Enzymatic degradation study of ^{111}In-labeled minigastrin peptides using cathepsin B enzyme and AR42J cancer cell line for the development of neuroendocrine tumor imaging radiopharmaceuticals

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Abstract: Neuroendocrine tumors (NET) are the rare tumors which often impose graveyard threat. These tumors are characterized by the overexpression of various G-protein coupled receptors including cholecystokinin (CCK) receptors-1 and 2 (A or B). Minigastrin peptides are being investigated for theranostic purposes of CCK-2 receptor positive NET. The minigastrin analogue (APHO70) was modified by engineering enzyme susceptible tetrapeptide sequence into APHO70 peptide to reduce the random degradation by lysosome enzymes which pave the way to random trafficking in patient's body and dipeptide addition at c-terminus. All the four modified minigastrin peptides (MG-CL1-4) were investigated for lysosome cathepsin B (catB) enzyme susceptibility and fate into AR42J cancer cell line. The indium-111 labeled MG-CL1-4 peptides were also studied for target (tumor) and non-target saccumulation by using tumor induced mice. The RP-HPLC analysis result showed nonspecific cleavage of standard ^{111}In-APH070 and ^{111}In-MGCL1 while specific cleavage was noted in case of ^{111}In-MGCL (2-4). The effect of specific and non-specific cleavage on biodistribution in tumor induced nude mice model indicates the promising accumulation of ^{111}In-MGCL2, ^{111}In-MGCL3, and ^{111}In-MGCL4 radiotracers while ^{111}In-MGCL1 showed less accumulation. ^{111}In-MGCL2 and ^{111}In-MGCL3 showed highest target-to-kidney ratio (T/K) i.e. 1.71 and 1.72, respectively whereas standard compound showed T/K 1.13. In conclusion, the two indium-111 labeled analogues i.e. ^{111}In-MGCL2 and ^{111}In-MGCL3 showed promising sensitivity for tumor and could be tested for further investigation to reach pre-clinical studies.

Keywords: Enzyme; peptide cleavage; tumor imaging; gastrin peptides; neuroendocrine tumor.

INTRODUCTION

In recent years, molecular imaging is gaining an ample attention due to its promising diagnostic potential of deep seated diseases at molecular level (Uliel, *et al.*, 2013). Whereas invasive radiological modalities such as x-rays, ultrasound, magnetic resonance imaging (MRI) and computed tomographic (CT) scan helps after certain morphological and entomological alteration in disease tissues. Molecular imaging works non-invasively through administration of a radionuclide labeled biologically active molecules such as antibody, peptides, or organic synthetic molecules followed by acquisition and quantification through single photon emission computed tomography (SPECT), or positron emission tomography (PET) (Rashid, *et al.*, 2017).

Radiolabeled regulatory peptides (RRP) have shown promising potential for tumor imaging and therapy due to its strong affinity for receptors which over express at the cancer cell surface – this gives the cancer therapy procedure called peptide receptor radionuclide therapy (PRRT) (Bodei, *et al.*, 2017; Lozza, *et al.*, 2013). The main advantages of this procedure lies in the rapid production of radiolabeled peptides, easy to administrate and cell endocytosis internalization, low immunogenicity and rapid clearance from non-target body organs (Dash, *et al.*, 2015). Radiolabeled peptides (RPs) reaches into cell lysosome following the binding to the peptide receptors at the tumor cell surface (Rosenbaum, *et al.*, 2009) where RPs are fragmented into labeled and non-labeled moieties. Non-specified fragmentation of RPs may produce daughter RPs which can accumulate non-specifically and consequently pose radiotoxicity. There in lysosome a significant number of enzymes of cathepsin family (cathepsin B, L, S, K, A, G, D, and E), work together or individually in reducing or acidic conditions (pH < 5). Out of different cathepsin enzymes, catB and L are widely distributed in specific cells which handle peptides and proteins to fragment into small sequences. The radiolabeled peptides also face the similar kind of fragmentation in lysosome (Naqvi, *et al.*, 2010). The aim of this study was to investigate the effect of catB enzyme susceptible peptide sequence (-Gly-Phe-Leu-Gly-), which

