Chapter 4

Development and characterization of the capture agents library targeting c-MET for in vivo cancer imaging

4.1. Introduction

As I mentioned in the previous chapter, positron emission tomography (PET) enables imaging of biological process in noninvasive and quantitative way[1], it is requested to develop new radio probes visualizing specific biomarkers, especially cancer markers to diagnose and stage the disease and guide therapeutic decisions[2]. For example, [4-^{18}F]fluorobenzaldehyde-conjugated aminooxy-protein scaffolds are used to detect tumoral Her2 expression[3], and [^{18}F]fluoroestradiol is used to stratify breast cancer patients by measuring estrogen receptor expression[4,5]. However, radio probes able to image-specific surface markers for cancer cells are still lacking.

Hepatocyte growth factor receptor (HGFR/c-MET) is a receptor tyrosine kinase whose expression has been shown to be associated with tumor invasion and metastasis. Since its discovery in mid-1980s[6], many reports elucidate that c-MET can be a cancer marker as well as a target of cancer therapy due to its high expression level in various tumors, such as lung, kidney, colon, gastric, thyroid, pancreas and prostate cancers[7-9]. As shown in Fig.4.1.[10], numerous oncogenic pathways are engaged downstream of c-MET, which may lend to multiple strategies for therapeutic inhibition. As recent clinical studies reported c-MET antibody improved the overall survival when it was treated to well stratified cancer patients, targeting c-MET shows promising prospect[11].

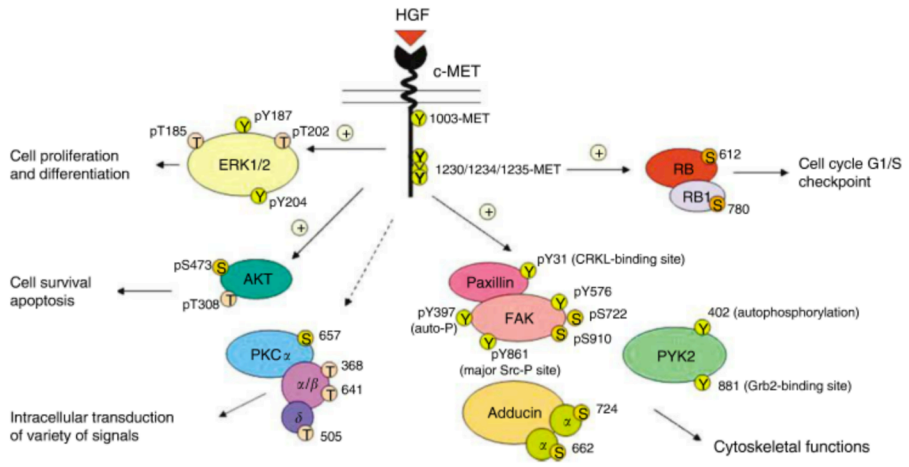

Fig. 4.1. c-MET pathway, adapted from *Br. J.*

In this chapter summarizing the current work, I present a novel library of capture agents synthesized from artificial amino acids to recognize extracellular domain of c-MET for avoiding disadvantages of antibodies, such as high cost, batch to batch variation and instability for long-term storage[12,13]. Researchers reported that these protein-catalyzed capture agents (PCC) could be synthesized for recognizing their specific target biomolecules, bCAII and Akt1, with high affinity compared to the commercialized antibody counterpart, and with solid stability for long-term storage at room temperature. Since it is also possible to synthesize the capture agents in bulk size by using standard solid-phase method, their production costs also have room to lower upon the demand[12,13].

4.2. Experimental Methods

4.2.1. Synthesis of PCC for c-MET

Multiligand protein-catalyzed capture agents (PCC) library can be generated by using in situ click chemistry, as shown in **Fig. 4.2.** Detailed synthetic method is explained in the previous paper[12,13]. In short, capture agents were generated by iteratively screening one-bead-one-compound (OBOC) peptide library on TentaGel beads against recombinant human c-MET extracellular domain (R&D Systems). The target protein was exploited to promote click reactions between individual azide- and alkyne-functionalized library elements, first to produce biligands and second to produce triligand candidates. At each screening stage, beads were selected both for binding to the target and evolution of a triazole-linked product. To remove beads that are prone to non-selective binding, an anti-screen was performed against human serum and detected via a labeled anti-human serum antibody. Only the peptides passing though both of positive and negative selections were employed as triligand c-MET capture agents.

