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ORIGINAL ARTICLE

Comparison of the binding and internalization properties of 12 DOTA-coupled and ^{111}In-labelled CCK2/gastrin receptor binding peptides: a collaborative project under COST Action BM0607

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Abstract
Purpose Specific overexpression of cholecystokinin 2 (CCK2)/gastrin receptors has been demonstrated in several tumours of neuroendocrine origin. In some of these cancer types, such as medullary thyroid cancer (MTC), a sensitive diagnostic modality is still unavailable and therapeutic options for inoperable lesions are needed. Peptide receptor radionuclide therapy (PRRT) may be a viable therapeutic strategy in the management of these patients. Several CCK2R-targeted radiopharmaceuticals have been described in recent years. As part of the European Union COST Action BM0607 we studied the in vitro and in vivo characteristics of 12 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-conjugated CCK2R binding peptides. In the present

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study, we analysed binding and internalization characteristics. Stability, biodistribution and imaging studies have been performed in parallel by other centres involved in the project.

Methods Determination of IC_{50} values was performed using autoradiography, with DOTA-peptides displacing ^{125}I-CCK from receptors on tissue sections from human tumours. Saturation binding and internalization experiments were performed using ^{111}In-labelled peptides. The rat AR42J cell line and the human A431-CCK2R transfected cell line were utilized for in vitro experiments; dissociation constants (K_d) and apparent number of binding sites (B_{max}) were determined. Internalization was determined in receptor-expressing cells by incubating with tracer amounts of peptide at 37 and 4°C for different times up to 120 min. Surface-bound peptide was then stripped either by acid wash or subsequent incubation with 1 μM unlabelled peptide at 4°C.

Results All peptides showed high receptor affinity with IC_{50} values ranging from 0.2 to 3.4 nM. Saturation experiments also showed high affinity with K_d values in the 10^{-9}–10^{-8} M range. B_{max} values estimated in A431-CCK2R cells ranged from 0.6 to 2.2×10^6 per cell. All peptides showed high levels of internalization when incubated at 37°C.

Conclusion All DOTA-conjugated peptides showed high receptor binding and internalization properties and appear suitable for further characterization, as described in other articles of this issue.

Keywords Gastrin · Cholecystokinin · Tumour · DOTA

Introduction

A number of cell surface receptor systems are being evaluated in order to develop peptide-based radiopharmaceuticals for diagnostic and therapeutic purposes in oncology [1]. Many of these receptor systems have been shown to be overexpressed in certain types of human cancers. Naturally occurring ligands for tumour-expressed receptors are often small peptides that can be readily radiolabelled and utilized for in vivo targeting. An excellent example of the successful clinical application of this approach has been the use of radiolabelled somatostatin analogues in a number of clinical studies on imaging and targeted radionuclide therapy of cancers that overexpress specific somatostatin receptor subtypes. Using peptides labelled with ^{111}In and ^{68}Ga, single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging of disease has been performed and afterwards therapy was applied using the same compounds labelled with β-emitters such as ^{90}Y or ^{177}Lu.

The cholecystokinin (CCK) family of receptors contains two major CCKR subtypes, CCK1R and CCK2R (also referred to as CCKAR and CCKBR or gastrin receptor, respectively). Overexpression of these receptor subtypes has been demonstrated in certain human tumours [2]. The CCK1R has been found to be overexpressed in a number of pancreatic adenocarcinomas as well as in cell lines derived from such tumours and to a smaller extent in gastroenteropancreatic (GEP) tumours. The CCK2R has been found to be overexpressed in >90% of medullary thyroid cancers (MTC) as well as in other tumours of neuroendocrine origin.

Development of CCK2R targeting radiopharmaceuticals for imaging and radionuclide therapy has gained great interest as there is an unmet demand for effective treatment options for this disease when in an advanced stage [3]. A number of CCK and gastrin derivatives showing high affinity for the CCK2R have been characterized over the past years for the purpose of in vivo receptor targeting for imaging and therapy. Béhé and Behr have evaluated diethylenetriaminepentaacetic acid (DTPA)- and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-coupled derivatives of minigastrin [4–6]. Peptides based on the CCK8 sequence have also been evaluated by different groups [7, 8]. von Guggenberg et al. have evaluated cyclized forms of minigastrin in order to improve stability [9]. Other approaches to improve peptide stability have been reported by Roosenburg et al. [10]. Mather and coworkers have recently evaluated and compared a number of gastrin and CCK derivatives [11], including a modified divalent gastrin peptide containing two receptor binding motifs in the same sequence [12]. Derivatives of minigastrin labelled with ^{99m}Tc have recently been evaluated as well [13] and the first clinical applications showed the great promise of this approach [14].

