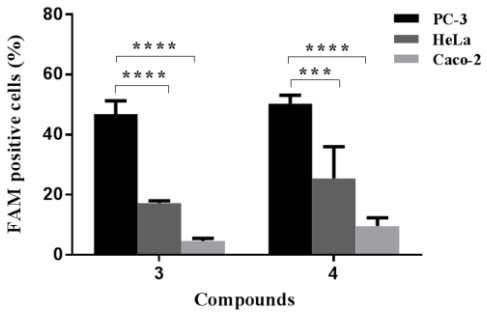

Figure 4. Comparing the cellular uptake of FAM-labelled BBN peptides **3** and **4** (at 5 μM concentration) in PC-3, HeLa, and Caco-2 cells. Data is presented as the mean percentage ± SD (n = 3) of FAM-positive PC-3 cells based on intracellular fluorescence after 4 h incubation for repeated experiments.

2.6 Confocal imaging of the internalisation of FAM-labelled BBN peptides 3 and 4 in PC-3 cells

To provide additional supporting evidence for the uptake of FAM-labelled BBN peptides **3** and **4** in PC-3 cells, live cell confocal microscopy was used to detect internalised peptides and to probe their subcellular localisation. For this experiment, the cells were incubated for 4 h with peptides **3** or **4** at 5 μM concentrations, washed, and stained with: Trypan blue to quench surface-associated fluorescence; Hoechst 33342 to label the nucleus; and LysoTracker® Red DND-99 to label endo(lyso)somes. Intracellular FAM-associated fluoresence was observed at 5 μM concentration for both peptides **3** and **4**, with fluorescence distributed throughout the cytoplasm (Fig. 5). The presence of punctate staining, colocalised with the LysoTracker dye (which stains acidic organelles, i.e. endo(lyso)somes)[33] suggests that BBN peptides are likely internalised by an endocytic pathway, with some entrapment in endosomes. Overall, this data demonstrates that the BBN peptides can be internalised by PC-3 cells, validating the results of our flow cytometry cellular uptake experiments.