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Table 1: Retention time of radio-labelled minigastrin peptides, nomenclature and sequence

Nomenclature	Radio-peptide sequence	Retention time (min)
^{111}In-APH070	^{111}In-DOTA-H-H----------E-A-Y-G-W-M-D-F	21.4
^{111}In-MGCL1	^{111}In-DOTA-H-H-G-F-L-G-E-A-Y-G-W-M-D-F	22.8
^{111}In-MGCL2	^{111}In-DOTA-D-D-G-F-L-G-E-A-Y-G-W-M-D-F	19.7
^{111}In-MGCL3	^{111}In-DOTA-L-L-G-F-L-G-E-A-Y-G-W-M-D-F	19.5
^{111}In-MGCL4	^{111}In-DOTA-N-N-G-F-L-G-E-A-Y-G-W-M-D-F	20.2

is reported for its specific susceptibility for cell lysosome enzymes (Lee, *et al.*, 2017), by introducing into the control minigastrin peptide (APHO70) between N-terminus glutamic acid and c-terminus di-histidine – further the fragmentation pattern was also studied by replacing c-terminus histidine dipeptide with di-aspartic acid, or di-leucine or di-asparagine peptide after indium-111 radiolabeling and biological evaluation.

MATERIALS AND METHODS

The chemical used in this study were of analytical grade. catB enzyme, L-cysteine, tri-isopropyl saline (TIS), acetonitrile (ACN), trypan blue sodium acetate anhydrous, ethylene diamine tetraacetic acid (EDTA), sodium acetate , trifluoroacetic acid (TFA); and SepPak® C-18 cartages were purchased from Sigma-Aldrich (Gillingham, UK). $^{111}InCl_3$ solution in 0.05 M HCl was obtained from Covidien (Petten, Netherlands).

Synthesis of ^{111}In-minigastrin

MGCL1-4 were synthesized using the procedure reported earlier, in which partial results of this study has already been discussed (Naqvi, *et al.*, 2010). Briefly, 20mL of $^{111}InCl_3$ solution, 4mL 1M ammonium acetate buffer (pH 5.5; 8.3 mg/mL gentistic acid) and 4-5mL peptide solution (1mg / mL aqueous solution) was added consecutively into polypropylene vial. The reaction mixture was incubated at 98°C for 15 minutes in a dry heating block. At the end of reaction period, 1.8 mL EDTA solution (0.1M) was added to quench the reaction.

Hydrolysis of ^{111}In-MGCL using cathepsin B enzyme

Hydrolysis of the RRP was carried following the procedure described earlier (Naqvi, *et al.*, 2010). In an eppendorf tube (1.5mL), containing 35µL ^{111}In-MGCL aliquot (1µg/10µL), subsequently added 10µL catB enzyme of 5 unit / mL sodium acetate buffer (20mM), 15 µL 1.0mM EDTA and 155µL L-cysteine buffer (5.0mM; pH 5). The reaction mixture was left for 15, 60 and 180 minutes at room temperature followed by RP-HPLC analyses.

HPLC Analysis

The enzyme treated ^{111}In-MGCL radiotracers were analysed with HPLC (with a Beckman 168 diode array ultraviolet (UV) detectors and a NaI radiochemical detector attached to a Raytest gamma-radioactivity monitor (Beckman, High Wycombe, England). The HPLC gradient system comprises of 0.1% TFA in water (solvent A) and acetonitrile (solvent B), at a flow rate of 1mL/minute was used. The gradient was performed as follows: 0-5 minute 95% solvent A and 5% solvent B), 5-20 minutes 40% solvent A and 60% solvent B), 20-25 minutes 0% solvent A and 100% solvent B) and finally, from 25-29 minutes 95% solvent A and 5% solvent B).

Degradation of radio-labeled peptide using cell lysosomes

The degradation of ^{111}In-MG-CL was carried out using AR42J cancer cell line. For this, ^{111}In-MG-CL solution (~ 4 MBq) was added to 1 mL AR42J cells (~1.3 million per mL). Peptides were allowed to internalise for 1.5 h at 37°C followed by twice washing in ice cold media and subjected to two 2×5 minutes acid washings in 20mM sodium acetate (pH 5) in Hank's balanced salt solution. Each washing step was followed by centrifugation at 1000 g, 4°C, for 5 minutes. The cell pellets following acid wash were lysed in internalisation media by two cycles of freeze-thawing-spinning at 20,000 g. The three lysates of each ^{111}In-MGCL were pooled and analysed on SepPak® C-18 cartridges.

