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14. ABSTRACT Tumors associated fibroblasts (TAFs) represent a major component of a PCa tumor, and play a critical role in tumor development. The purpose of this proposal is to utilize fibroblast activation protein alpha (FAP) expression on TAFs within the tumor stroma for the diagnostic imaging of PCa using novel radiopharmaceuticals and innovative multimodal imaging platforms. This progress report describes the <i>in vivo</i> biodistribution analysis of radiolabeled FAP conjugates, which are 10-20 fold more potent as FAP inhibitors than similar inhibitory peptides described in the literature. Radiopharmaceutical retention in blood was low at early time points suggesting rapid clearance from the blood pool. Radioactivity was retained in kidney tissue and suggests that this is the primary route of excretion. Tumor targeting was inefficient.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	05
2. Keywords.....	05
3. Overall Project Summary.....	05
4. Key Research Accomplishments.....	07
5. Conclusion.....	07
6. Publications, Abstracts, and Presentations.....	07
7. Inventions, Patents and Licenses.....	07
8. Reportable Outcomes.....	07
9. Other Achievements.....	07
10. References.....	08

1.0. Introduction

Tumor microenvironment is as important to the development of the malignant phenotype as the genetic mutations accumulated by cancerous cells over their lifetime [1]. Tumor-associated fibroblasts (TAFs), which express the serine protease fibroblast activation protein alpha (FAP), are up-regulated in the tumor stroma in 90% of all epithelial cancers including prostate cancer (PCa) [2-10]. **This proposal seeks to utilize FAP expression on TAFs within the tumor stroma for the diagnostic imaging of PCa using novel radiopharmaceuticals and innovative multimodal imaging platforms.** We have developed peptides that are specific for the FAP active site, conjugated them to the cross-bridged macrocycle 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (CB-TE2A), and radiolabeled them with ^{64}Cu , which has favorable decay characteristics ($t_{1/2} = 12.7\text{ h}$; β^+ : 19%; $E_{\beta^+ \text{ max}}$, 0.656 MeV; EC: 41%; β^- : 40%) for PET imaging [11]. This report summarizes the experiments completed and the progress made during the current funding period.

2.0. Keywords

PET, prostate cancer, FAP, molecular imaging, peptide, copper-64, FRET, radiopharmaceutical, radiotracer, tumor microenvironment, stroma, mesenchymal stem cell, tumor associated fibroblast, serine protease

- (1) Lys(R)-**Thr-Ser-Gly-Pro-Asn**-Glu-CONH₂
- (2) Lys(R)-**Thr-Ala-Gly-Pro-Asn**-Glu-CONH₂
- (3) Lys(R)-**Ala-Ala-Gly-Pro-Asn**-Glu-CONH₂
- (4) Lys(R)-**Thr-Ser-Gly-Pro-Ser**-Glu-CONH₂

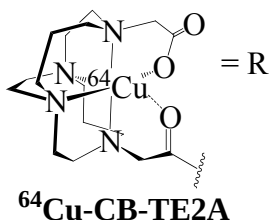


Figure 1. Radiotracers evaluated as FAP substrates. Each tracer contains a Gly-Pro sequence, which undergoes cleavage at the FAP active site. All peptides are radiolabeled with the ultra-stable ^{64}Cu -CB-TE2A reporter.

3.0. Overall Project Summary

Despite the important role of FAP in tumor biology, PCa and cancer therapy, there remains a dearth of molecular probes designed to detect and quantify FAP *in vivo*. A peptide-based, diagnostic PET agent that can detect FAP *in vivo* would be of great value to the medical community since PET is superior to SPECT in terms of sensitivity and resolution and it is not influenced by the same limitations, which hinder optical imaging methods [12]. Furthermore, peptide based agents demonstrate greater design flexibility, greater tolerance to diverse reaction conditions during preparation and better biokinetic and clearance properties when compared to antibodies, which can result in enhanced contrast and reduced patient burden during imaging. Moreover, agents designed to exploit FAP expression would serve as non-invasive imaging agents for the early detection of cancer, the stratification of patients for FAP therapy and the monitoring of response to treatment [13].

During the current funding period, a major research focus was the *in vivo* evaluation of the radiolabeled peptides depicted in **Figure 1**. We optimized radiolabeling conditions including time, temperature and pH, for conjugates **1-4**. Reaction of each conjugate in ammonium acetate buffer under basic conditions for 30 minutes at 95°C yields the desired radiolabeled conjugate in high radiochemical purity. High temperature and basic conditions are necessary due to the proton sponge behavior of the cross-bridged macrocycle [14, 15]. Radiolabeled conjugates can be prepared with a specific activity of 37 MBq (1 mCi)/ μg , which is consistent with the specific activity of other ^{64}Cu -CB-TE2A conjugates