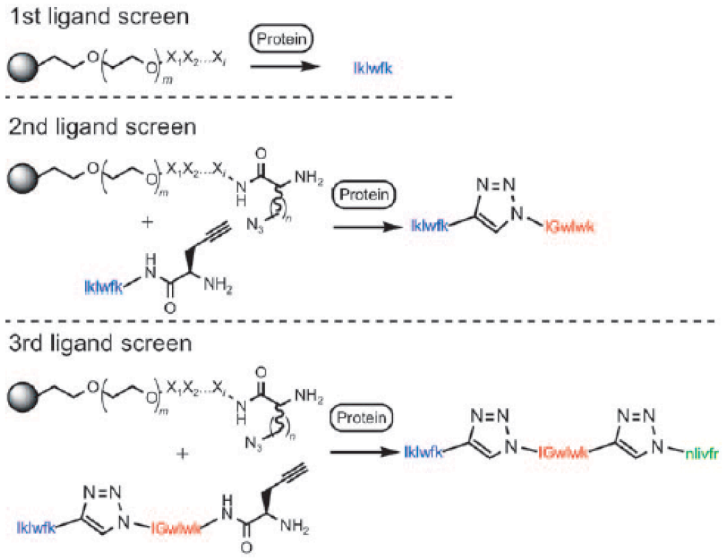

Fig. 4.2. Scheme of capture agent synthesis by in-situ click chemistry, adapted from *Angew. Chem. Int. Ed.* (2009).

Once several candidates were found, these triligand c-MET capture agents were prepared in bulk quantities by using solid-phase synthesis of the individual ligands followed by copper(I)-catalyzed ligation of azide and terminal alkyne[14]. Final products were purified by HPLC and characterized by mass spectrometry.

4.2.2. Functionalization of capture agents.

To work as in vivo imaging probes, the capture agents synthesized as above were labeled with a radioactive atom. However, it is not practical to characterize the radio-labeled capture agents at the beginning, because their short life time limits available time for experiments and their radioactivity demands extra safety and special equipment for handling and visualizing. Hence, the characterization of capture agents was achieved with dye-labeled materials, and the fluorescent signals from the capture agents were quantified

first. Only candidates showing superior performance to the commercialized c-MET antibody was selected for the next step, the labeling with a radioactive atom.

As a dye molecule, fluorescein isothiocyanate (FITC) was used for its ease of functionalization. The N-terminus of the capture agent was labeled with FITC (AnaSpec) as a final step in its solid-phase synthesis. The reaction proceeded as follows **Fig. 4.3.**

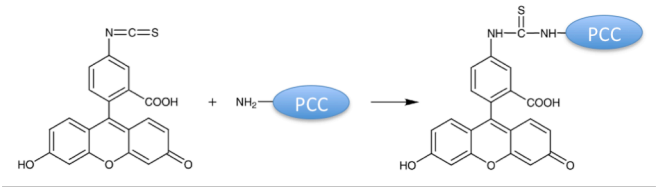

Fig. 4.3. Scheme of FITC labeling, provided by the manufacturer.

For the radio-labeling of capture agents, 4-[^{18}F]fluorobenzaldehyde was synthesized first, ([^{18}F]FB-CHO), then it was conjugated with the N-terminus of the capture agent as shown in **Fig. 4.4.** as described in the literature[15]. After the synthesis, the product was rapidly purified by HPLC and its radioactivity was measured with a gamma counter.

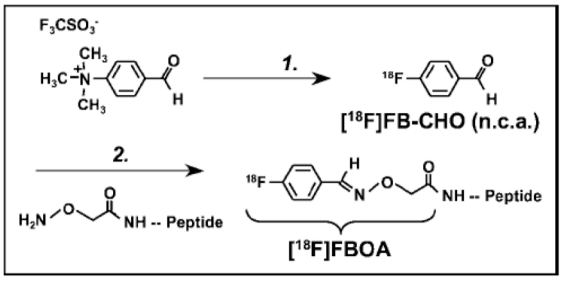

Fig. 4.4. Scheme of ^{18}F labeling, presented in *J. Nucl. Med.* (2004)

4.2.3. Preparation of cells

In order to verify the binding capacity of capture agents, human prostatic carcinoma cell lines, PC3, DU145, LNCaP and 22Rv1 cells, were purchased from ATCC as described in the literature[16]. All cell lines were cultured in RPMI medium 1640, supplemented with 10% FBS and 1% pen/strep solution, in a 37 °C incubator.