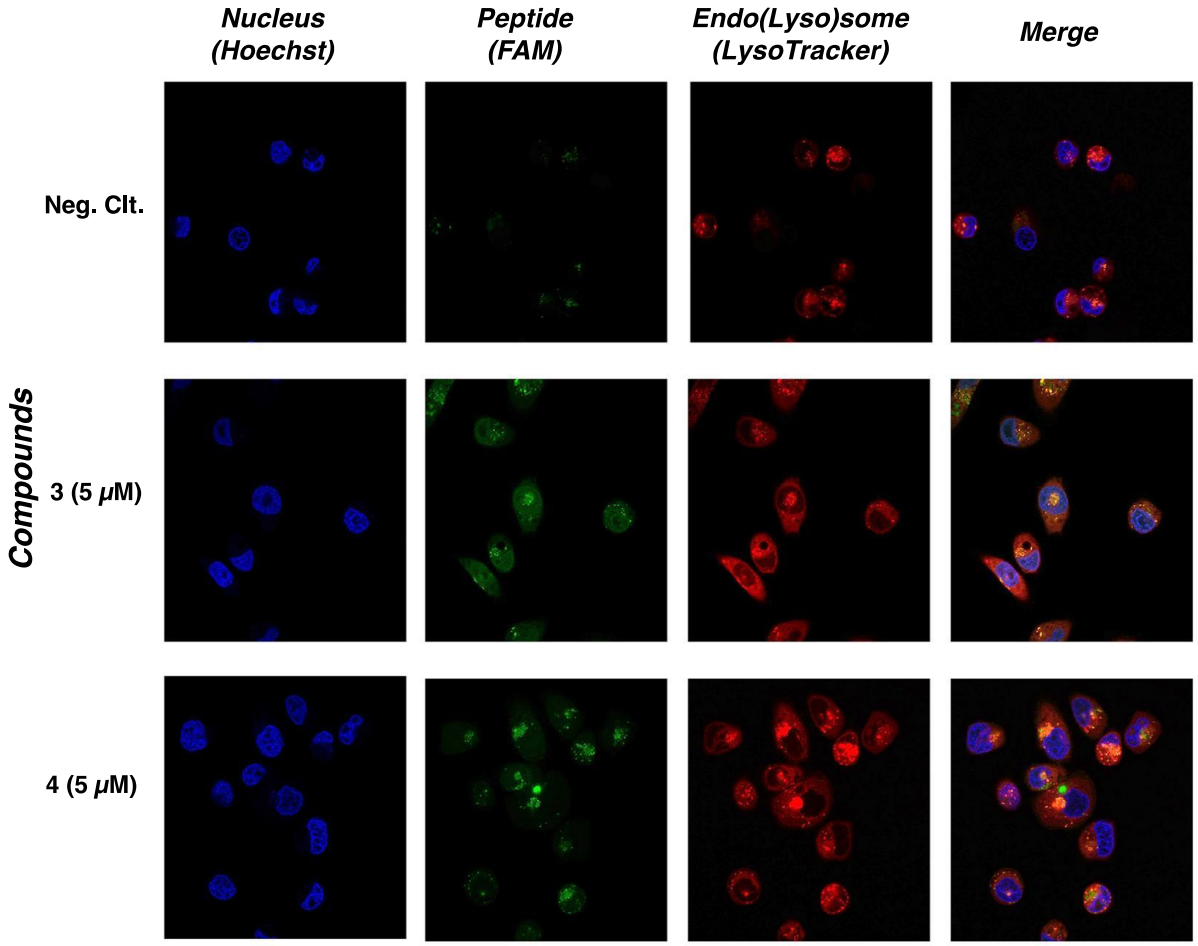

Figure 5. Live-cell confocal imaging of the uptake and subcellular localisation of FAM-labelled BBN peptides **3** or **4** (at 5 μM concentration) after 4 h incubation with PC-3 cells. Untreated PC-3 cells were used as a negative control (neg. ctl.). Imaging was performed at 63× magnification, using an oil emersion lens.

3. Conclusion

The tissue-specific targeting of therapeutic molecules provides a useful means to improve drug efficacy by enabling reductions in the dose of therapeutic molecules that are delivered, and offering the potential to reduce adverse effects. These advantages of targeted drug delivery are of immense interest for the treatment of various cancers. Herein, we aimed to characterise the potential of GRPR, which is overexpressed on many types of tumours, to be targeted by BBN peptides in order to improve the targeted delivery of anticancer therapeutics. A key finding from this work was the evaluation of GRPR expression on a library of different cultured cancerous (Caco-2, HeLa, MDA-MB-231, and PC-3) and non-cancerous (WPMY-1) cell lines. This information indicated that of these cell lines, PC-3 cells demonstrated

significantly higher overexpression of GRPR than the other assessed cell lines, with Caco-2 cells demonstrating the lowest GRPR expression. Using this information, the capacity for wildtype BBN and truncated BBN(6-14) peptides to act as ligands for GRPR overexpressing cell lines was assessed. In these experiments the level of BBN peptide uptake was found to positively correlate with the measured level of GRPR expression in each cell line assessed. Further, both BBN analogues (**3** and **4**) were observed to exhibit similar internalisation by flow cytometry and confocal microscopy. This data therefore suggests that the truncated BBN(6-14) peptide, owing to its shorter sequence, would be preferable for incorporation into GRPR targeted drug delivery systems. Finally, the ability to competitively inhibit the uptake of labelled BBN peptides with unlabelled BBN peptides or reduced temperatures suggested that the uptake of these peptides was through GRPR rather than non-specific mechanisms. Overall, the information gained herein on GRPR expression levels and the uptake of BBN peptides in various cancerous and non-cancerous cell lines will prove of great utility for the engineering of GRPR targeted drug delivery systems, and for the *in vitro* assessment of their targeting capacity.