SepPak ® C-18 Analysis

SepPak® C-18 cartridges were used for SepPak column analysis. The column was primed with 5 mL methanol and equilibrated with 5 mL double distilled water, followed by the loading of lysate fraction of ^{111}In labeled MGCL peptides (with or without excess competitor). The column was first eluted with 5 mL water to elute unbound (hydrophilic) cleaved radio-fragments and collected. Hydrophobic radio-fragments were subsequently eluted with 4mL each of 10, 20, 30, 40, 50, 60 and 80% acetonitrile in water supplemented with 0.1% TFA and collected each fraction separately. All fractions were counted in a gamma counter.

Biodistribution

Biodistribution study was carried out using tumor induced nude mice. For this 4.0 MBq of the radiochemical was diluted up to 1.5mL with phosphate buffered saline (PBS) in a 1.5mL vial. Then administration of 200 µL of labeled peptide through tail vein of nude female mice (bred in-house at Cancer Research UK) was performed and sacrificed at 4 h post injection. After each sacrifice, both kidneys, the complete intestine, the liver, the pancreas, the heart, the spleen, the lungs, the stomach, the muscle, the tumour, a sample of blood, and the tail from each animal

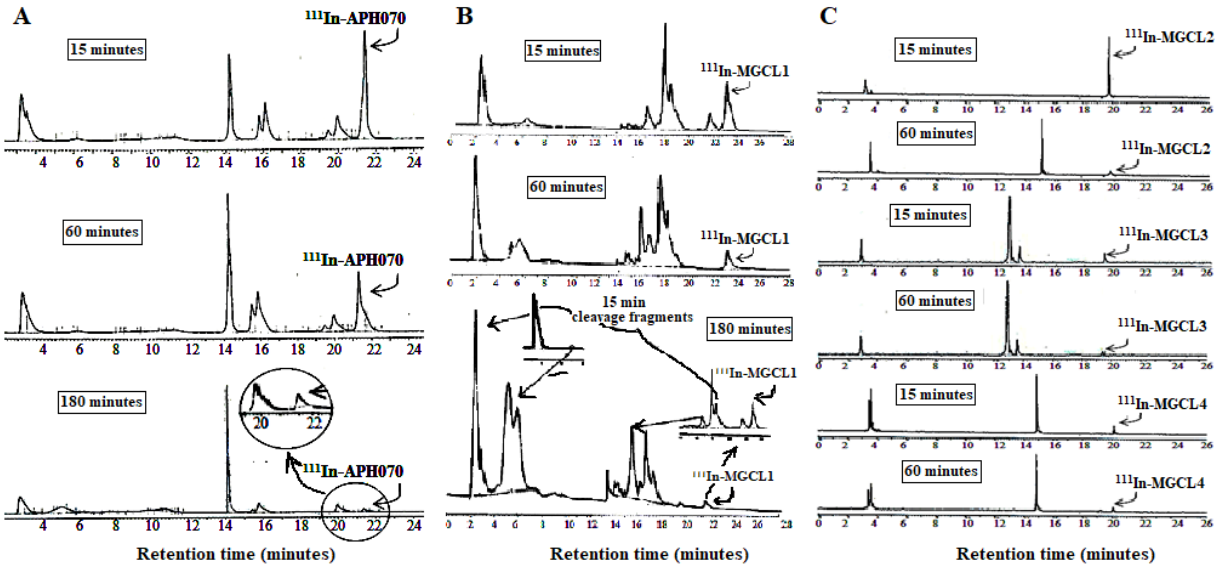

Fig. 1: A) RP-HPLC analysis of ^{111}In-APH070 treated with catB; 15, 60 and 90 minutes time point fragmentation chromatograms; B) RP-HPLC analysis of ^{111}In-MGCL1 treated with catB; 15, 60 and 180 minutes time point fragmentation chromatograms; C) RP-HPLC analysis of ^{111}In-MGCL2, ^{111}In-MGCL3 and ^{111}In-MGCL4 treated with catB; 15 and 60 minutes time point fragmentation chromatograms

