WAVELENGTH ELECTRONICS

Fluorescent Hypoxia Detection Based on HIF-1α ODD Domain

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ABSTRACT

Functional imaging of tumor cells provides critical information about the size and location of the tumor, as well as the micro-environment. Many tumors grow faster than blood vessels can form, leading to a lack of oxygen. HIF-1α then regulates the creation of new vascular networks. Hence HIF-1α can be used to identify tumor aggressiveness, proliferation, and likelihood to metastasize. Researchers from Spain have reported a fluorescent hypoxia biosensor based on the oxygen-dependent degradation domain of HIF-1α. This probe is stabilized under hypoxia, properly degrades under normoxia, and retains the oxygen sensing capability of HIF-1α. The developed hypoxia sensor contributes to a deeper view and understanding of the tumor micro-environment that modern imaging techniques cannot provide.

TUMOR CELL BIOLOGY

Visualizing tumors and the effects they have in their micro-environments (**Figure 1**) is critical to monitoring biological processes. Not only can imaging help locate and provide information about the size of the tumor, it can provide important treatment and recovery information. As some tumor types have disorganized and rapid growth, low oxygen and poor development of blood vessels within a tumor occur. Because of the lack of nutrition and oxygen that all cells need, tumor cells continually activate pathways that regulate neoangiogenesis – causing the normally controlled blood vessel system to continually sprout new vessels that help sustain the growing tumor cells. Angiogenesis, or the development of new blood vessels, is a vital process in adult growth as well as wound healing and forming the tissue on the wound. However, when activated by the tumor cell, angiogenesis can turn the benign tumor into a malignant tumor. The tumor cells activate the angiogenesis switch, constantly sustaining the expansion.[1,2]

Hypoxia, the lack of oxygen as a result of the existence of the tumor cells, is the main cause for the activation of the angiogenesis switch. Hypoxia is a poor-prognosis micro-environmental hallmark of solid tumors and affects treatment and therapy.[3] In response to this lack of oxygen, a protein coding gene starts working. This gene helps encode the alpha subunit of the transcription factor hypoxia-inducible factor-1 (HIF-1). The alpha subunit, HIF-1α, helps regulate the cellular and developmental response to hypoxia. However, in scenarios involving tumor cells, HIF-1α is over-expressed, making the tumor or cancer cells more aggressive and invasive. In this case, HIF-1α can be used as a prognostic marker of tumor cell reproduction and ability to spread to other areas of the body.[4]

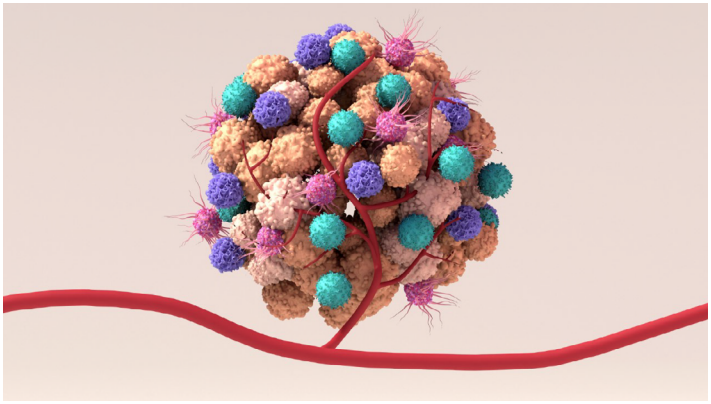

Figure 1. Tumor micro-environment, normal cells, molecules, and blood vessels surrounding and feeding a tumor cell.

PROBLEMS AND GOALS

Clearly, stopping the growth of a tumor and the neoangiogenesis is top priority in treating and monitoring the state of the problem. With optical imaging, specifically functional imaging, monitoring biological processes can be achieved. Processes such as metastasis and hypoxia are of the most important. Some researchers use near-infrared fluorescence to enable real-time imaging. However, among researchers, fluorescence and bioluminescence imaging are popular due to the availability of a variety of proteins and dyes.