4.1 Experimental

4.1 Materials

All chemicals used were of analytical grade or equivalent, unless otherwise noted. Rink amide *p*-MBHA resin (100-200 mesh: 0.36 mmol/g), *N*,*N*-dimethylformamide (DMF), piperidine and trifluoroacetic acid (TFA) were obtained from Merck Millipore (Kilsynth, VIC, Australia). Fmoc (Fluorenylmethoxycarbonyl)-L-amino acids and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) were purchased from Mimotopes (Clayton, VIC, Australia). *N*,*N*-Diisopropylethylamine (DIPEA), and L-pyroglutamic acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM), Foetal Bovine Serum (FBS), and Opti-MEM® I reduced serum medium were purchased from Gibco (Mulgrave, VIC, Australia). Fmoc-Lysine(ivDde)-OH was bought from ChemPep inc. (Wellington, FL, USA). YoYo-1, 1kb Plus DNA ladder, Hoechst 33342, LysoTracker DND 99, Lipofectamine® LTX with Plus™ reagent, and FluoroBrite™ DMEM medium were obtained from Invitrogen, Life technologies. (Mulgrave, VIC, Australia). All other reagents were purchased from Sigma Aldrich at the highest available purity.

4.2 Equipment

Analytical RP-HPLC analysis was conducted using a Vydac C18 analytical column (218TP54, 5 µm, 4.6 x 250 mm) at 1 mL/min flow rate with detection at 214 nm using linear gradients over 30 min as described in table 1. Preparative HPLC was performed using a Vydac C18 preparative column (218TP1022, 10 µm, 22 x 250 mm) at 20 mL/min with detection at 214 nm. All purifications were performed using linear gradients of solvent A [0.1% (v/v) TFA-H_2O] and solvent B [90% (v/v) MeCN-0.1% (v/v) TFA-H_2O] over 30 min. Electrospray ionization-mass spectrometry (ESI-MS) was performed on a Perkin Elmer-Sciex API3000 triple quadrupole instrument using Analyst 1.4 software (Applied Biosystems - MDS Sciex, Framingham, MA, USA). Confocal microscopy was performed using Zeiss LSM 710 confocal microscope. Data were analysed in Image J software. Flow cytometry was performed on a BD Accuri C6 flow cytometer (BD; New Jersey, USA).

4.3 Solid phase peptide synthesis

Peptides **1-4** (Fig. 1) were prepared by stepwise Fmoc-solid phase peptide synthesis[34] on Rink amide 4-methylbenzhydrylamine (MBHA) resin (100-200 mesh, 0.38 mmol/g) at 0.125 mmol scale. Fmoc removal was achieved using 20% (v/v) piperidine in DMF for 3 × 5 min at RT. Fmoc-amino acids (4.2 eq to resin) were activated with 0.5 M HATU in DMF (4 eq) and DIPEA (6 eq) and coupled at RT for 30 min per cycle. A Kaiser test[35] was performed after each amino acid coupling to evaluate coupling efficiency. Where this test demonstrated the presence of free amines, the amino acid was recoupled. L-Pyroglutamic acid (4.2 eq to resin) was coupled at RT using 0.5 M HOBt (4 eq) and DIC (4 eq) in DMF, with 2 min preactivation at 4 °C followed by 15 min rotation at RT before its addition to the resin for the synthesis of wild type bombesin (**2**). For the synthesis of fluorophore-labelled peptides (**3** and **4**), 5(6)-carboxyfluorescein (2 eq) was coupled through a lysine spacer to peptides **1** and **2** using 0.5 M HOBt (2 eq) and DIC (2 eq) in DMF, with 15 min preactivation as above. The coupling was performed overnight, in the dark to minimize photobleaching. Each peptide was cleaved from the resin using TFA/EDT/H_2O/triisopropylsilane (TIPS) (94/2.5/2.5/1) for 4 h at RT. The peptides were then precipitated using ice-cold diethyl ether, pelleted by centrifugation, dissolved in 1:1 MeCN-H_2O containing 0.1% (v/v) TFA, and lyophilised to yield crude peptides **1-4**.