Specific targeting to CCK2R has been demonstrated with most compounds tested to date, but there remain a number of issues that need improvement, particularly for therapeutic applications. One major issue encountered in the development of therapeutic peptides targeting the CCK2R is the elevated kidney accumulation especially of gastrin-based analogues, which may cause severe toxicity. This appeared to be a significant problem with DTPA- and DOTA-coupled minigastrin analogues applied in the clinic [15], but co-infusion of polyglutamic acid has been shown to greatly reduce renal radioactivity accumulation [16]. On the other hand, kidney accumulation appeared much lower for CCK derivatives [7, 17] and truncated minigastrin analogues [18].

As part of a European Union COST project (BM0607: Targeted Radionuclide Therapy) an effort has been made to evaluate and compare in a standardized way 12 peptide derivatives designed for CCK2R targeting with the final

goal of developing a ligand for clinical radionuclide therapy. Peptides have been synthesized and shared by groups throughout Europe and the same groups have provided know-how and scientific tools aimed at testing all the peptides using standardized protocols in side by side comparison studies. This approach should provide conclusive data on which peptides will be selected for further clinical development.

All peptides have been coupled to the DOTA chelator which is widely used for applications in targeted radionuclide therapy with β-emitting radionuclides such as ^{90}Y or ^{177}Lu. In this initial evaluation we have compared the biological behaviour of the ^{111}In-labelled derivatives in in vitro experiments aimed at determining binding affinities and internalization properties of the peptide analogues in human tumour tissues and in tumour cells in culture. Separate manuscripts will focus on stability and metabolism [19], biodistribution [20] and small animal imaging properties [21] of the conjugates being presented.

Materials and methods

Peptide conjugates

Peptide synthesis was carried out in different laboratories taking part in COST Action BM0607. All peptides were synthesized on a solid support using Fmoc strategy and coupled to DOTA at the N terminus. Sequences and molecular weights of the conjugates are reported in Table 1 along with references to previous publications characterizing the same or similar peptide conjugates. The crude compounds were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC). Mass spectral analysis was carried out on matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF). The desired compounds were obtained at HPLC purity higher than 95% and with a final yield between 30 and 40%.

Radiolabelling

Peptide conjugates were dissolved at concentrations ranging from 50 to 200 μg/ml. The concentration of the stock solutions was determined using a UV-Vis spectrophotometer. Extinction coefficients at 280 nm used were based on tryptophan, tyrosine and cysteine content (14,060 cm^{-1} for MGD5, 12,660 cm^{-1} for sargastrin, 5,690 cm^{-1} for SA106 and 6,890 cm^{-1} for the remaining peptides). Labelling was performed in 0.1 M hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer pH 5.5 at 10 μM peptide concentration incubating the peptide solution with $^{111}InCl_3$ (Covidien, Petten, The Netherlands, 37–185 MBq) for 30 min at 95°C. In order to avoid sulfoxide formation selenomethionine was added (≈ 1,000 times molar excess). When labelling for saturation binding experiments, 1.5 equivalents of $^{nat}InCl_3$ was added and the final solution incubated again at 95°C for 30 min in order to obtain structurally homogeneous ligands. This last step was omitted when labelling for internalization experiments. Radiochemical purity was assessed by thin-layer chromatography and was always ≥97%.