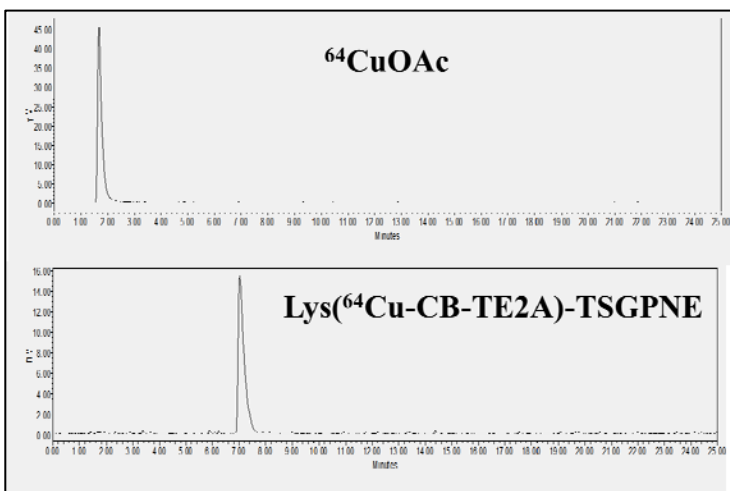


Figure 2. Representative radio-HPLC chromatograms of ^{64}Cu -based radiotracers. Upper Panel: Copper-64 acetate (standard control). Bottom Panel: K(^{64}Cu -Cb-TE2A)-TSGPNE.

that have been reported in the literature and used in *in vitro* and *in vivo* studies [16-19]. Importantly, optimization of these parameters has eliminated the need for solid phase purification, and reduced the

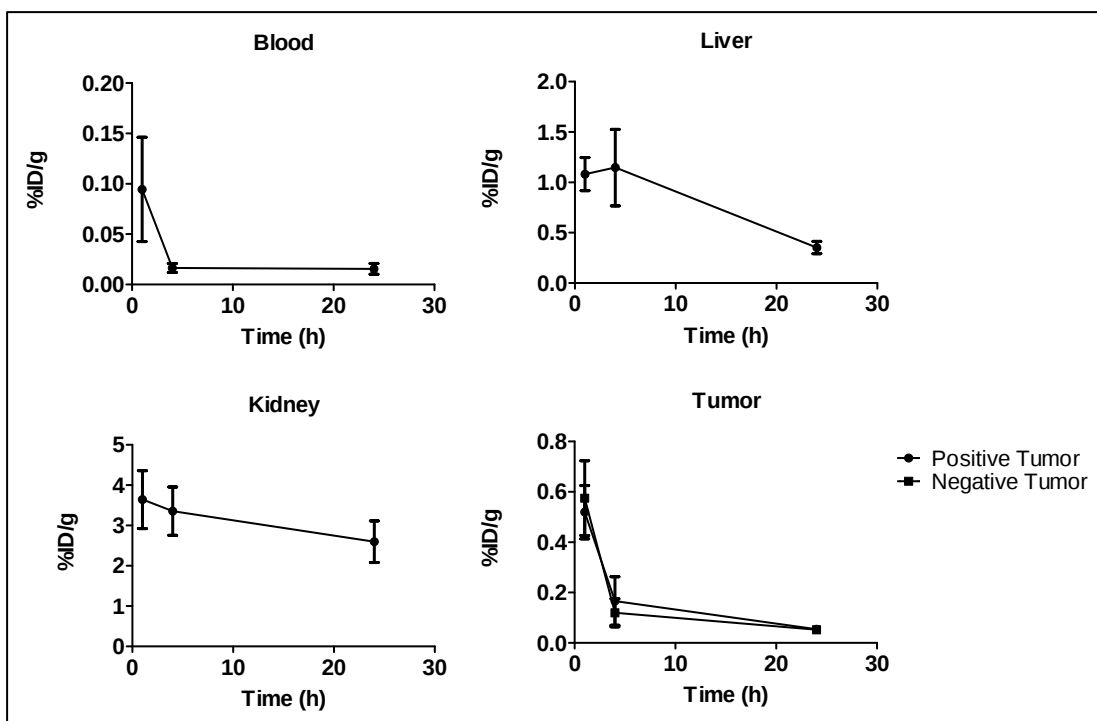


Figure 3. Acute biodistribution results of K(⁶⁴Cu-Cb-TE2A)-TSGPNE. The radiopharmaceutical demonstrated efficient blood and liver clearance. Kidney clearance and tumor retention was poor.

production time of these radiopharmaceuticals to 30 minutes. Additionally, we have evaluated the stability of each conjugate over time in saline and in human serum. **Figure 2** depicts the stability of K(⁶⁴Cu-Cb-TE2A)-TSGPNE in saline. In this system, unchelated or transchelated ⁶⁴Cu elutes at 2 min. while the intact conjugate moves elutes at 7-8 min. As can be observed, K(⁶⁴Cu-Cb-TE2A)-TSGPNE is remarkably

stable even after 24 h and reflects the kinetically inert nature of the ⁶⁴Cu-CB-TE2A

complex [15]. All complexes demonstrate the same stability properties.