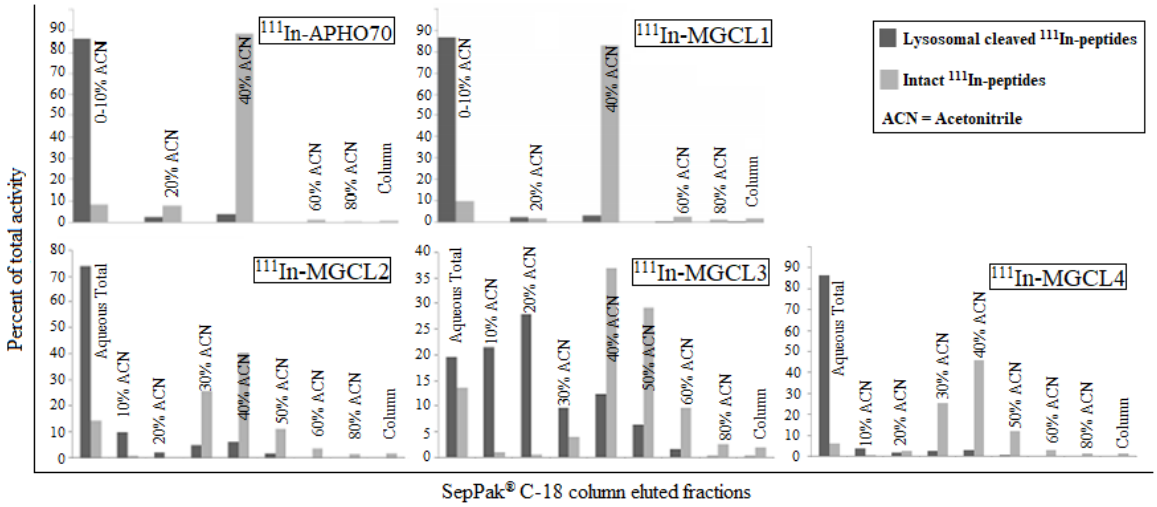

Fig. 2: SepPak® C-18 analysis of ^{111}In-APH070, ^{111}In-MGCL1, ^{111}In-MGCL2, ^{111}In-MGCL3 and ^{111}In-MGCL4 incubated with AR42J cell line for lysosomal fragmentation pattern at 90 minutes.

were separated and placed in pre-weighed gamma counting tubes and sealed. This was done with all ^{111}In-MG-CL solutions; the tubes were placed in gamma counter and counted for four minutes using the ^{111}In calibration setting. The recorded data was then used to calculate the present injected-dose per gram organ (%ID/gm organ).

RESULTS

Radiosynthesis and HPLC analysis

The ^{111}In-labeled MGCL (1-4) peptides were synthesized using the protocol reported earlier (Naqvi, *et al.*, 2013). The HPLC analysis revealed >85% labeled compounds with small fraction of oxidized radiolabeled compound. The radiochemical purity was achieved more than 90% after filtering through 0.4µm membrane filter. The retention time of each radiolabeled peptide recorded through RP-HPLC are shown in table 1.

Action of catB enzyme on ^{111}In-MGCL peptides

To evaluate the susceptibility of tetra peptide sequence, inserted into the control peptide (APHO70) sequence, were noted either by incubating the radiolabeled minigastrin peptides with most active lysosomal enzyme (catB) or living cell lysosome using AR42J cancer cell

line. Fragmentation pattern, as recorded by HPLC, of ^{111}In-APH070 and ^{111}In-MGCL1 radiolabeled fractions incubating with catB enzyme at 15, 60 and 90 minutes time point are shown in fig. 1A&B. The HPLC chromatograms of ^{111}In-MGCL2, ^{111}In-MGCL3 and ^{111}In-MGCL4 incubated with catB analysed at 15 and 60 minutes are shown in fig. 1C.