In a clinical setting, the difficulties with biosensors using large proteins include the need for transfection and over-expression, relatively low photostability, and large size that leads to interference, poor biodistribution, or immune response.[1] [Transfection is the process of introducing nucleic acids (DNA or RNA) into cells, altering

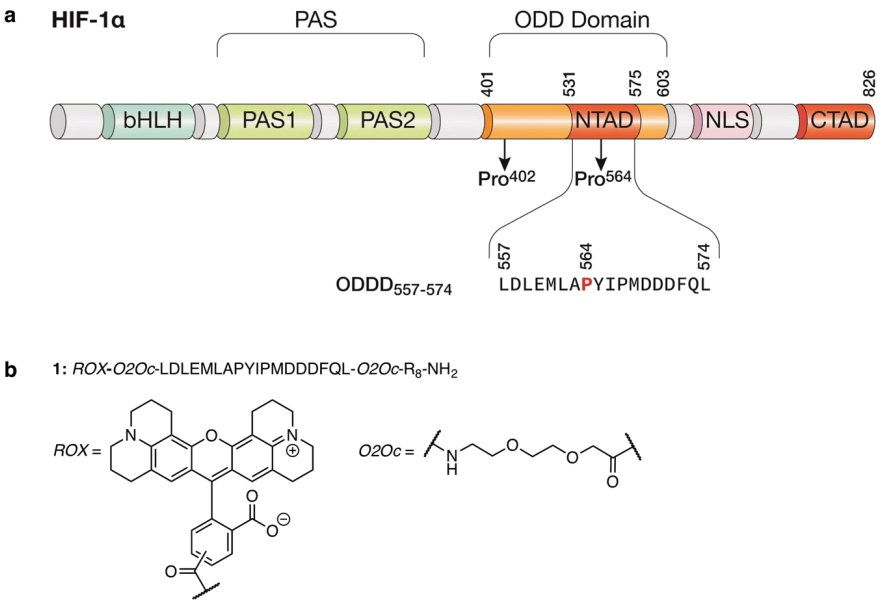

Figure 2. Biosensor design rationale. (a) Domain structures of HIF-1α. The ODD domain regulates the stability of HIF-1α via recognition by the E3 ubiquitin ligase pVHL. (b) Chemical structure of sensor 1.[1]

the properties of the cells.] One of the most important parameters is how the sensor is detected, mainly through fluorescent spectroscopy. For best results, the biosensor needs to have relatively low photostability or a low resistance to change under the influence of light or radiant energy.

Using smaller peptides provides easier integration. These short chains of amino acids, linked by peptide bonds, offer higher stability, lower immunogenicity, ease of synthesis, and the simplicity for molecular engineering. With better biodistribution and lower immunogenicity, these peptides can be easily tracked without provoking an immune response.

METHOD

Researchers from Spain have developed a compact sensor consisting of a fluorescently-labeled peptide, corresponding to a small fraction of the oxygen-dependent degradation (ODD) domain of HIF-1α, that mimics the behavior of HIF-1α under hypoxia conditions. This enables application for the monitorization of hypoxia activity with potential clinical application.[1]

To develop new hypoxia tracers, a fluorescent peptide needs to imitate the effect of hypoxia on the half-life of HIF-1α. HIF-1α is rapidly degraded in normoxic cells, or cells with normal levels of oxygen, upon hydroxylation of two proline residues: Pro^{402} and Pro^{564}. These are located in the ODD domain of HIF-1α in **Figure 2**. From the HIF-1α ODD domain, the short 16-residues peptide (Leu^{557} to Leu^{574}) is used as it has been previously reported to lead to pVHL-mediated degradation and retains the oxygen-sensing properties of HIF-1α.

The new sensor contains three sections with different functions as seen in **Figure 2**:

1. An octa-arginine peptide: This controls the cell internalization.
2. A central domain: This is from the HIF-1α degradome able to sense low oxygen levels (^{557}LDLEMLAPYIPMDDDFQL^{574}).
3. The ROX fluorochrome: This long-wavelength rhodamine is characterized by good stability, high quantum yield (0.92), and it acts as the fluorescent reporter for both *in vitro* and *in vivo* imaging.

In contrast with other fluorescent sensors, the sensing mechanism relies on the higher lifetime of the ODD domain sequence under hypoxic conditions than under normoxia. Thus, peptide 1 was synthesized, and excitation and emission spectra were recorded with a fluorescent spectrometer.[1]

To test the capabilities and characteristics of peptide 1, human breast cancer cells (MDA-MD 231) were treated with peptide 1. Some of the cells were incubated with the hypoxia-mimetic agents $CoCl^2$ or L-Mimosine to test the stability and degradation of the hypoxia probe.

