Bright idea in cancer

By Kai-Jye Lou, Staff Writer

Researchers at the **University of Tokyo** and the **NIH**'s **National Cancer Institute** have developed pH-sensitive fluorescent probes that can selectively label viable cancer cells *in vivo*.[1] These probes may be useful as visualization aids in surgery and imaging procedures as well as in drug efficacy studies.

In a paper published in *Nature Medicine*, researchers described boron-dipyrromethene-based probes conjugated to a mAb targeting cell surface proteins. Following internalization, the conjugated probes become activated only within the acidic microenvironment of lysosomes—a hallmark of viable cells. Damaged or dying cells cannot maintain a low-pH lysosomal microenvironment, and probes within such cells will not activate.

In a proof-of-concept study, a Herceptin-conjugated probe was used to image HER2 (ERBB2)-overexpressing lung metastases in mice. In a second experiment in probe-labeled, tumor-bearing mouse lungs, killing viable cells with pure ethanol significantly lowered relative fluorescence intensity compared with that seen in lung regions labeled with a constitutively active control probe ($p<0.0001$).

Herceptin trastuzumab, a humanized mAb against HER2 from **Genentech Inc.** and **Roche**, is marketed to treat breast cancer.

"There are three issues that arise with whatever you label: you want your label to be specific, you have to wait for the label to accumulate specifically at the target and you have to wait for what doesn't accumulate to wash away," said Jeffrey Peterson, VP of applied biology at **VisEn Medical Inc.** What Peterson likes about the new technique, he said, is "getting the probe into the cell without having to wait for what doesn't accumulate to wash away. What's circulating is not fluorescent so it shouldn't interfere with your signal."

VisEn markets fluorescence imaging agents and the Fluorescence Molecular Tomographic imaging systems, which provide *in vivo* 3D analysis and quantification of biological changes in deep tissue.

The company's fluorescence imaging agents include a series of cathepsin-activated probes called ProSense. Cathepsins are primarily lysosomal enzymes that become activated in a low-pH environment.

"Eliminating background is always an important issue in cellular analysis and particularly so for *in vivo* imaging applications where signals may be low," said Magnus Persmark, a senior product manager in the Cell Systems Division at **Life Technologies Corp.**

Life Technologies, which was renamed from Invitrogen Corp. after Invitrogen completed its acquisition of Applied Biosystems Group in November 2008, markets multiple molecular imaging agents, including pHrodo, a rhodamine-based, red-shifted dye that increases in fluorescence as the pH of its surroundings becomes more acidic.

According to Hisataka Kobayashi, coauthor of the *Nature Medicine* paper and chief scientist in the molecular imaging program at the NCI, most pH-sensitive probes are activated by increases in pH, which makes them generally unsuitable for *in vivo* imaging because significant increases in pH seldom occur under physiological conditions.

The few pH-sensitive probes that do activate under acidic conditions have their own problems, including emission of light with too short of a wavelength, which requires the use of cytotoxic ultraviolet light for visualization. Other issues with pH-sensitive probes include too much hydrophobicity, which can alter pharmacokinetics and lead to off-target labeling, the lack of a tagging moiety that would facilitate conjugation with a targeting molecule and relatively high background fluorescence at neutral pH.

Kobayashi said the conjugated, pH-sensitive probes described in the *Nature Medicine* paper were designed to avoid those problems while offering advantages over other activated fluorescence probes. For example, he said results from imaging studies using certain enzyme-activated probes can be confounded by high background fluorescence when the activating enzyme is also present in the extracellular environment.

"Our method is very specific for the surface molecule," Kobayashi told *SciBX*. "For enzyme-activated probes, it is hard to say if the probe is actually being activated within a cell or outside of the cell." He added that enzyme-activated probes generally undergo irreversible activation.

According to Kobayashi, the versatility of the conjugated, pH-sensitive probes should allow them to be used in surgical, diagnostic and preclinical settings. He said the two primary applications are in endoscopic and fluorescence-guided surgery systems and that his research group already has developed an endoscopic system for mice that uses these probes.