Measurement of binding affinities by autoradiography

Binding affinity measurements using autoradiography were performed as previously described [8] in surgically extracted human tumour tissues selected from previous experiments to express CCK2 receptors [2]. The peptide DTyr-Gly-[(Nle28,31)CCK-26-33] was purchased from

Table 1 Amino acid sequences of the peptide conjugates

Name	Sequence	MW (g/mol)	Reference
G-CCK8	DOTA-Gly-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	1,507.0	[7]
SA106	DOTA-DAsp-Phe(*p*-CH_2SO_3H)-HPG-Gly-Trp-HPG-Asp-Phe-NH_2	1,482.6	[10]
MG0	DOTA-DGlu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	2,037.0	[6]
Sargastrin	DOTA-Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	2,483.6	[26]
MG11	DOTA-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	1,531.0	[6]
APH070	DOTA-His-His-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	1,677.8	[11]
PP-F10	DOTA-DGln-DGln-DGln-DGln-DGln-DGln-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	2,042.9	[27]
PP-F6	DOTA-DGln-DGln-DGln-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	1,658.7	[27]
PP-F16	DOTA-DGln-DGlu-DGln-DGlu-DGln-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	2,045.9	-
PP-F11	DOTA-DGlu-DGlu-DGlu-DGlu-DGlu-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	2,048.8	-
Cyclo-MG1	DOTA-cyclo[γ-DGlu-Ala-Tyr-DLys]-Trp-Met-Asp-Phe-NH_2	1,455.7	[9]
MGD5	DOTA-Gly-Ser-Cys-(Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2)$_2$	2,782.9	[12]

HPG homopropargyl glycine

Research Plus, Inc. (Bayonne, NJ, USA) and labelled with ^{125}I (Anawa, Wangen, Switzerland, 2,000 Ci/mmol). Increasing amounts of unlabelled CCK2R binding DOTA-peptide conjugates were added to incubation medium containing fixed concentrations of the ^{125}I-labelled peptide to generate competitive inhibition curves. Tissue slides were exposed to Biomax MR films (Kodak) for 1–7 days. Tissue standards were used as reference for quantification (Amersham, UK).

Saturation binding experiments in cells

Cell binding assays were performed on A431 cells that had been stably transfected with the plasmid pCR3.1 containing the full coding sequence for the human CCK2 receptor (A431-CCK2R) [7]. Cells were maintained in medium containing the neomycin analogue G418 at a concentration of 500 μg/ml. Binding assays were performed on cells that had been plated at a density of 100,000–200,000 cells/well in 12-well multi-well plates 2 or 3 days prior to the experiments. These conditions allowed for the cells to be almost confluent at the time of the assay. To assess binding affinity of the different compounds, triplicate wells were incubated with serial dilutions of labelled conjugate in culture medium at 4°C for 1 h, with concentrations ranging from 0.5 to 500 nM. To assess nonspecific binding, binding curves with the same concentration of labelled peptide in the presence of 100-fold excess unlabelled peptide were obtained. Cell-associated radioactivity was recovered from the wells by trypsinization after two rapid washes in ice-cold phosphate-buffered saline (PBS). Radioactivity was then counted, normalized for the number of cells and nonspecific binding was subtracted. Binding curves were analysed using GraphPad Prism (version 4.00 for Macintosh, GraphPad Software, San Diego, CA, USA). Dissociation constants (K_d) and apparent number of binding sites per cell (B_{max}) were derived by fitting the data to the following equation:

$$\text{Bound} = (B_{max} * [\text{peptide conjugate}]) / (K_d + [\text{peptide conjugate}]).$$

Cell internalization experiments

Cellular internalization was first determined by comparing differences in accumulation of the compound incubated with A431-CCK2R or AR42J cells at 4°C, a temperature at which most metabolic processes are blocked, and at 37°C, when all metabolic processes including receptor internalization are active. Cells were incubated with a fixed concentration of the peptide conjugate (10 nM in culture medium) at both temperatures for 60 and 120 min. After 120 min of incubation some wells were rinsed with PBS to remove unbound radioactivity and were subsequently incubated for an additional hour at 4°C with 1 μM cold peptide, in order to displace any surface-bound radioactivity. Specific binding was assessed by co-incubating with a 100-fold molar excess of unlabelled peptide in parallel wells. Cell-associated radioactivity was recovered from the wells by trypsinization after two rapid washes in ice-cold PBS.

