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13. SUPPLEMENTARY NOTES

14. ABSTRACT We continue to derivatize and test the collagen mimetic peptides (CMPs) for imaging. We continue to work on: (1) Dual radio- and fluorescent labeling of CMPs retaining high-affinity and specificity for intact and digested collagen (type I) films; (2) Validation of dual-labeled CMPs that display high affinity and specificity for stromal collagens in frozen PCa xenografts; and, (3) Measurement of pharmacokinetics and *in vivo* imaging of dual-labeled CMPs in mouse subcutaneous PC-3 xenograft (and pancreatic xenograft) models. Efforts have been focused on achieving a biologically stable CMP with both gamma-emitting and fluorescent labels. We have employed several strategies utilizing the previously biologically validated, high affinity basic CMP peptide core, which may contain chelators, radioiodine moieties and fluorescent dyes. While the radiochemistry aspect of the project has proved challenging, we have made substantial progress biologically with the optical agents as detailed below.

15. SUBJECT TERMS
molecular imaging, tumor microenvironment, collagen mimetic peptide, SPECT, near-infrared imaging

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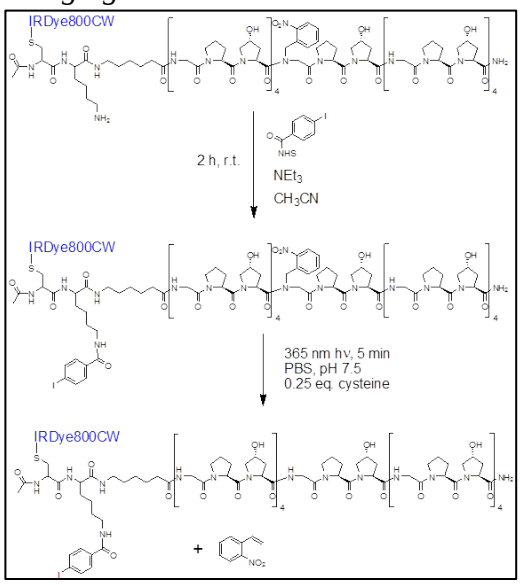
INTRODUCTION

Small collagen mimetic peptide (CMPS) that mimic the amino acid sequence and three dimensional structure of collagen were shown to have specific binding affinity to type I collagen fibers. Although the exact mechanism of binding is not known fully, evidence is accumulating that supports the idea that the CMP is binding to partially denatured domains of natural collagen by triple helical hybridization. Here we use CMP as a collagen targeting agent that will allow imaging of prostate cancer (PCa). Since CMP binds to unstructured collagen domains more readily, it is expected to exhibit selective affinity to metastatic PCa known to contain processed and denatured collagens. This is the first time that the remodeled ECM of tumor microenvironment is targeted for cancer imaging which is an entirely new way to image PCa with a potential to revolutionize the cancer community with respect to imaging and possibly treating PCa and its microenvironment.

BODY

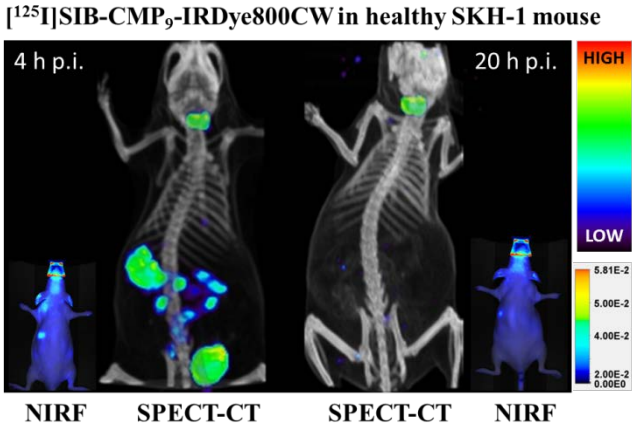
In year two, we synthesized several mono- and dual-labeled CMP analogs and tested them *in vivo* for their pharmacokinetic profiles. As we observed what appears to be widespread metabolism in each analog, we have continued our efforts to install and retain a radionuclide label to allow for tomographic and translatable *in vivo* studies of the tumor microenvironment. Our approach has centered on placing a lysine residue C-terminal to a cysteine residue directly conjugated to a bulky IRDye through a maleimide-thiol linkage. **Scheme 1** shows conjugation of [^{125}I]SIB-NHS ester to the lysyl-CMP-IRDye scaffold. Para-iodo-benzoyl amide linkages are both chemically and biologically robust and this analog was expected to perform well *in vivo*.