Another probe, 1-SS, was created with a disulfide bridge with the purpose of reducing the half-life of the probe. Two female mice were injected in the tail-vein with either probe 1 or probe 1-SS. Fluorescence pictures were taken every

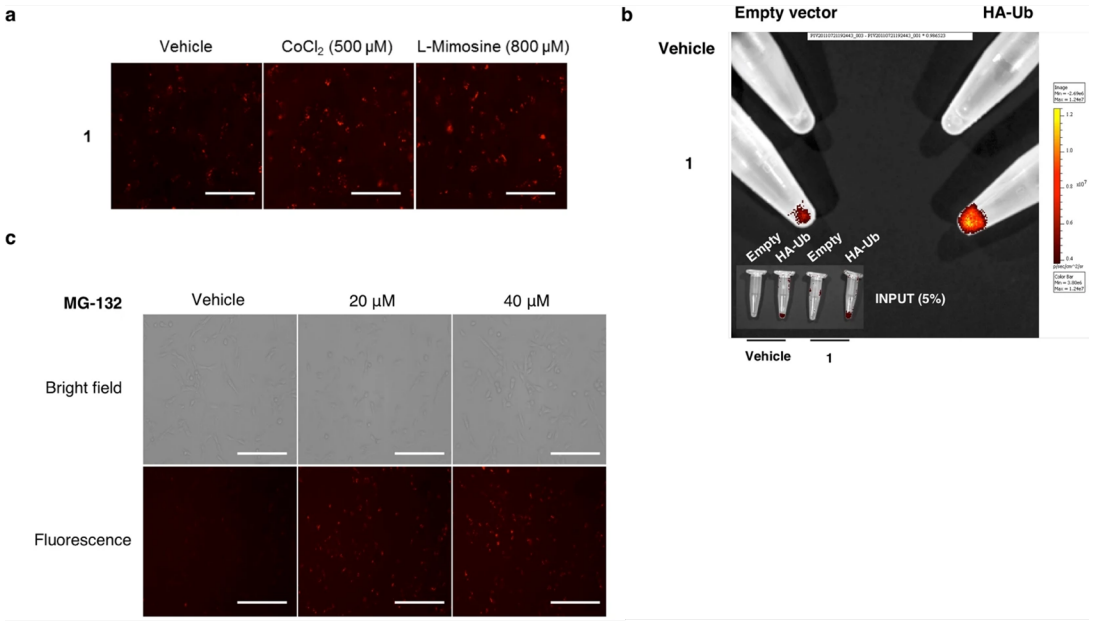

Figure 3. (a) Hypoxia mimetics inhibit sensor degradation. Cells were incubated with hypoxia sensor 1 in the presence of $CoCL_2$ (500 µM) and L-Mimosine (800 µM). Scale = 50 µm. (b) Ubiquitination of hypoxia sensor 1 is demonstrated by immunoprecipitation of HA-tagged ubiquitination substrate. (c) Proteasome degrades sensor 1. Proteasome inhibitor MG132 inhibits in a dose response manner the degradation of hypoxia probe 1 in MDA-MB 231 cells. Scale = 100 µm.[1]

two hours. For excitation and emission images, the 570 nm and 620 nm filters were used, respectively, for specific fluorescence images. Background fluorescence used the 465 nm and 620 nm filters.

RESULTS

Once the cancer cells were treated with peptide 1, fluorescence spectra were recorded with the vehicle and the two hypoxia-mimetic agents, $CoCl_2$ and L-Mimosine. **Figure 3a** shows the fluorescent peptide 1 exhibiting stable qualities in the presence of both molecules that mimic hypoxia. This first test validates the hypothesis for the probe design. This new sensor, compared to the level of signal in vehicle-treated cells, shows an improved stabilization with the $CoCl_2$ and L-Mimosine-treated cells.

For the peptide probe to act similar to HIF-1α, it needs to be destroyed or eliminated in the same manner. HIF-1α is degraded by the proteasome upon hydroxylation of the ODD domain as previously discussed. To confirm similar behavior with the peptide probe, cells were transfected with a plasmid encoding anti-hemagglutinin (HA)-tagged ubiquitin. These cells were treated with probe 1 as shown in **Figure 3b**. This image shows that the degradation of sensor 1 is tied to the ubiquitin proteasome. Similarly to HIF-1α, this is what regulates the degradation.