In another set of experiments, subconfluent cell cultures of A431-CCK2R and AR42J were transferred to six-well plates (about 10^6 cells/well) 24 h before internalization experiments. Cells were rinsed twice with 2 ml warm PBS (pH 7.4) and 1 ml 10^{-9}M ^{111}In-labelled peptides (containing 100–180 kBq) in RPMI culture medium supplemented with 20 mM HEPES and 1% bovine serum albumin was added and incubated in triplicate for 1 h at 37°C. Cellular uptake was stopped by removing radiopeptides from the cells and rinsing twice with 2 ml of ice-cold PBS. Cell surface-bound radioactivity was removed by adding 1 ml of 20 mM sodium acetate in PBS (pH 5.0) and collected after incubation for 10 min at room temperature, after which 1 ml of 1 M NaOH was added to lyse the cells. The lysates (internalized fractions) and surface-bound fractions were counted separately in a gamma counter (Perkin Elmer, Wallac, 1480 Wizard 3", Turku, Finland). To test receptor specificity of internalization, 10^{-6} M gastrin was added to the 10^{-9}M radiolabelled CCK/gastrin analogues to compete with the binding of radiolabelled CCK/gastrin analogues to the CCK2 receptor and also tested in triplicate (referred to as "block"). For comparison of uptakes in different cell lines, counts were expressed as percentage of added radioactivity per milligram protein of cell lysate, the latter determined using a commercially available colorimetric assay (Bio-Rad, Veenendaal, The Netherlands).

Results

Displacement experiments performed with unlabelled DOTA-peptide conjugates on tissue samples and evaluated using quantitative autoradiography are reported in Table 2. All peptide conjugates showed high affinity displacement of ^{125}I-CCK8 with IC_{50} values between 10^{-10} and 10^{-9} M.

Saturation binding experiments performed with ^{111}In-labelled DOTA-peptide conjugates on A431-CCK2R cells showed saturable binding for all peptides with similar K_d values in the range of 10^{-9} and 10^{-8} M. Accordingly the apparent number of binding sites per cell was also similar among different peptides in a range between 0.6 and 2.2 × 10^6 sites per cell (Table 3).

In order to assess and compare the rate and level of internalization of the different peptide conjugates, experiments were performed incubating cells with small amounts

Table 2 IC_{50} values obtained with the unlabelled DOTA-peptide conjugates by quantitative autoradiography using human tumour tissue samples

Conjugate	IC_{50}±SEM (nM, *n* in parentheses)
G-CCK8	NP
SA106	1.1±0.35 (3)
MG0	0.4±0.12 (3)
Sargastrin	0.4±0.17 (3)
MG11	1.0±0.42 (3)
APH070	0.6±0.15 (3)
PP-F10	1.1±0.06 (3)
PP-F6	3.4±0.36 (5)
PP-F16	0.6±0.11 (4)
PP-F11	0.5±0.07 (5)
Cyclo-MG1	0.7±0.12 (3)
MGD5	0.2±0.04 (3)

NP not performed

of labelled peptide (10 nM) and comparing cell retention at 4 and 37°C, two incubation conditions generally accepted as having no active internalization of receptor and active receptor turnover, respectively. These experiments were conducted independently in two laboratories.

Experiments performed on A431-CCK2R cells showed progressive accumulation in the cells of all labelled radiopeptides tested. The rate at which activity in cells increased over time is shown in Fig. 1a. Values are normalized to the average maximum amount of radioactivity reached after 2 h of incubation for each peptide at 37°C. The amount bound to cells at 4°C (30 min of incubation), which is assumed to be the starting point for subsequent internalization of peptide, is set as the 0 min time point. The time course of cell-associated radioactivity for cells incubated at 4°C over the 2-h observation period (not shown) showed no significant increase in uptake over time for all peptides.

After 2 h of incubation with labelled peptide at both temperatures, cells were incubated with 1 μM unlabelled peptide in order to displace any surface-bound radioligand still present. Figure 1b shows that cells previously incubated at 37°C retained most radioactivity after displacement of surface-bound peptide. Conversely, cells incubated at 4°C show that most radioactivity was displaced from the cell surface to background levels with a certain degree of variability in the residual nonspecific binding to cells. These results show that internalization of the peptides occurred for all conjugates tested. The increase in cell-associated activity for cells grown in internalizing conditions (37°C) compared to cells where internalization was inhibited by incubation at 4°C was in the range of 5–20 times at 2 h, suggesting that internalization likely will play an important role in increasing the amount of radiopeptide targeted to tumours in vivo.

Figure 2 shows the internalization results obtained in the second set of internalization experiments. In accordance with the data shown in Fig. 1, all compounds showed a high internalization rate in A431-CCK2R cells and AR42J cells after 60 min incubation (Fig. 2a). Cell-associated activity was significantly reduced after blockade with unlabelled gastrin for all compounds, indicating receptor-specific binding and internalization. One compound (cyclo-MG1) appeared to show a much lower total uptake in rat AR42J cells, expressing the rat receptor, than in A431-CCK2R cells, expressing the human CCK2 receptor (Fig. 2b).