Scheme 1. *Conjugation of [^{125}I]SIB to Ac-C(IRDye800CW)-K-CMP$_9$-CONH$_2$ and photo decaging.*



In vivo testing of this dual-modality compound utilizing both SPECT-CT and near IR fluorescence imaging (NIRF) revealed that both the radioiodine label was quickly metabolized and the CMP scaffold performed poorly as shown in **Figure 1**. We speculate that the use of acetonitrile and triethylamine, vital for deprotonating the epsilon amine of the lysine and solubilizing the SIB group, may have chemically decaged the CMP and perturbed the structure of the dye. A color change in the CMP solution after purification supports alteration of the dye structure.

Figure 1. *SPECT-CT and NIRF imaging of [^{125}I]SIB-CMP$_9$ in a healthy SKH-1 mouse.*



At 4 hours post-injection, extensive free radioiodine uptake is observed in stomach, GI and thyroid while NIRF imaging shows stomach uptake without characteristic kidney uptake. By 20 hours post-injection, virtually all SPECT and NIRF signals have cleared except for thyroid retention of radioiodine.

Previous attempts to stably dual-label using In-111 nuclide met without success and we tried a simple addition of re-melting the purified labeled solution following photodecaging in case radiometallation resulted in chemical decaging of the nitrobenzoyl group with resultant refolding. Addition of an 80° C melt step immediately prior to injection into a healthy mouse did improve binding of CMP to skeleton compared with photodecaging alone. **Figure 2** shows the results of this experiment. Because the NIRF imaging showed little of the expected uptake along the spine, which has been previously demonstrated with analogs labeled with IRDye680RD, it would appear that the chemistry and/or purification used to introduce indium may also interfere with the stability of this dye structure.

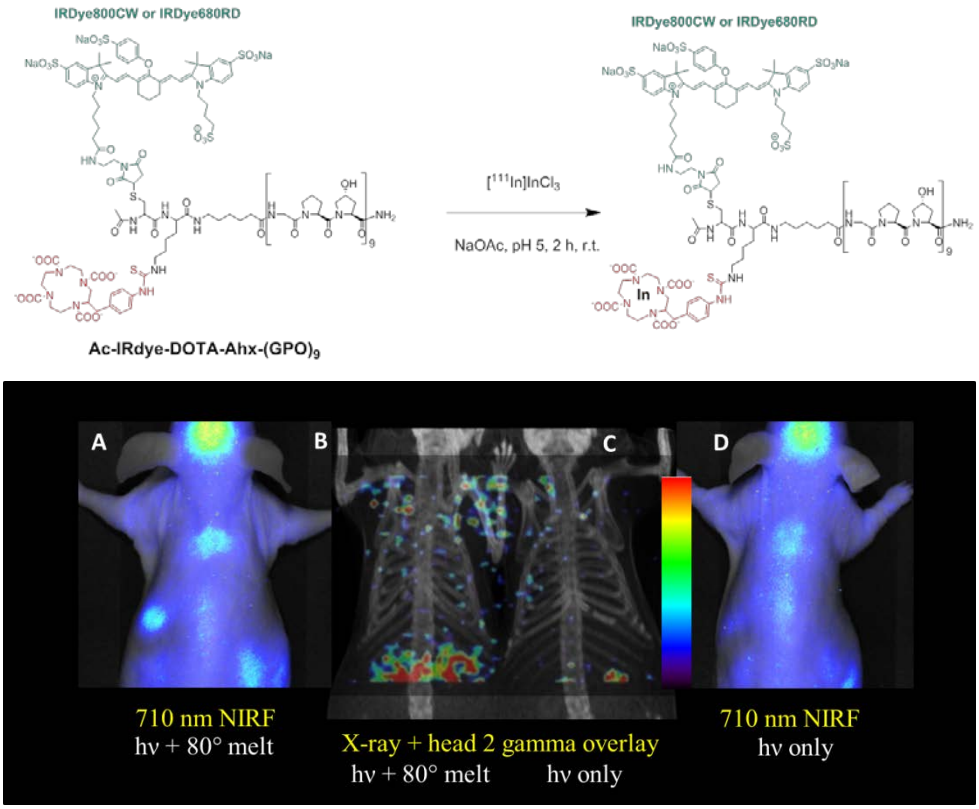
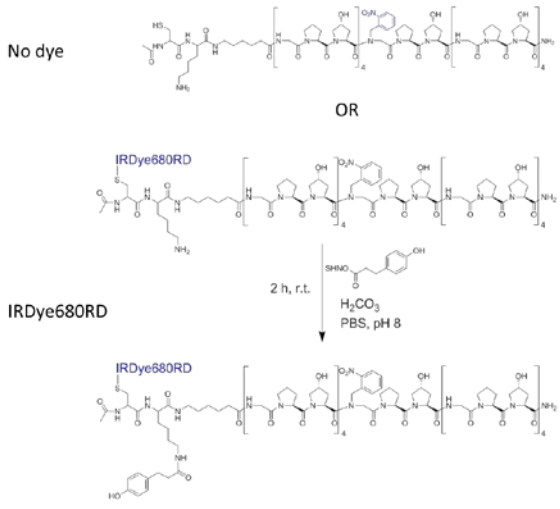