Once radiolabeling and stability were verified the second goal of this progress period was to examine the acute biodistribution of the radiolabeled complexes in athymic mice bearing FAP⁺ and FAP⁻ tumors, and representative results of these studies are depicted in **Figure 3**. Radiopharmaceutical retention in blood was low at early time points suggesting rapid clearance from the blood pool. By 24 h, 84% of the injected activity was excreted. This is expected given the low molecular weight of the radiotracer. Liver retention over time was modest with 67% of the 1 h activity excreted by the end of the study. The extraordinary stability of the ⁶⁴Cu-CB-TE2A complex is believed to contribute to the low retention of radioactivity in hepatic tissue. Elevated levels of radioactivity were observed in kidney tissue suggesting that these radiotracers are excreted renally. By 24 h, 71% of the injected activity was observed to still be present, and suggests that these radiotracers are retained here without excretion. This may be a consequence of the cationic nature of the radiopharmaceuticals. Tumor retention was modest at every time point, and radioactivity in the FAP⁺ tumors was not statistically different from retention in FAP⁻ tumors. This suggests that the radiopharmaceuticals did not demonstrate effective targeting of FAP *in vivo* and that the retention and clearance of radioactivity in the FAP⁺ tumors was dominated primarily by perfusion. This phenomenon was observed for all radiopharmaceuticals evaluated (Figure 1).

Since none of the peptides demonstrated efficient tumor targeting, PET imaging will not proceed. Rather we will attempt to derive more appropriate substrates using a focused, one-bead one-compound (OBOC) combinatorial peptide library. This library would be synthesized at the Bioanalytical Laboratory Core Facility, which is a shared resource of the Comprehensive Cancer Center of Wake Forest School of Medicine. The OBOC approach allows many copies of the same peptide to be synthesized on a single bead [20, 21], and its

context specific nature will allow for the rapid identification of FAP specific ligands [22]. For example, when considering conjugate **1**, molecular modeling and computational studies have determined that the G-P amino acid sequence is critical for FAP cleavage and the remaining amino acids are necessary for optimum secondary enzyme-substrate interactions. Currently there are more than 10 commercially available derivatives for the amino acids, Thr, Ser, Asn and Gly and 5 commercially available Pro derivatives (ANASPEC, Inc. Fremont, CA) [23]. If these amino acid derivatives are used, a focused library with more than 50,000 compounds would be developed. This focused library would be screened by MALDI-TOF MS, and Edmund's degradation to fully characterize the FAP cleavage products and parent peptides originally prepared using on-bead synthesis [24]. Once peptides have been obtained, FRET and biodistribution analysis will occur as described in the original proposal [21, 25-27] to find lead candidates for PET imaging.

4.0. Key Research Accomplishments

SOW Task 3C: Radiolabel CB-TE2A-conjugates with ^{64}Cu . Each conjugate will be labeled with ^{64}Cu in high purity and high specific activity. Purity will be assessed using a Waters analytical reversed-phase high performance liquid chromatography (HPLC) system. The complex $^{64}\text{Cu}(\text{OAc})_2$ will be used as a standard control.

SOW Task 3D: Perform biodistribution studies and analyze data

Tumor bearing mice will be injected with a ^{64}Cu -CB-TE2A-peptide, and blocking studies will also be performed using the ^{64}Cu -CB-TE2A-peptide along with the respective radiopharmaceutical. Animals will be sacrificed and organs of interest and tumor will be removed, weighed and counted on a gamma counter. The percent injected dose per gram (%ID/g) and percent injected dose per organ (%ID/organ) will be counted and compared to a weighed, counted standard for both groups.

5.0. Conclusions

Targeting stromal elements such as tumor associated fibroblasts (TAFs) within the tumor microenvironment represents a novel way to detect and image PCa. TAFs express FAP, which is strictly regulated to the surface of TAFs found in the tumor stroma, but is not observed on PCa cells, normal fibroblasts or other benign tissues. Despite the important role of FAP in tumor biology, PCa and cancer therapy, there remains a dearth of molecular probes designed to detect and quantify FAP *in vivo*. In the current funding period, we have evaluated our radiolabeled peptides as FAP substrates in acute biodistribution studies. During these studies we observed efficient blood and liver clearance. Radioactivity was retained in kidney tissue and suggests that this is the primary route of excretion. Tumor targeting was inefficient. Current efforts include a further biodistribution studies to confirm the results obtained during the funding period, and to elucidate better substrates using a medicinal chemistry approach.

6.0. Publications, Abstracts, and Presentations

Nothing to report

7.0. Inventions, Patents and Licenses

Nothing to report

8.0. Reportable Outcomes

The biodistribution of several radiolabeled FAP substrates have been evaluated, but did not demonstrate FAP selectivity.

Other Achievements

During the current funding period an NIH, NCI grant application was submitted and is pending.

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