Effect of lysosome enzymes on ^{111}In-MGCL peptides degradation

To analyse the actual fate of minigastrin peptide, inserted with catB susceptible tetra peptide sequence, in living cancer cell lysosome, the radiolabeled peptides were subjected to incubate with AR42J cancer cell line. The fractions were analysed through SepPak® C-18 cartridge. The fragmentation fractions obtained are shown in fig. 2.

Biodistribution

To investigate the effect of structural modification in control minigastrin peptide, the biodistribution of ^{111}In-labeled peptides in tumor induced nude mice models were assessed. The mice were administrated with 200µL of labeled peptide through tail vein and then sacrificed at 4 h post injection. The each organ were separated, washed with PBS and counted for radioactive fraction uptake. The uptake in the form of % ID / g organ by different organs at 4 hour post injection are shown in fig. 3.

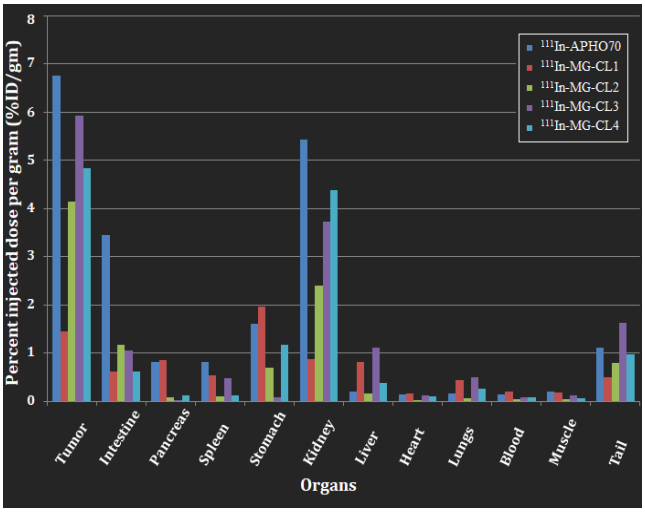

Fig. 3: Biodistribution study of ^{111}In-APH070, ^{111}In-MGCL1, ^{111}In-MGCL2, ^{111}In-MGCL3 and ^{111}In-MGCL4 in tumor induced nude mice at 4 h post injection time point

DISCUSSION

Neuroendocrine tumors are rare and commonly inoperable with high chronic potential and morbidity rate. Peptide receptor radionuclide therapy (PRRT) is known for its excellent potential to take the patient out from risk. Minigastrin peptides are known to accumulate at CCK2 receptors, these receptors are overexpressed at neuroendocrine cancer cells (Naqvi, *et al.*, 2013). The radiolabeled minigastrin peptides are being evaluated for obtaining maximum sensitivity and negligible non-target accumulation in the patient body. A series of four MG-CL peptides (1 to 4) were tested for specific cleavage by carrying out modification in control minigastrin peptide (APHO70). Random fragmentation, typically the basic factor for non-targeted accumulation and subsequently lest specificity. In cell lysosome, catB is the principal enzyme which hydrolysis the proteins and peptides (Verma, *et al.*, 2016).

The incubation of control and modified ^{111}In-labeled minigastrin peptides with catB, reveals it is not only enzyme susceptible sequence which control the fragmentation pattern but also the amino acid or peptide sequences which are directly bonded to -Gly-Phe-Leu-Gly-. The HPLC chromatogram of ^{111}In-APHO70 and ^{111}In-MGCL1 showed more than one peaks at 15 min time point. The intact peak of both peptides at 21.4 min and 22.8min, respectively, gradually disappeared in late chromatograms obtained at 60 and 180 min. In contrast to ^{111}In-MGCL1 chromatogram the ^{111}In-APHO70 showed only one main peak at 180 min while ^{111}In-MGCL1 showed numerous peaks which indicate that the agent may distribute itself in different organs randomly and not fit for imaging procedure. The other radiolabeled peptides i.e. ^{111}In-labeled MGCL(2-4) showed very simple chromatogram with one main peak at 60 min time point which indicates if there is no other factor to affect then ^{111}In-MGCL 2-4 radio-peptides will show negligible nonspecific accumulation and high target specificity.