Discussion

The 12 CCK2R/gastrin binding peptides evaluated appear to be very similar with regard to receptor binding and internalization properties. The displacement experiments performed by quantitative autoradiography showed high affinity displacement for all peptides tested with IC_{50} values in the same range as for similar peptides evaluated

Table 3 K_d and B_{max} values obtained from saturation binding experiments with ^{111}In-labelled DOTA-peptide conjugates in A431-CCK2R cells

Conjugate	K_d±SEM (nM)	B_{max}±SEM (nM)	B_{max}±SEM sites/cell × 10^6
G-CCK8	15.7±2.9	0.40±0.02	0.8±0.5
SA106	14.6±2.7	0.20±0.01	0.7±0.4
MG0	13.5±2.9	0.83±0.07	1.7±0.2
Sargastrin	13.2±4.6	0.31±0.04	0.6±0.1
MG11	16.0±1.4	0.36±0.01	0.7±0.2
APH070	21.4 ±1.6	1.49±0.05	2.2±0.1
PP-F10	9.4±1.0	0.21±0.06	0.6±0.2
PP-F6	4.8±2.4	0.38±0.06	1.1±0.1
PP-F16	9.0±4.2	0.30±0.04	0.9±0.1
PP-F11	15.9±2.2	1.21±0.06	1.8±0.2
Cyclo-MG1	13.0±1.6	0.42±0.03	1.3±0.1
MGD5	11.5±1.2	2.13±0.08	2.1±0.1