Figure 2. *Labeling scheme (top) and in vivo imaging (bottom) of [^{111}In]Ac-C-K-CMP$_9$-CONH$_2$.*

Radiolabeling proceeded with a modest 40% yield followed by a C18 sep-pak purification. A color change was noted following formulation for injection. Planar scintigraphic imaging was performed and overlayed with x-ray and shows more bone uptake in the photodecaged and melted solution versus decaged only solution. NIRF imaging shows weak uptake in spine, suggesting either perturbation of the IRDye or CMP scaffold.

Current work is focused on introducing a synthon that is biologically stable and requires gentle, aqueous, pH 8 conjugation conditions to avoid perturbing the IRDye label and nitrobenzoyl cage group. To that effect, we have recently prepared two analogs, Ac-K-CMP$_9$-CONH$_2$ and Ac-C-(IRDye680RD)-K-CMP$_9$-CONH$_2$, for conjugation with water soluble Bolton-Hunter reagent. Both of these have been prepared according to **Scheme 2** and will be simple to label with radioiodine under gentle conditions. These analogs await further chemical and biological characterization.

Scheme 2. *Conjugation of water soluble Bolton-Hunter reagent to CMPs under aqueous conditions in mild base.*



Biological studies proposed under Aim 2 using optically labeled CMP to map collagen remodeling signatures *in vivo* and within a frozen tumor xenograft library have begun with comparison to MMPase activity, which is involved in collagen remodeling. Male athymic nude mice were subcutaneously implanted with a selection of human prostate cancer cell lines to form xenografts ranging from 3.5-7 mm in diameter. Each mouse was intravenously injected with 4 nmol of photodecaged CMP$_9$-IRDye800CW and 24 h prior to imaging, the mice were also injected intravenously with MMPSense-680™ to concurrently measure the activity of MMPases 2, 3, 9 and 13. As reported in

our previously published pilot study (PMID 22927373), CMP uptake overlaps with MMPSense activation but imperfectly. This is expected since cathepsins and other proteases also play large roles in collagen cleavage and remodeling. **Figures 3-6** show both the *in vivo* NIRF imaging of concurrent CMP and MMPSense uptake and the high resolution distributions of both optical probes within representative tumor sections for each xenograft type. CMP distribution was consistent within each cohort of mice with a particular xenograft type and CMP binding was dense and distributed throughout the xenografts of fast-growing tumor lines (PC-3 isoforms) while slower growing xenograft types (DU-145, HP LNCaP) displayed only scattered focal or tumor rim uptake of CMPs. MMPSense uptake was widely and abundantly present in all tumor xenograft lines as well as in the benign but enlarged lymph nodes of mice, where CMP uptake was absent. In essence, CMP uptake correctly distinguished all tumors from enlarged lymph nodes.