4.4 Purification and characterisation

Each crude peptide was purified by RP-HPLC on a Vydac C18 column (218TP1022, 22 mm x 250 mm) at 20 mL/min using solvent B gradients over 30 min (Table 1) with detection at 214 nm. Each fraction was analysed for purity by analytical HPLC on a Vydac C18 column (218TP54, 4.6 mm × 250 mm, 5 μm) using the same gradient over the same duration used for purification. Fractions that contained pure peptide were combined and lyophilised to yield pure peptides **1**-**4**. Each peptide was characterised by RP-HPLC for purity and ESI-MS for identity (Table 1).

4.5 Cell culture

Human prostate cancer cell lines PC-3, and LNCaP, and the non-cancerous prostate cell line WPMY-1 were cultured in RPMI-1640, supplemented with 10% FBS. Human breast cancer cell line MDA-MB231 was cultured in RPMI-1640, supplimented with 10% FBS and 8 mg/L insulin (Actrapid® Neutral insulin 100 IU/ml, Novo Nordisk). The human cervical cancer cell line HeLa and colon cancer cell line Caco-2 were cultured in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% FBS and 1% non-essential amino acids. All cells were cultured in T-75 flasks at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. All cell lines were harvested at confluence and verified by short tandem repeat (STR) profiling using a Promega Cell ID kit.

4.6 Western blotting

Various cancerous (Caco-2, HeLa, LNCaP, MDA-MB-231 and PC-3) and non-cancerous (WPMY-1) cell lines were collected on ice after washing with ice-cold PBS, followed by scraping of adherent cells into 5 mL of ice-cold PBS. The cell suspensions were then collected into a 15 mL conical tube, and pelleted (1000 RPM, 5 min, 4 °C). The supernatant was then discarded and the cells were resuspended in 0.5 mL RIPA buffer (150 mM NaCl, 1 % IGEPAL CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris, pH 8.0) and gently aggitated for 30 min at 4 °C. The cell suspensions were then transferred into microcentrifuge tubes, pelleted (12k RPM, 10 min, 4 °C), the supernatant collected, and the protein content of the supernatant determined using a BCA assay against a bovine serum albumin (BSA) standard curve. Equal amounts (15 μg/lane) of protein from each cell line were then loaded onto a 10 % reducing SDS-PAGE gel and run at 100 V for 10 min and then 150 V until the loading dye reached the end of the gel. The proteins were then electrotransferred to PVDF membrane (0.2 μm pore size) using Towbin buffer (25 mM Tris

base, 192 mM Glycine containing 10% MeOH) at 100V for 1 h at 4 °C. The membrane was blocked with 5 % (w/v) skim milk powder in TBS-T (20 mM Tris base, 150 mM NaCl containing 0.1 % Tween, pH 7.6) for 1 h at RT, and subsequently probed with polyclonal rabbit α-GRPR antibody (Abcam ab39963) diluted 1:300 in 5 % (w/v) skim milk powder in TBS-T for 1 h at RT. The membrane was then washed for 3 × 5 min with TBS-T, followed by probing with goat α-rabbit IgG-peroxidase conjugate (Sigma A6154) secondary antibody diluted 1:15000 in 5 % (w/v) skim milk powder in TBS-T for 1 h at RT. The membrane was then washed for 3 × 5 min with TBS-T, followed by detection using ECL prime (GE Healthcare) enhanced chemiluminescence reagent using a Bio-Rad ChemiDoc™ Touch Imaging System. Visualization of protein transfer, and the GAPDH loading control[27] was performed using coomassie R-250 staining of the PVDF membrane.

4.7 *In vitro* cellular uptake experiments

4.7.1 *Flow cytometry quantification of cellular uptake*

Twenty-four hours prior to the study, PC-3 cells were seeded into a 48 well plate at a density of 4×10^4 cells/well and cultured as previously described. Upon reaching ~80 % confluence, the cells were washed with phosphate buffered saline (PBS) and then incubated with 200 µL /well Opti-MEM® I reduced serum medium containing 0, 1, 5 and 10 µM of fluoroscently-labelled peptides (**3** or **4**) for 4 h at 37 °C. The cells were then washed three times with 2 % (v/v) FBS in PBS (250 µL vol/wash), followed by trypsinisation with 50 µL/well of TrypLE™ Express (1×) for 5-7 min at 37 °C. Subsequently 250 µL of 2 % (v/v) FBS in PBS was added to each well, and the cell suspension was transferred into a microcentrifuge tube. To quench any cell surface bound fluorescence, the cells were treated with trypan blue to 0.1 % final concentration 2 min prior to analysis by flow cytometry (BD Accuri C6 flowcytometer, 488 nm excitation and 515-545 nm emission filter). Each assay was carried out in triplicate and 10,000 events were collected.