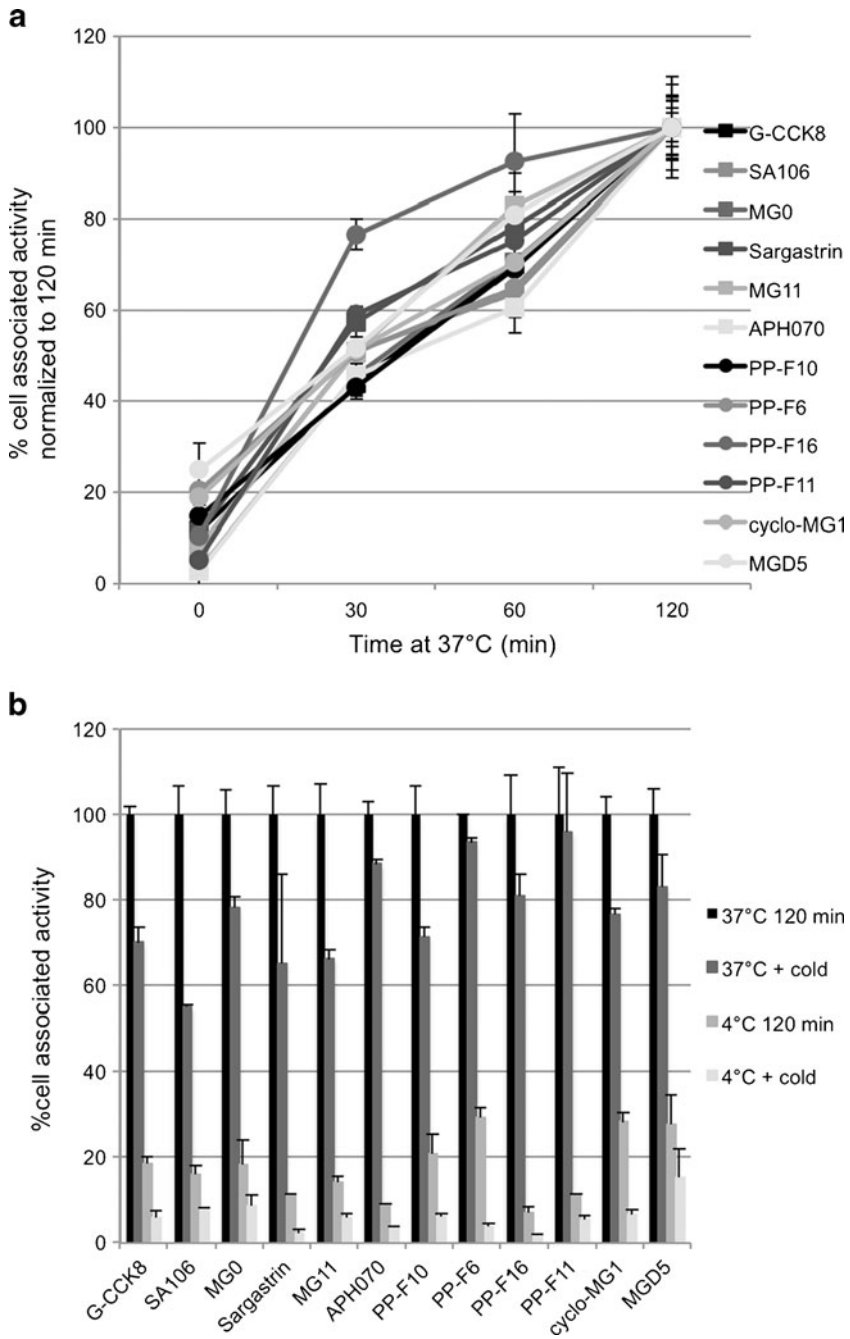

Fig. 1 **a** Time course of cell-associated activity in A431-CCK2R cells incubated at 37°C for the 12 radiopeptides tested. Uptake values are normalized to uptake at 120 min that was set at 100%. **b** Displacement of surface-bound radioactivity by subsequent addition of unlabelled peptide (1 μM) after incubation with radiolabelled peptides for 120 min at 37 and 4°C

using the same method in previous publications [8]. All the peptides tested have a common Trp-X-Asp-Phe-NH_2 motif at the C terminus of the peptide and most have the Ala-Tyr-Gly triplet immediately to the N-terminal side. There is structural evidence of a direct interaction of the Trp-Met sequence of CCK8 with the extracellular portion between transmembrane domains 6 and 7 of the CCK2 receptor [22]. Given the small differences in the IC_{50} values derived it appears that these sequences are important for adequate interaction with the CCK2 receptor and none of the modifications performed in other areas of the peptide significantly impact the ability to have high affinity displacement.

Saturation binding experiments similarly showed K_d values all within a fairly narrow window of values in the 10^{-8} M range. The K_d values reported show slightly lower affinity compared to previously reported values for similar peptides tested on other cell culture systems or tissue samples [23], but are comparable to values found for similar peptide conjugates tested on the same tissue culture system [7, 24, 25]. The cell culture model in question is the human A431 epidermoid carcinoma cell line where