Figure 3c shows that if the cells are incubated with hypoxic sensor 1 and are treated with a proteasome inhibitor (MG-132), a dose-dependent effect on proteasome degradation of the fluorescent signal is demonstrated.[1]

With the introduction of the disulfide bridge in the new probe, 1-SS, the reduced half-life needed to be established and tested. **Figure 4a** shows the chemical structures of a second probe, peptide 2, and the probe with the disulfide bridge, probe 1-SS. The separation of the disulfide bond in the cytosol, once the compound is internalized, should facilitate the degradation of the probe.

Probe 1-SS was placed in the presence of $CoCl_2$ and L-Mimosine. **Figure 4a** (lower panel) shows a faster reduction of the fluorescence intensity. This indicates an increased rate of degradation, better relating to HIF-1α. A reduction in background fluorescence and an enhanced sensitivity with increasing concentrations of $CoCl_2$ were also observed with probe 1-SS when compared to probe 1.

This reduction in background fluorescence signal was also observed when probe 1 and 1-SS were administered *in vivo* to mice. In **Figure 4b**, probe 1-SS showed a lower signal at 8 hours post administration than probe 1 at 24 hours. The fast elimination of fluorophore results in a strong fluorescence signal in the bladder when probe 1-SS was administered as seen in the bottom right image.

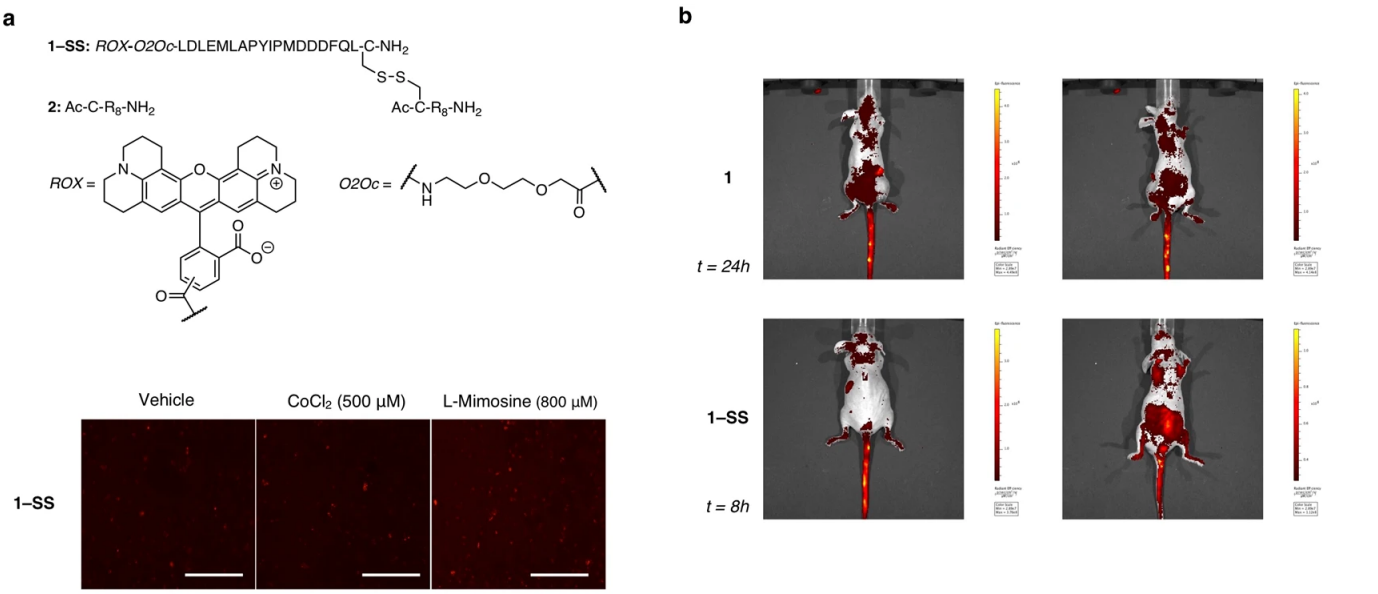

Figure 4. (a) (upper panel) Chemical structures of peptide 2 and probe 1-SS. (lower panel) Hypoxia mimetics also inhibit sensor 1-SS degradation. Cells were incubated with hypoxia sensor (1-SS) in the presence of $CoCl_2$ (500 µM) and L-Mimosine (800 µM). Scale = 50 µm. (b) Comparison between the clearance time of sensor 1 (up) and 1-SS (down) *in vivo*. Images in prone (left) and supine position (right) were taken every two hours during the first eight hours upon sensor administration and one final time at 24 hours post-injection. Images shown correspond to the last time point where specific signal from the sensor was detected.[1]