In HPLC most of the fragments were eluted with high aqueous concentration of mobile phase. The SepPak® C-18 cartridge elution of ^{111}In-APH070 and four ^{111}In-MG-CL radiolabeled peptides also advocated the hydrophilic characteristics of radiolabeled fragments as shown in fig. 4 with one major and two minor fragments in case of ^{111}In-APH070 and ^{111}In-MG-CL1 and populated fragmentation elution in case of ^{111}In-MG-CL2, ^{111}In-MG-CL3 and ^{111}In-MG-CL4. This might be due to two main reasons; one, catB not alone responsible for degradation and the second the SepPak® C-18 does not work with reliable sensitivity as HPLC works.

Biodistribution study revealed that ^{111}In-MGCL1 and ^{111}In-MGCL4 at 4 h post injection showed low uptake of activity by different organs (<1% ID/g organ) except kidneys which showed highest uptake. Blood clearance was noted quickly, at 4 h post injection blood counts showed <0.18%±0.15% ID/g blood. Whereas, ^{111}In-MGCL2 and ^{111}In-MGCL3 biodistribution showed encouraging results; very low non-specific accumulation and high tumor up-take. The organs including heart, spleen, liver, lungs, pancreas, intestine, muscle and blood showed uptake within the range 0.0319±0.001 to 0.675±

0.021% ID/g organ. Both peptides were also cleared from blood extremely quickly as compared to the ^{111}In-APHO70 and ^{111}In-MGCL 1&4 radiopeptides with high target accumulation. The most sensitive organ in PRRT is kidney whose care must be taken, the uptake of ^{111}In-APH070, ^{111}In-MGCL1 and ^{111}In-MGCL4 in kidneys was noted 5.17±1.56, 3.092±1.86 and 4.477±1.57% ID/g organ, respectively; whereas ^{111}In-MGCL2, ^{111}In-MGCL3 uptake in kidneys was noted 2.39±0.86 and 2.80±0.16% ID/g organ, respectively. On the other hand ^{111}In-MGCL2 and ^{111}In-MGCL3 showed promising accumulation (4.08±0.87 and 4.82±0.74% ID/g organ, respectively) was seen at tumour than any other organ. In comparison, ^{111}In-APHO70 also showed high affinity for tumour, but tumour to kidney ratio 1.13 of APHO70 indicates higher relative retention in the kidneys compared to the ^{111}In-MGCL2 (T/K = 1.71) and ^{111}In-MG-CL3 (T/K = 1.72).

The ^{111}In-MGCL1 varies from control peptide only through catB tetra-peptide sequence inserted between receptor susceptible peptide sequence (-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-) and –His-His- dipeptide (having positive charges side chain) of APHO70, and both showed more or less similar enzyme degradation and biodistribution pattern. The ^{111}In-MGCL4 was different from ^{111}In-MGCL1-3 through c-terminus dipeptide sequence i.e. presence of -Asn-Ser- (having neutral side chain). It showed one fragment during enzyme degradation study while biodistribution similar to ^{111}In-MGCL1. The other two peptides i.e. ^{111}In-MGCL2 and ^{111}In-MGCL3 with –Asp-Asp- (having negative charge side chain) and –Leu-Leu- (having hydrophobic side chain) c-terminus dipeptide sequence, respectively showed promising results in context of enzymatic degradation and biodistribution. The study reveals that it is not only the enzyme susceptible peptide sequence which can control the biodistribution and target accumulation of radiolabeled peptides but also the c-terminus peptide sequence which control the kinetics and subsequently the accumulation pattern. Further the c-terminus dipeptide sequence, which responsible for kinetics of the distribution, work more favourably if the c-terminus peptide is acidic or hydrophobic in nature.

CONCLUSION

The development of radiolabeled peptides for the diagnosis and therapy of rare NET is highly important to manage NETs. The results of this study described the effect of engineering of lysosome catB enzyme susceptible -Gly-Phe-Leu-Gly- peptide sequence into CCK2 receptor seeking minigastrin radio-peptides with neutral, positive, hydrophobic and acidic c-terminus dipeptide sequence. The acidic and hydrophobic dipeptide sequence containing radiolabeled minigastrin peptides (^{111}In-MGCL2&3) showed results more or less according to the hypothesis and could further be evaluated for developing radiopharmaceuticals of choice for theranostic purposes of NETs.

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