The probes also could be useful for studying intracellular kinetics of receptor-ligand complexes, Kobayashi said, as well as for the real-time monitoring of cellular responses to cancer therapy.

Probing for utility

Companies contacted by *SciBX* agreed that the pH-sensitive probes could potentially lead to improved specificity and accuracy in imaging studies, but they suggested that the probe's targeting mechanism and its fluorophore have limitations.

"In a clinical setting we believe that acid-activated pH probes will turn out to be superior to the nonactivated and fluorescein-based probes," Persmark told *SciBX*. "The reason for this belief is that nonactivated probes, such as tetramethylrhodamine, require wash steps and result in background staining, and fluorescein-based probes become less fluorescent rather than more fluorescent as the environment becomes more acidic, resulting in signals that are increasingly difficult to detect as the physiological event of interest becomes more significant," he said.

Lars Abrahmsén, CSO of **Affibody AB**, told *SciBX* that "this concept is useful for *in vitro* studies in cells and for animal imaging applications." However, he was less optimistic on the clinical side, noting that poor tissue penetration of the fluorescence signal may limit their clinical applicability.

Affibody's ABY-025 is a HER2-binding molecular imaging agent in preclinical testing to diagnose breast cancer. The company also markets antibody-based research reagents, including anti-HER2 antibodies.

Kobayashi acknowledged that the green light emitted by the current fluorophore does not penetrate tissue very well and that using a core that emits a longer wavelength light may be necessary for deep tissue imaging. He said his group and collaborators are trying to modify the fluorophore core to emit a stronger signal upon activation.

"You need to know your target. You can only use this fluorophore with antibodies that are taken into the cell by the cellular target."
—Jeffrey Peterson, VisEn Medical Inc.

Kobayashi did note that increasing the wavelength of emitted light will weaken the signal strength from the probe. This can make the emitted light invisible to the eyes and less noticeable on a camera. "It's a tradeoff, because a strong signal is always good," he said.

He also noted that use of the probe as a visualization aid in fluorescence-guided surgery and endoscopy applications does not require deep light penetration.

Kjetil Hestdal, president and CEO of **PhotoCure ASA**, added that the probes may be useful for *in vitro* diagnostic techniques or in fluorescence-assisted diagnostic procedures for organs where fluorescence can be visually monitored—like the bladder, colon and cervix.

In Europe, PhotoCure markets Hexvix hexaminolevulinate, a fluorescence-based optical imaging agent to diagnose bladder cancer during cystoscopy.

According to Hestdal, the advantage of the probes is that "the fluorescence signal is linked to a specific uptake of the antibody and a specific intracellular routing—ending up in the lysosome." He said conjugated probes based on this principle will lead to increased selectivity of antibody-based diagnostics.

Because the probes described in the *Nature Medicine* paper become activated in the lysosome, which is intracellular, VisEn's Peterson said conjugation options for the probe's cell surface protein–targeting moiety will be limited. "You need to know your target. You can only use this fluorophore with antibodies that are taken into the cell by the cellular target," he told *SciBX*.

Hestdal wanted to see additional testing that demonstrates the specificity of the conjugated, pH-sensitive probes for its cell surface target. He noted that expression of the probe-targeted proteins may vary between different tumors and individuals, and he said it is necessary to demonstrate the reliability of the probe in such contexts.

If there is a large variation in target expression between patients, Hestdal said, "the clinical usefulness of the method is reduced."

Peterson wanted to see safety studies that detail the fate of the antibody-probe complex after it enters the lysosome and how the imaging agent is metabolized.

Multiple patent applications have been filed covering the design and synthesis of the fluorophore, the pH-sensitive probe and their uses in detecting biologically active cells, including tumors, *in vivo*. The probes are available for licensing from the University of Tokyo Technology Licensing Organization and the NIH Office of Technology Transfer.

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