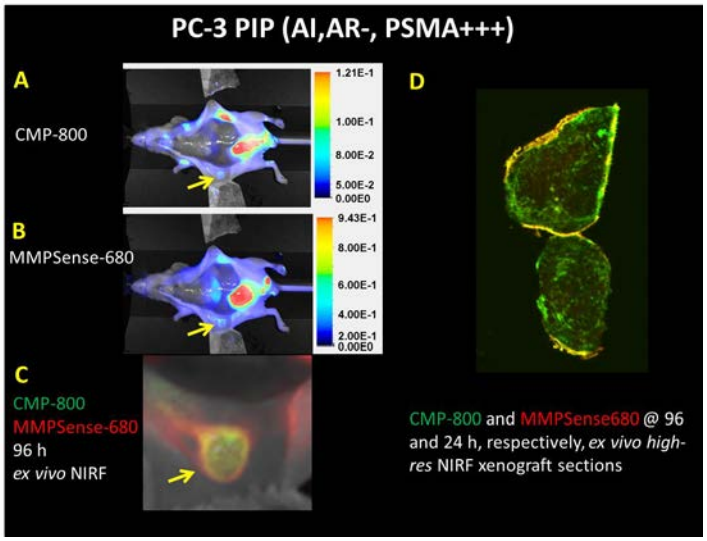


Figure 3. Ex vivo *NIRF imaging of PC-3 PIP xenograft*. AI = androgen independent, AR = androgen receptor negative, PSMA = prostate-specific membrane antigen expression. **A**. CMP9-IRDye800CW uptake showing tumor uptake (yellow arrow). **B**. MMPSense uptake showing uptake mostly at tumor rim. **C**. a magnified composite of A and B. **D**. High-resolution NIRF imaging of frozen tumor sections with in situ optical probes showing dense CMP accumulation throughout this fast growing xenograft.

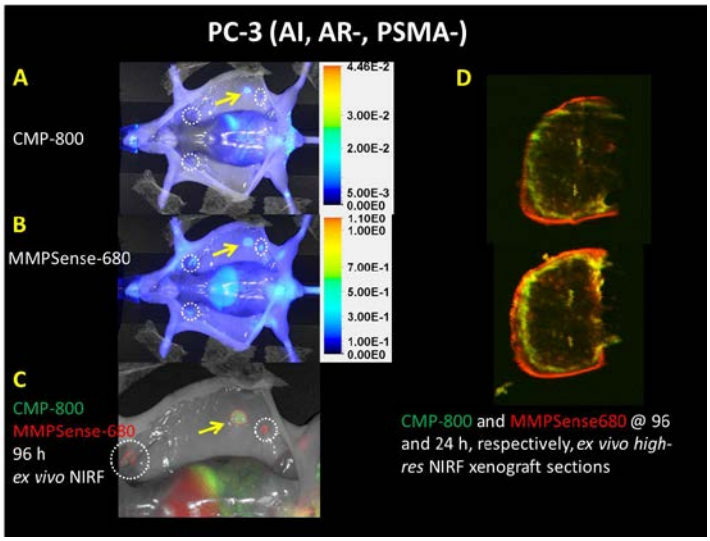


Figure 4. Ex vivo *NIRF imaging of PC-3 (wild type) xenograft*. Abreviations and legend exactly as in **Fig. 3**. Yellow arrow indicates the tumor location. White circles delineate benign lymph nodes that are positive for MMPSense but negative for CMP uptake. High-resolution NIRF imaging of frozen sections (D) with *in situ* optical probes show uptake of CMP (green) throughout the tumor but with increased uptake at the tumor rim. MMPSense (red) uptake is focal throughout the tumor but concentrated at the external edge of the tumor rim.

