

Effective Bone Labeling for *In Vivo* NIR Noninvasive Imaging in Nude Mice

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ABSTRACT

Calcium-chelating compounds have been used effectively for the detection of bone mineralization, growth, and morphological changes, including tetracycline derivatives, xylenol orange, alizarin, calcein, and fluorescein. These compounds contain iminodiacetic acid groups that can form chelating complexes with apatite and provide a certain level of native fluorescence in the visible spectrum as the complexes become incorporated in mineralizing bone. We chose to exploit these characteristics to produce a near infrared (NIR) optical bone marker for small animal imaging. By conjugating compounds to IRDye[®] 800CW or IRDye 680, we have extended the effective fluorescence signal detection to the NIR region without affecting the compound's ability to function as a marker of the mineralization process. Initial screening of multiple compounds was performed using MC3T3-E1 (osteoblasts) in an *in vitro* cell-based assay. Two of the seven compounds exhibited signal intensities approximately 3–6X higher than the others. Subsequent *in vivo* testing of Compounds A and F demonstrated effective skeletal labeling for imaging which is unabated several weeks post-administration. The ability to visualize bone anatomy/structures for an extended period will facilitate use of the bone targeting agent in conjunction with a second optical agent specific for a primary target (*i.e.*, tumor tissue). We demonstrate a multiple probe application with the administration of IRDye 680 bone marker one week prior to implantation of prostate tumor cells in the flank of a nude mouse. Animals received weekly intravenous injections of IRDye 800CW 2-deoxyglucose and were imaged 24 hours post-injection over a six-week period, using a prototype small animal imaging system. The results provide evidence that NIR labeled conjugates are useful for multiple probe/localization applications in small animal imaging.

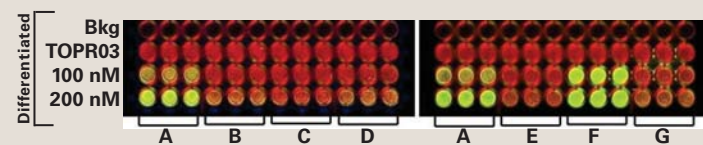


Figure 1. Preliminary *in vitro* cell-based analysis of the test compounds utilized MC3T3-E1 osteoblast cells. Results from a 24-hour treatment of seven test compounds labeled with IRDye 800CW reveals two compounds (Compounds A and F) preferentially labeling the bone cells. Images were generated with the Aeries Imaging System. Red represents the 700 nm signal for normalization and green represents the 800 nm signal from the test compounds.

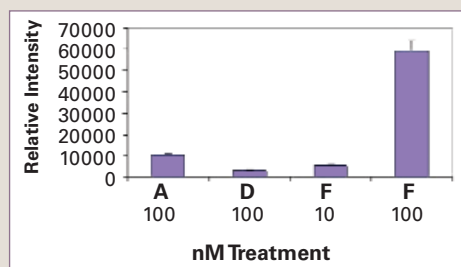


Figure 2. In a similar assay, osteoblasts were incubated with three of the test compounds and signal intensity was quantified to compare magnitude of labeling specificity. Approximately 6-fold increase in signal intensity was evident for Compound F when compared to Compound A at equal concentrations.

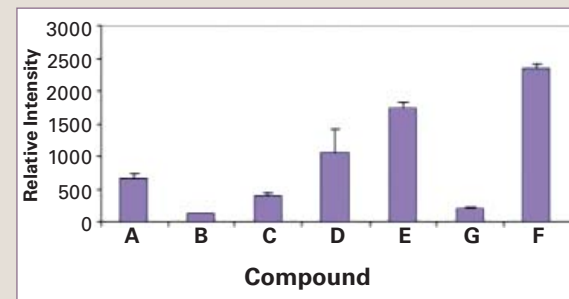


Figure 3. Specificity of the compounds for bone cell binding was verified relative to an epithelial carcinoma cell line (A431). Although the highest level of non-specific binding was noted for Compound F, this signal intensity represents only ~4% of the signal noted on the osteoblasts treated with the same concentration.