Also, HGF, the ligand of c-MET, was added to cells in c-MET inhibition experiment to check that synthetic capture agents actually bind to c-MET to form a competitive relationship with the original ligand, HGF. In this case, just before the harvest of cells, cells were treated with recombinant HGF (Sigma) at the concentration of 25ng/ml for 15 min at the cell incubator as suggested in a previous work[17].

4.2.4. Confocal experiment for 3D imaging of live cells incubated with capture agents

Synthetic capture agents must bind to the extracellular domain of c-MET for working as imaging probes. In order to confirm the binding site, c-MET expressing cells were incubated with FITC-conjugated capture agents to be analyzed with a confocal microscope. As a control, cells were also incubated with a commercial FITC labeled antibody for the visualization, and the 3D images from both conditions were compared with ImageJ (NIH).

The detailed procedure will be as follows: 100,000 cells were seeded into each well of microwell slides with 2ml of cell culture media 24 hour before the imaging experiment. On the next day, cell culture media was aspirated, and cells were stained with 100μl of cell culture media containing different concentration of dye-conjugated capture agent or

antibody for 1 hour at cell incubator to find an optimal condition. After washing with cell culture media twice, cells were on top of LSM510 microscope (Zeiss) for imaging.

4.2.5. Flow cytometry for the quantification of binding affinity

Flow cytometry is a powerful tool to compare the interactions between ligands and receptors on the surface of cells as well as to find an optimal concentration of materials for staining[18]. To quantify the binding capacity of capture agents to c-MET on the surface of cells, the fluorescence intensity of cells incubated with FITC-labeled capture agents were analyzed with a flow cytometer by following procedures:

Cells were harvested and aliquoted into 1.7ml falcon tubes at the volume of 100μl having 10million/ml concentration in 1% BSA-PBS. In order to make calibration curves, each aliquot was treated with serial dilutions of FITC-conjugated capture agents and antibody, as a control, for 1hr on ice. After the treatment, by using a centrifuge, cells were washed and re-suspended into 0.5ml of 1% BSA-PBS. Data was obtained and analyzed with FACSCalibur (BD bioscience) and Flow Jo (Tree Star), respectively.

4.2.6. In vitro radioassay with RIMChip

In order to do an in vitro radioassay, RIMChip was fabricated with PDMS material as explained at the previous Chapter 3. Cells were prepared at 3×10^6 cells per ml and injected into the RIMChip. Cell numbers were counted on an optical microscope. For the HGF inhibition studies, 25ng/ml of HGF in RPMI 1640, supplemented with 10% FBS, was added to the cells for 15 min. 100 μCi of radio-labeled capture agents were pipetted into the RIMChip microchannels, and the RIMChip were then incubated for 1 hour at cell culture

incubator and flushed with PBS twice. Finally, a betabox having beta camera was assembled for the measurement of radioactivity.

4.2.7. In vivo treatment and imaging with microPET/CT

To demonstrate the PCC work as imaging probes, in vivo experiment with mice is required. All animal studies will be done according to the guideline of the office of laboratory animal resources at Caltech. SCID mice will be injected with 1 million PC3 cells, and tumors will be allowed to grow for one week. Mice will be killed after imaging on day 14 for biodistribution studies. For imaging, mice will be injected with 100 μCi of radio-labeled capture agents. Data will be obtained in a dynamic scan for 1.5 hours after the probe administration by using Siemens Preclinical Solutions microPET focus 220 and a MicroCAT II CT instrument as described previously[2].

4.3. Results and Discussion

4.3.1. Synthesis of capture agents for c-MET

IN-CT-1025, the triligand capture agent against c-MET, was discovered and synthesized by Indi Molecular in Culver City, CA (data unpublished, **Fig. 4.5**).

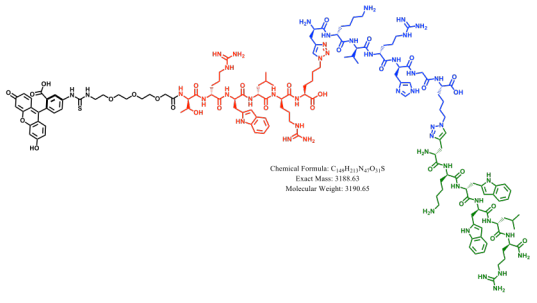

Fig. 4.5. Molecular structure of the 1st generation capture agent for targeting human c-MET, conjugated with FITC, IN-CT-1025.