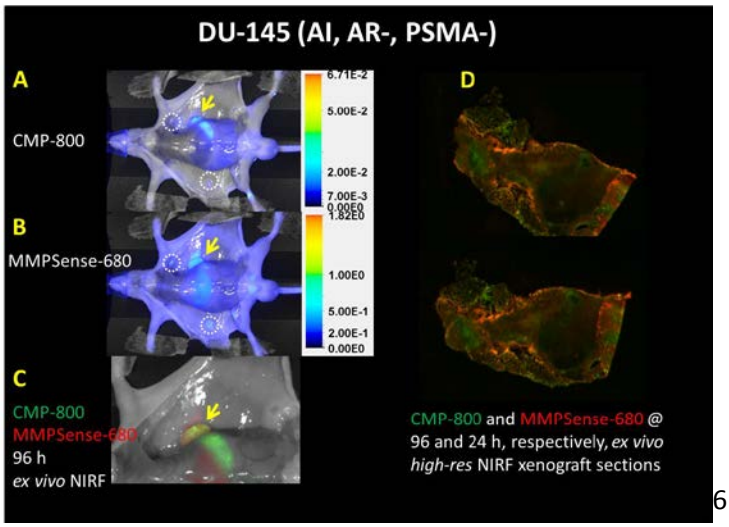


Figure 5. Ex vivo *NIRF imaging of DU-145 xenograft*. Abreviations and legend exactly as in Fig. 3. DU-145 is also AR- (and therefor AI) but is relatively slow growing. **A**. CMP uptake is strongly focal (detail in **C**) while (**B**) MMPSense uptake is distributed throughout the tumor. This was consistently observed in each DU-145 xenograft studied. **D**. High-resolution NIRF images show the weaker, more focal uptake of CMP (green) in tumor sections while MMPSense (red) uptake is distributed throughout the tumor.

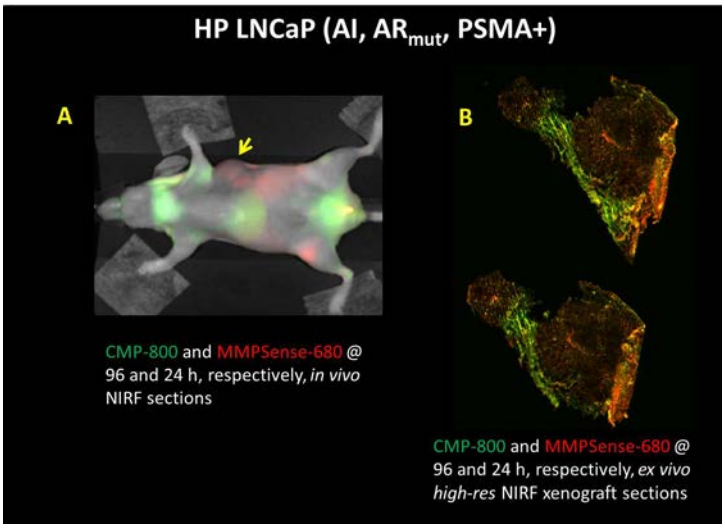


Figure 6. Ex vivo *NIRF imaging of HP LNCaP xenograft*. ARmut = mutant androgen receptor but androgen insensitive for growth. High passage LNCaP line (JHU isolate) grows relatively slowly as a subcutaneous tumor but has a 100% spontaneous metastasis rate to lymph nodes from this site (manuscript in progress). HP LNCaP tumors are highly hemorrhagic within the tumor and up to 2 cm away in the subcutaneous space. HP LNCaPs only display tumor rim-bound CMPs (green) (**B**) despite intense MMPSense uptake (red) within the tumor and the surrounding site of bruising (**A**, red).

Finally, we compared CMP and MMPSense distributions of the current prostate cancer library described above with a few pancreatic cancer xenografts grown under the same conditions to ascertain whether CMP uptake and distribution was also correlated to growth kinetics for pancreatic cancer lines. We tested two fast growth (subcutaneous implantation) cell lines (SK1990 and Panc-198) and one slow growth cell line (Panc-1), none of which metastasize spontaneously from this anatomic site. Indeed, CMP uptake was robust and distributed throughout the tumors of the two rapid growth lines (left column, bottom two) while very little CMP accumulation occurred within the slow growth xenograft (top left). This correlates with the CMP uptake patterns observed in the prostate cancer lines. **Table 1** lists quantitative values of CMP distribution among sections and confirms qualitative observations of CMP densities among xenograft types.