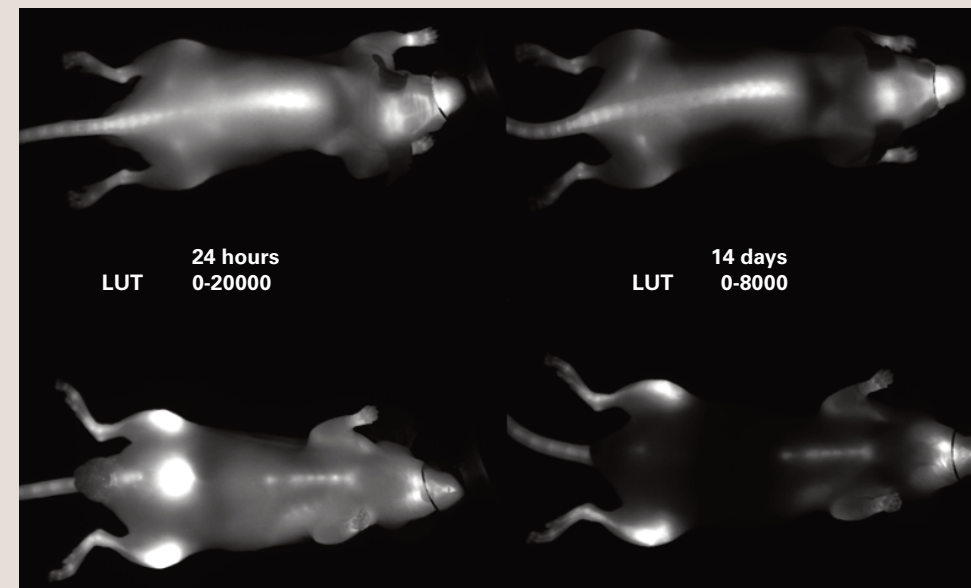


Figure 5. The duration of signal detected in the bone from a single injection (2 nmol) was evaluated longitudinally in nude mice injected intra-venously with IRDye 800CW Compound F. Skeletal structures were clearly visible at 2 weeks and longer.

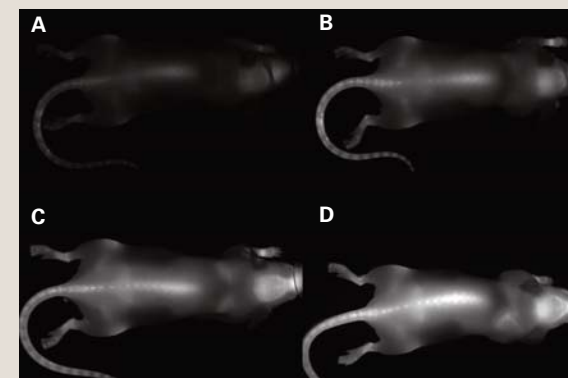


Figure 4. Nude mice were injected intravenously with increasing concentrations of IRDye 680 Compound F as follows: A) 1 nmol, B) 2 nmol, C) 3 nmol, and D) 4 nmol. Images were acquired 24 hours post-injection using a research prototype NIR small animal imaging system optimized for NIR fluorescence detection (700 and 800 nm). Sufficient signal intensities were obtained with a 4 nmole dose.

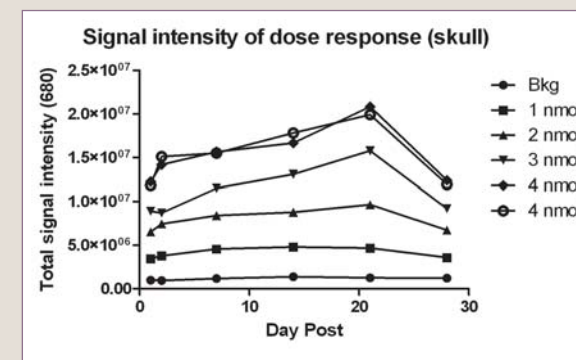


Figure 6. Mice were injected with a single dose of IRDye 680 Compound F (as indicated) and imaged over a 4-week period. An ROI encompassing the skull was used to calculate total signal intensity values at 700 nm, which are graphically represented. A dose-dependent increase in signal intensities was noted in mice and maintained over time. In addition, two mice receiving 4 nmole IRDye 680 Compound F demonstrated excellent reproducibility in signal intensity.

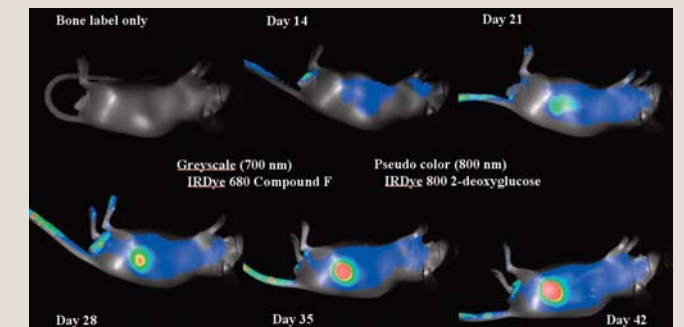
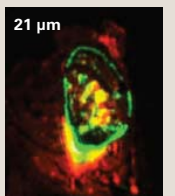


Figure 7. Two NIR optical imaging agents are used to monitor tumor growth longitudinally over a 6-week time period in nude mice. Day stamp on images represent the time post-tumor cell implantation. Images were collected in two wavelengths, 700 nm and 800 nm. The pseudo color images have been normalized to the same LUT.

Figure 8. Femurs were decalcified and paraffin embedded for tissue section evaluation of signal deposition. A representative femur cross-section was scanned on the Odyssey System (LI-COR Biosciences) illustrating the presence of IRDye 800CW Compound A (green) in the compact and trabecular bone tissues. The red signal represents autofluorescence from residual tissue and/or paraffin media.



CONCLUSIONS

- Effective labeling of bone tissue was demonstrated *in vitro* with Compounds A and F. Compound F exhibited 6-fold higher signal intensities at the same concentration.
- Low non-specific binding to a non-bone cell line was verified.
- Signal to noise levels were excellent 24 hours post-injection.
- Dose response results suggest that 4 nmole is an effective working dose.
- Compound F proved to be equally effective for bone imaging whether labeled with IRDye 680 or IRDye 800CW.
- Femur cross-sections show the deposition of the marker in the compact and trabecular bone tissues.
- Dual probe assays are possible.

REFERENCES

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