4.3.2. Cell-based fluorescence imaging assays

In order to find the best positive and negative controls for the cell-based assays, the expression level of c-MET in four prostate cancer cell lines, PC3, DU145, LNCaP and 22Rv1, was first examined by Western blot with lysates of those cell lines as shown in **Fig. 4.6.A,** which is accordance with the previous literature[17]. To check the affinity of IN-CT-1025 to c-MET on the surface of prostate cancer cells, with a confocal microscope, we did a proof-of-concept experiment by measuring fluorescent signals from four kinds of living prostate cancer cell lines applied with IN-CT-1025. As presented in **Fig. 4.6.B**, the fluorescent signals were comparable with the result from Western blot, which indicates that IN-CT-1025 is able to recognize the target protein, c-MET, at the extracellular domain of cell membrane.

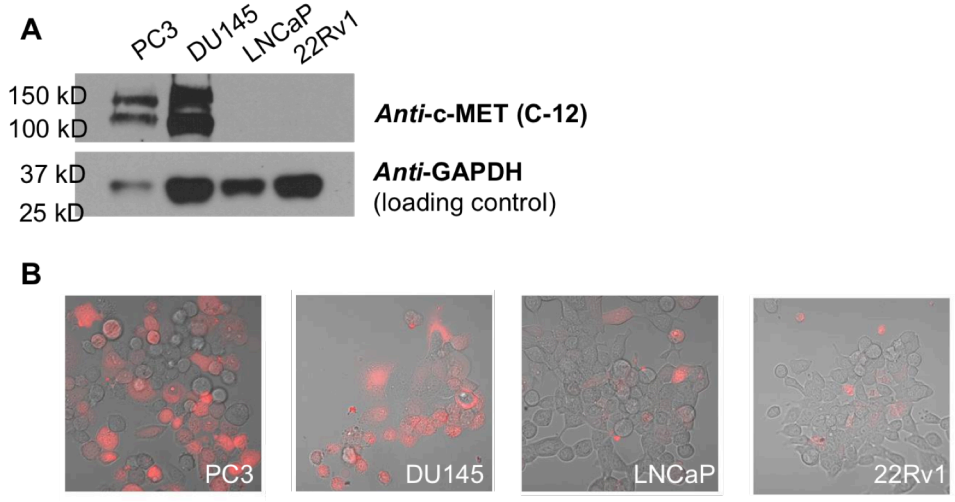

Fig. 4.6. Expression level of c-MET in different prostate cancer cell lines. (A) The result of Western blot with lysates of prostate cancer cell lines. (B) The result of confocal microscopy experiments with live prostate cancer cell lines applied to IN-CT-1025.

After visualizing the binding of IN-CT-1025to cells, its affinity to prostate cancer cells was quantitatively measured with a flow cytometer. By titrating the concentration of IN-CT-1025 with 1 million PC3 and 22Rv1 cells as positive and negative controls, the mean fluorescent signals from each sample were obtained to make a plot as shown in **Fig. 4.7.** A commercial c-MET antibody (eBioscience) was also applied to cells for the comparison, but we could not explore the region of high concentration of antibody (more than 375nM) due to its high cost.

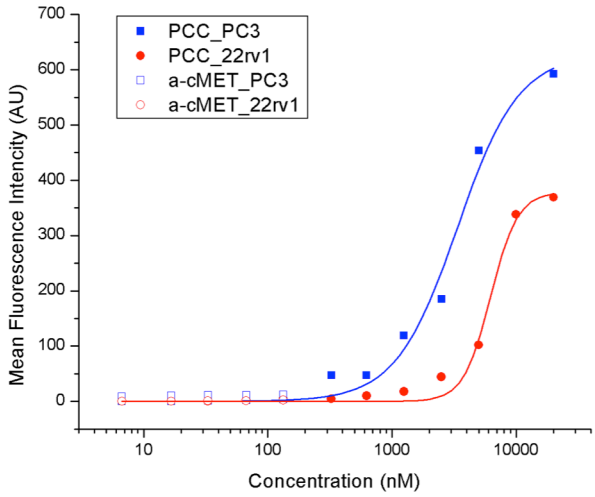

Fig. 4.7. Result of titration experiment with IN-CT-1025. With flow cytometry, the binding affinity of IN-CT-1025 to PC3 cells and 22Rv1 cells, employed as positive and negative controls, was measured. Commercial anti c-MET antibody were also applied to cells for the comparison.

The data was fitted with Hill function which gives the dissociation constant, Kd, of IN-CT-1025 with PC3 cells to be 3.36μM while Kd of IN-CT-1025 with 22rv1 cells is 6.23μM. Interestingly, compared to the dynamic range of the commercial antibody, 30 (11.84 to 0.39), IN-CT-1025 showed much wider dynamic range of about 2,800 (592.7 to

0.21), which implies that IN-CT-1025 can work as a better imaging probe to distinguish c-MET positive and negative cells than antibodies.