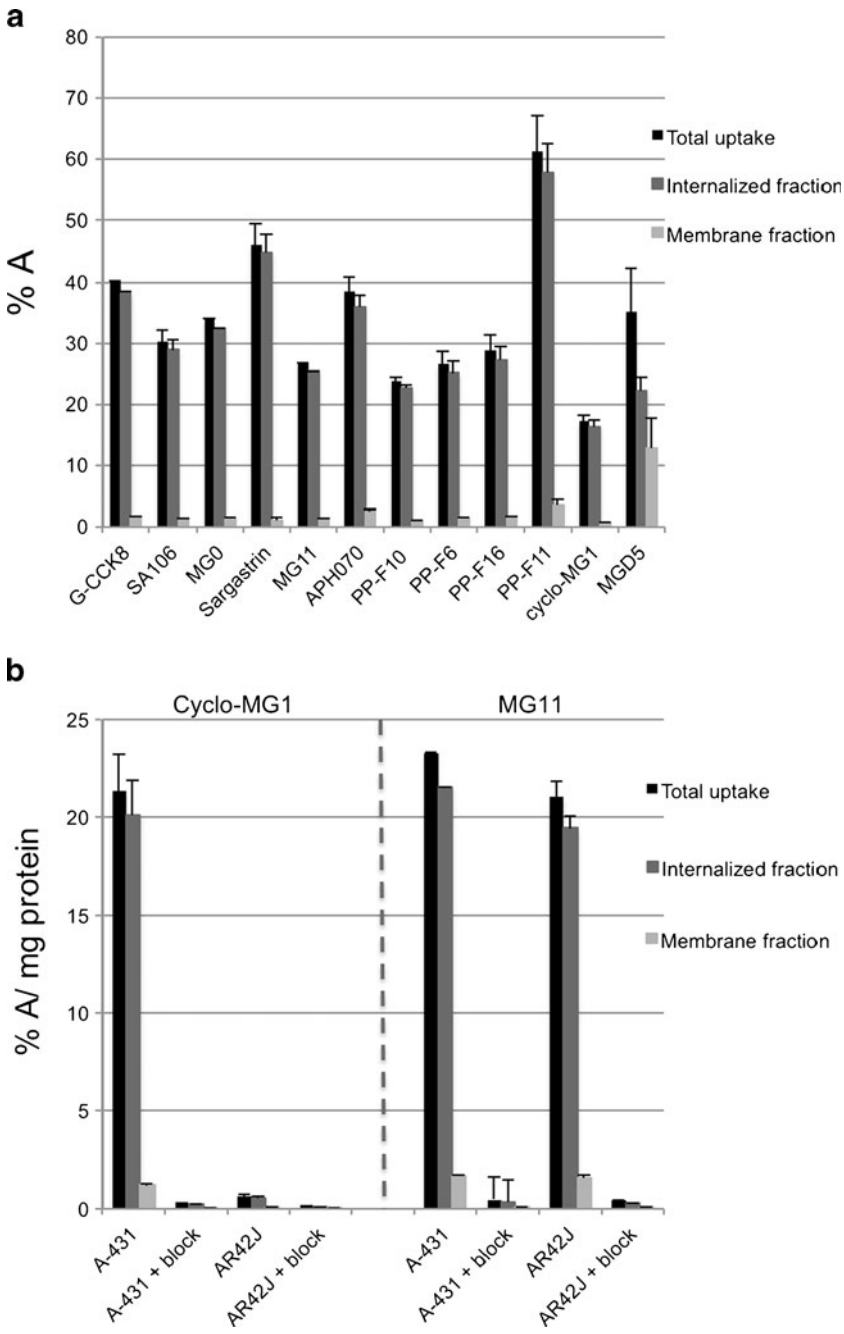

Fig. 2 **a** Comparison of cell-associated radioactivity, expressed of % of the total added radioactivity (*% A*), for all 12 radiopeptides after 60 min incubation at 37°C using A431-CCK2R cells. Total uptake is the sum of internalized fraction and membrane-bound fraction. **b** Comparison of cell-associated radioactivity, expressed as % of the total added radioactivity per mg cell protein (*% A/mg protein*), for two radiopeptides after 60 min incubation at 37°C using A431-CCK2R cells versus AR42J cells. Total uptake is the sum of internalized fraction and membrane-bound fraction

expression of the receptor was obtained by cellular transfection [7]. Although the human coding sequence was utilized to transfect these cells, it is conceivable that some of the post-translational modifications to the protein are missing in the A431 cells relative to cells of neuroendocrine origin where the protein is physiologically expressed. This may contribute to the slightly lower affinity values observed when using these cells. Nevertheless, given the high level binding and active internalization properties displayed by the cells they are an adequate model for testing binding and internalization properties of CCK2R peptides.

Indeed, internalization experiments performed using two different protocols showed a high internalization rate of all peptides in the CCK2R transfected A431 cell line again indicating that the differences in the peptide sequences presented have minimal impact on internalization of the receptor-peptide complex.

Internalization experiments performed in the rat AR42J cell line also produced similar results. One exception was the internalization of cyclo-MG1 (Fig. 2b). In this cell line, very little specific interaction and internalization was observed with ^{111}In-DOTA-cyclo-MG1. This may be due to the differences in the binding domain of the rat versus

the human receptor. It should be noted that this same peptide analogue coupled to a different chelating system for labelling with ^{99m}Tc [9] was previously characterized in this same cell line and showed saturable binding with a K_d of approximately 20 nM in the AR42J cells. The structural peculiarity of this cyclized peptide, which places certain conformational constraints on peptide-receptor interaction, may have contributed to the lower receptor affinity found for the DOTA-coupled conjugate currently being evaluated towards the rat receptor.

Conclusion

All peptide conjugates tested showed high affinity binding and active internalization into CCK2R-expressing cells. Based on the presented in vitro data there were no candidates that showed markedly superior properties compared to the others for further clinical evaluation. All peptide sequences tested appeared suitable for further characterization as described in the other articles of this issue [19–21].

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Conflicts of interest None.

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