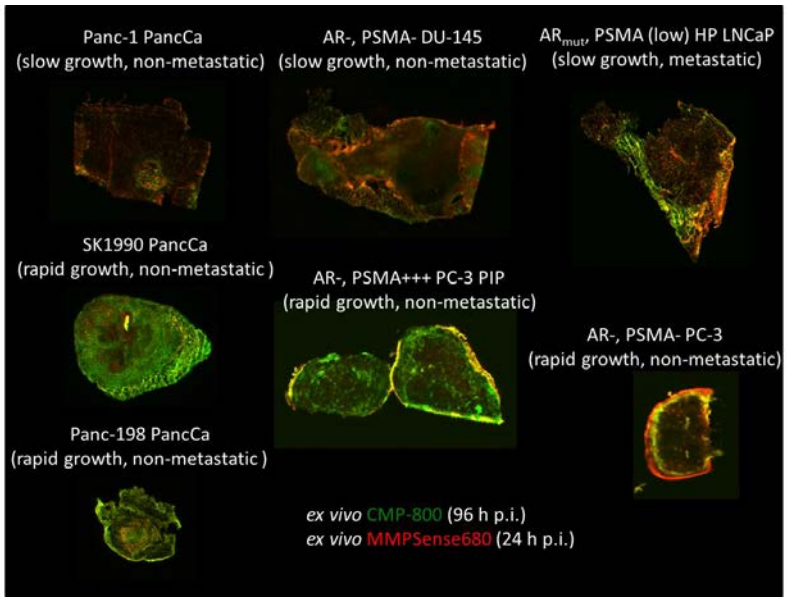


Figure 7. High-resolution NIRF imaging of the prostate cancer library alongside imaging of a few pancreatic cancer xenografts for comparison. Frozen tumor sections with *in situ* optical probes are shown as indicated.

Table 1. Relative optical densities of CMP-800 uptake in tumor xenograft sections.

xenograft line	subQ growth kinetics	O.D. center	O.D. rim/focal ROI
PC-3	rapid	0.25 ± .09	1.21 ± 0.40
PC-3 (PSMA+) PIP	rapid	0.26 ± .12	NA
DU-145	slow	0.01 ± 0.01	0.06 ± 0.02
HP LNCaP	moderate*	0.03 ± 0.01	0.43 ± 0.18
SW1990	rapid	0.16 ± 0.02	0.23 ± 0.04
Pan-198	moderate	0.08 ± 0.05	NA
Panc-1	slow	0.03 ± 0.02	0.08 ± 0.03
* requires Matrigel to grow; O.D. = optical density of CMP fluorescence			

KEY RESEARCH ACCOMPLISHMENTS

1. Four new CMP analogs were synthesized, two were radiolabeled and evaluated *in vivo*.
2. A library of four prostate cancer xenograft lines were evaluated in vivo and ex vivo for CMP-IRDye800CW binding and MMPSense-680 binding.
3. Three additional pancreatic cancer xenograft lines were tested to confirm CMP binding trends observed for xenograft growth kinetics.

REPORTABLE OUTCOMES

We have not yet achieved a biologically stable mono- or dual-modality CMP. We have determined that CMP binding does clearly distinguish between tumor and benign enlarged lymph nodes in our mouse model library of xenograft lines. Additionally, CMP uptake appears to distinguish between rapidly growing and slowly growing xenografts while MMPSense probe does not.

CONCLUSIONS

Chemistry conditions for the conjugation or radiometallation of IRDy-labelled CMPs appear to greatly affect the integrity of the dye and/or the nitrobenzoyl photo cage group. Deviations away from pH 6-8 and heating are to be avoided. Our current strategy of using water soluble Bolton-Hunter group for subsequent aqueous radioiodinations is likely to afford an intact labeled CMP that is also biologically stable.

Experiments using CMP_9-IRDye800CW to map collagen remodeling signatures within mice bearing a range of selected subcutaneous prostate cancer xenografts resulted in the observation of a trend in which CMP-800 accumulates with higher density in rapidly growing tumors. This trend was also observed in similarly prepared mice bearing subcutaneous xenografts of pancreatic cancer origin. We are currently finishing the probing of our existing prostate cancer library and anticipate the trend of CMP binding to growth kinetics to be conserved.

The concurrent binding of MMPSense™, reporting on the enzymatic activities of MMPs 2, 3, 9 and 13, to each of the tumor models described above revealed no trend in discerning tumor growth kinetics, propensity to metastasize and equally bound to tumor xenografts and benign inflamed lymph nodes while CMP-800 bound to tumors but not benign inflamed lymph nodes.

References

No publications are submitted for this work yet.