4.4. Conclusions and Future Directions

As written above, c-MET is highly overexpressed in cancer patients. Having a specific imaging probe to this cancer marker, diagnosis and/or staging of cancer will be more accurate and lead to more rapid therapeutic decision making. With the first-generation protein-catalyzed capture agent, IN-CT-1025, we checked the possibility of PCC as an in vivo imaging probe by measuring its binding affinity to prostate cancer cells through cell-based fluorescent imaging assays. Compared to a commercialized c-MET antibody, IN-CT-1025 is shown to be a better agent to discriminate c-MET positive and negative cells due to its wide dynamic range. With this PCC, we will examine its capacity to work as an in vivo imaging probe by using an in vitro radio assay with RIMChip and Betabox as described in the previous Chapter 3, which will be then followed by the in vivo experiments with mice imaging on micro PET/CT.

4.5. References

1. Phelps, M. E. Positron emission tomography provides molecular imaging of biological processes. *P Natl Acad Sci USA* **97,** 9226–9233 (2000).
2. Laing, R. E. *et al.* Noninvasive prediction of tumor responses to gemcitabine using positron emission tomography. *P Natl Acad Sci USA* **106,** 2847–2852 (2009).
3. Cheng, P. *et al.* Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *Journal of Experimental Medicine* **205,** 2235–2249 (2008).
4. Linden, H. M. *et al.* Quantitative fluoroestradiol positron emission tomography

imaging predicts response to endocrine treatment in breast cancer. *J. Clin. Oncol.* **24,** 2793–2799 (2006).

5. Peterson, L. M. *et al.* Quantitative imaging of estrogen receptor expression in breast cancer with PET and 18F-fluoroestradiol. *J Nucl Med* **49,** 367–374 (2008).
6. Cooper, C. S. *et al.* Molecular cloning of a new transforming gene from a chemically transformed human cell line. *Nature* **311,** 29–33 (1984).
7. Ma, P. C. *et al.* Expression and mutational analysis of MET in human solid cancers. *Genes Chromosomes Cancer* **47,** 1025–1037 (2008).
8. Liu, N., Furukawa, T., Kobari, M. & Tsao, M. S. Comparative phenotypic studies of duct epithelial cell lines derived from normal human pancreas and pancreatic carcinoma. *Am J Pathol* **153,** 263–269 (1998).
9. Jacobsen, F. *et al.* High c-MET expression is frequent but not associated with early PSA recurrence in prostate cancer. *Exp Ther Med* **5,** 102–106 (2013).
10. Ma, P. C., Tretiakova, M. S. & Nallasura, V. Downstream signalling and specific inhibition of c-MET/HGF pathway in small cell lung cancer: implications for tumour invasion. *British journal of Cancer* (2007).
11. Gherardi, E., Birchmeier, W., Birchmeier, C. & Vande Woude, G. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* **12,** 89–103 (2012).
12. Agnew, H. D. *et al.* Iterative in situ click chemistry creates antibody-like protein-capture agents. *Angew Chem Int Ed Engl* **48,** 4944–4948 (2009).
13. Millward, S. W. *et al.* Iterative in Situ Click Chemistry Assembles a Branched Capture Agent and Allosteric Inhibitor for Akt1. *J. Am. Chem. Soc.* **133,** 18280–18288 (2011).
14. Rostovtsev, V. V., Green, L. G., Fokin, V. V. & Sharpless, K. B. A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective ‘ligation’ of azides and terminal alkynes. *Angew Chem Int Ed Engl* **41,** 2596–2599 (2002).
15. Poethko, T., Schottelius, M. & Thumshirn, G. Two-step methodology for high-yield routine radiohalogenation of peptides: 18F-labeled RGD and octreotide analogs. *J Nucl Med* (2004).
16. Aggarwal, S., Singh, P., Topaloglu, O., Isaacs, J. T. & Denmeade, S. R. A dimeric peptide that binds selectively to prostate-specific membrane antigen and inhibits its

enzymatic activity. *Cancer Res* **66,** 9171–9177 (2006).

17. Van Leenders, G. J. L. H. *et al.* Activation of c-MET induces a stem-like phenotype in human prostate cancer. *PLoS ONE* **6,** e26753 (2011).

18. Sklar, L. A. & Finney, D. A. Analysis of ligand-receptor interactions with the fluorescence activated cell sorter. *Cytometry* **3,** 161–165 (1982).