This study has shown that the stabilization effect of hypoxia on the half-life of HIF-1α can be exploited for designing fluorescent biosensors of hypoxia. The original synthetic peptide biosensor was modified with an octa-arginine cell-penetrating peptide and a fluorescent dye. This biosensor's break-down stability is controlled under hypoxia, and the fluorescence signal depends on the oxygen availability in the immediate micro-environment. Further, the probe is confirmed to degrade by the proteasome upon hydroxylation of the ODD domain. This is also accelerated by the addition of a disulfide bridge between the octa-arginine vector and the ODD domain sensing unit. This probe, unlike other similar concepts, combines excellent cell internalization with faster clearance rates, improving sensitivity and applicability in monitoring hypoxic environments. Not only could this be utilized to finding tumor masses, but it could also provide vital information about the tumor's potential to spread and how aggressively. The developed hypoxia sensor contributes to a deeper view and understanding of the tumor micro-environment that modern imaging techniques cannot provide.[1]

WAVELENGTH'S ROLE

Due to the highly sensitive nature of fluorescence spectroscopy, the spectra were recorded with a fluorometer coupled to Wavelength Electronics' LFI-3751 temperature controller. The high performance and high precision LFI-3751 temperature controller ensured stable wavelength and power of the emitted light. Because the integration time was minimal (0.50 s), proper performance of the system was crucial. The measurements with the fluorometer were made with an increment of 1 nm and slit width of 2 nm, so narrow linewidth measurements were critical. As the fluorescence signal depended on the oxygen available, the fluorometer system needed to accurately and precisely collect multiple spectra to gain a deeper view on the environment around the tumor, accessing more information than typical imaging techniques.

The LFI-3751 temperature controller enables linear stability of as low as 0.001ºC with a drive current of up to ±5 A to either thermoelectric coolers or resistive heaters. Autotune PID allows for a sophisticated algorithm to optimize the PID control parameters of the load.

By using the LFI-3751 temperature controller, researchers were able to maintain narrow linewidth, stable wavelength, and accurate and repeatable results with fluorescence spectroscopy of the hypoxia sensor.

GLOSSARY

- Angiogenesis - The development of new blood vessels
- HIF-1α - The alpha subunit of the Hypoxia-Inducible Factor that helps regulate the cellular response to lack of oxygen
- Hypoxia - Lack of oxygen
- Neoangiogenesis - The mechanism, specifically in cancer or tumors, responsible for the formation of new blood vessels to sustain and help the tumor cells grow
- ODD - Oxygen-Dependent Degradation
- Photostability - Resistance to change under the influence of radiant energy and especially of light
- Transfection - The process of artificially introducing nucleic acids (DNA or RNA) into a cell to modify the cell

REFERENCES

1. Iglesias, P., Penas, C., Barral-Cagiao, L., Pazos, E., Costoya, J. A. A Bio-inspired Hypoxia Sensor using HIF1a-Oxygen-Dependent Degradation Domain, *Sci Rep* **9**, 7117 (2019). https://doi.org/10.1038/s41598-019-43618-4
2. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: the Next Generation. *Cell* **144**, 646–674 (2011). https://doi.org/10.1016/j.cell.2011.02.013
3. Fluegen, G. et al. Phenotypic Heterogeneity of Disseminated Tumour Cells is Preset by Primary Tumour Hypoxic Microenvironments. *Nat. Cell Biol.* **19**, 120–132 (2017). https://doi.org/10.1038/ncb3465
4. LaGory, E. L. & Giaccia, A. J. The ever-expanding role of HIF in tumour and stromal biology. *Nat. Cell Biol.* **18**, 356–365 (2016). https://doi.org/10.1038/ncb3330

USEFUL LINKS

- LFI Temperature Controller Product Page

PERMISSIONS

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PRODUCTS USED
LFI-3751

KEYWORDS
Hypoxia, biosensor, HIF-1a, fluorescence, fluorescent spectroscopy, peptide, photostability, oxygen-dependent degradation, ODD, angiogenesis, temperature controller, LFI-3751

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REVISION	DATE	NOTES
A	November 2021	Initial Release