4.7.2 The effect of temperature on cellular uptake

To further investigate the mechanism of uptake for peptides **3** and **4**, energy-dependant endocytosis was inhibited by conducting the uptake assay 4.7.1 at 4 °C, and comparing the results with uptake at 37 °C using flow cytometry. For the 4 °C group, the cells were incubated at 4 °C for 30 min prior to performing the uptake experiment.[36]

4.7.3 Comparing bombesin uptake in Caco-2, HeLa, and PC-3 cells

The uptake of fluorophore-labelled bombesin peptides **3** and **4** were compared in Caco-2, HeLa, and PC-3 cell lines. Each cell line was seeded into 48-well tissue culture plates at a cell density of 2×10^4 cells/well for Caco-2 and HeLa cells, and 4×10^4 cells/well for PC-3 cells. The cells were then grown to 80% confluence prior to conducting the experiment. On the day of the experiment, the media was replaced with 200 µL/well of Opti-MEM® I reduced serum medium containing peptides **3** or **4** at 5 µM concentration, and the cells were subsequently incubated at 37 °C for 4 h. The cells were subsequently washed, trypsinised, stained with trypan blue, and analysed by flow cytometry as described under 4.7.1.

4.7.4 Investigating cell uptake in the presence of excess bombesin peptides

To further examine the mechanism of cellular uptake, a competative study was performed, where an excess of unlabelled bombesin peptides **1** or **2** were added at 50 µM (10× concentration) to compete for uptake with the fluorescently-labelled peptides **3** or **4** (at 5 µM) respectively. Briefly, PC-3 cells (4×10^4 cells/well) were grown to 80 % confluence in 48 well plates. The media was then replaced with 200 µL/well Opti-MEM® I reduced serum medium and the cells were incubated with a 10× excess of peptides **1** or **2** (to 50 µM final concentration) or no peptide (control) for 1 h at 37 °C. Subsequently, fluorophore-labelled bombesin peptides **3** or **4** were added (to 5 µM final concentration). The cells were then incubated at 37 °C in the dark for 4 h, and subsequently washed, trypsinised, stained with trypan blue, and analysed by flow cytometry as described under 4.7.1.

4.7.5 Confocal microscopy

The intracellular localisation of fluorophore-labelled bombesin receptor ligand peptides **3** and **4**, after uptake into PC-3 cells, was assessed by confocal microscopy.[37] PC-3 cells were seeded at a density of $3 \times 10^4/cm^2$ into ibidi µ-slide 8 well plates. After 24 h, the cells were incubated with 5 µM concentration of compounds **3** or **4** in 200 µL/well Opti-MEM® I reduced serum medium at 37 °C for 4 h. The cells were then washed 3 times with PBS, followed by the addition of Lyso Tracker Red DND 99 (6 µg/mL) for endosome staining and Hoechst 33342 (10 nmol/mL) for nuclear staining, followed by incubation at 37 °C for 20 min. To quench the cell surface fluorescence, the cells were treated with 0.2 % trypan blue for 2 min. Finally, the cells were washed 3 times with PBS and covered with complete

FluoroBrite media. Real time confocal microscopy was then performed on a Zeiss LSM 710 confocal microscope at 63× magnification using an oil emersion lens.

4.7.6 Statistical Analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. GraphPad Prism 6 was used for statistial analysis. (ns, $p > 0.05$; *, $p < 0.05$; **,$p < 0.01$; ***, $p < 0.0001$; ****, $p < 0.0001$).

Acknowledgements

The authors would like to acknowledge the technical support of Dr Michael Nefedov for flow cytometry and Dr Steven Mason for confocal imaging. This work was supported by Australian Research Council (ARC) grant DP130100952. Anjuman Ara Begum, Peter Moyle and Istvan Toth were financially supported by Endeavour postgraduate scholarship, NHMRC postdoctoral training fellowship (569869) and ARC Australian Professorial Fellowship (DP 110100212). PC-3, LnCap and WPMY-1 cell lines were kindly supplied by Mr Carson Stephens at the Australian Prostate Cancer Research Center-Queensland (APCRC-Q).

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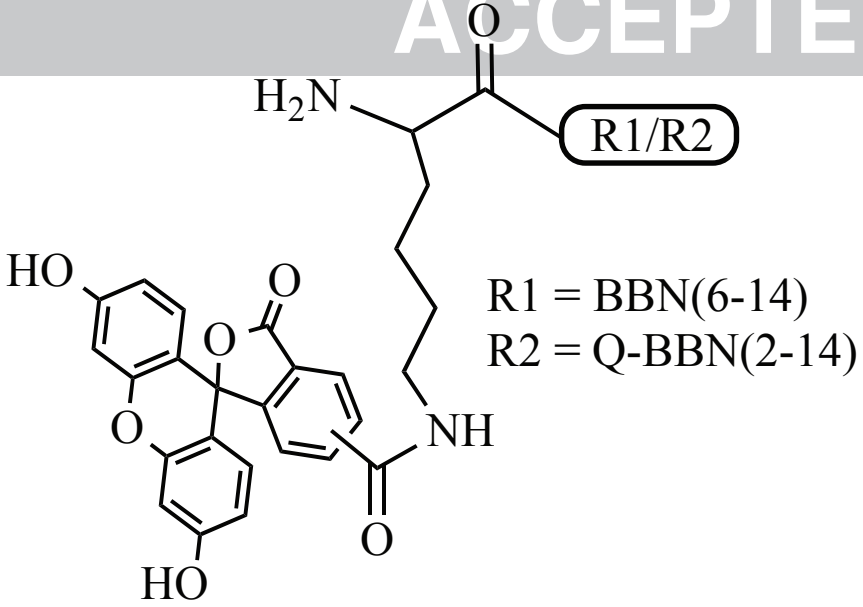
Graphical Abstract



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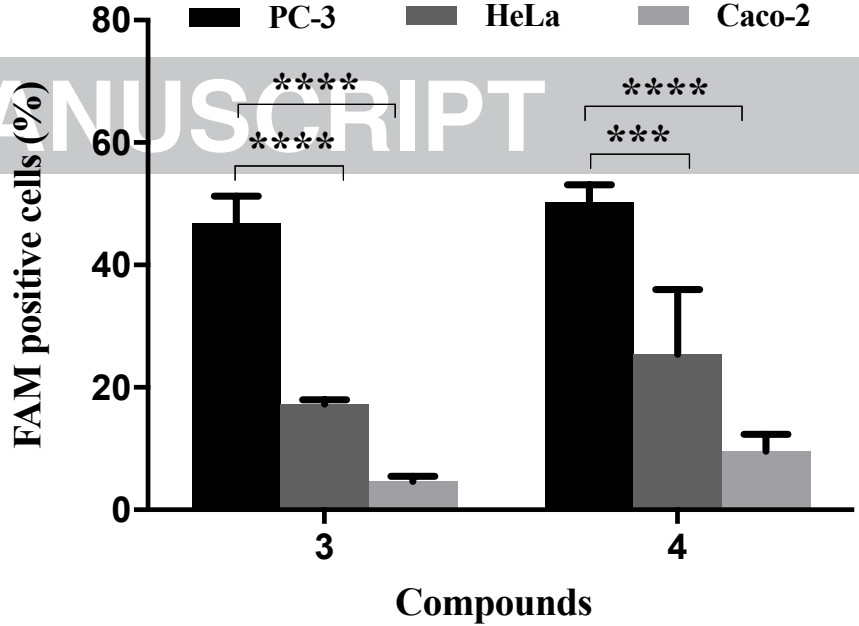
R2 = Q-BBN(2-14)



FAM-K-BBN(6-14) (3)
FAM-K-Q-BBN(2-14) (4)