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A New Prodrug-Derived Ratiometric Fluorescent Probe for Hypoxia: High Selectivity of Nitroreductase and Imaging in Tumor Cell

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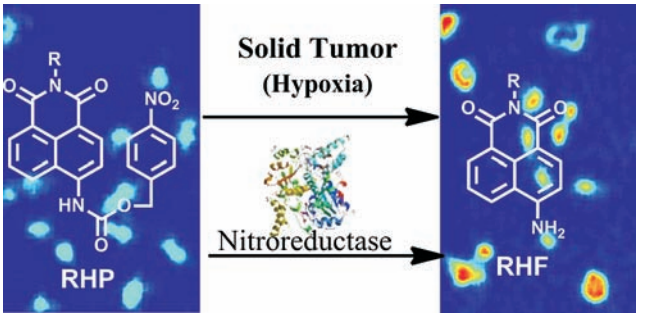
ABSTRACT



Based on the hypoxia prodrug moiety of *p*-nitrobenzyl, a selective ratiometric fluorescent sensor (RHP) for the detection of microenvironment hypoxia was designed and synthesized. RHP can be selectively activated by bioreductive enzymes (NTR) and results in an evident blue to green fluorescent emission wavelength change in both solution phases and in cell lines, which might be the first fluorescent ratiometric probe for hypoxia in solid tumors.

Hypoxia is usually observed in solid tumors and affects the therapy of tumor cells by preventing the proper metabolism of various anticancer drugs.[1] Clinical research found that a hypoxic environment in tumor cells has a positive correlation with their resistance to treatment and metastasis.[2] In cervix cancer and head and neck squamous cancer, hypoxia has been considered as a poor progression-free survival signal for detection.[3] Therefore, it is of considerable clinical significance to measure the hypoxic fraction in tumors.[4]

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(1) (a) Williams, K. J.; Albertella, M. R.; Fitzpatrick, B.; Loadman, P. M.; Shnyder, S. D.; Chinje, E. C.; Telfer, B. A.; Dunk, C. R.; Harris, P. A.; Stratford, I. J.. *Mol. Cancer Ther.* **2009**, *8*, 3266–3275. (b) Shinohara, E. T.; Maity, A. *Curr. Mol. Med.* **2009**, *9*, 1034–45.

(2) (a) Hockel, M.; Schlenger, K.; Aral, B.; Mitze, M.; Schaffer, U.; Vaupel, P. *Cancer Res.* **1996**, *56*, 4509–4515. (b) Rofstad, E. K.; Rasmussen, H.; Galappathi, K.; Mathiesen, B.; Nilsen, K.; Graff, B. A. *Cancer Res.* **2002**, *62*, 1847–1853.

(3) (a) Fyles, A.; Milosevic, M.; Pintilie, M.; Syed, A.; Levin, W.; Manchul, L.; Hill, R. P. *Radiother. Oncol.* **2006**, *80*, 132–137. (b) Kaanders, J. H.; Wijffels, K. I.; Marres, H. A.; Ljungkvist, A. S.; Pop, L. A.; van den Hoogen, F. J.; de Wilde, P. C.; Bussink, J.; Raleigh, J. A.; van der Kogel, A. J. *Cancer Res.* **2002**, *62*, 7066–7074.

(4) (a) He, F.; Deng, X.; Wen, B.; Liu, Y.; Sun, X.; Xing, L.; Minami, A.; Huang, Y.; Chen, Q.; Zanzonico, P. B.; Ling, C. C.; Li, G. C. *Cancer Res.* **2008**, *68*, 8597–8606. (b) Iglesias, P.; Costoya, J. A. *Biosens. Bioelectron.* **2009**, *24*, 3126–3130. (c) Lehmann, S.; Stiehl, D. P.; Honer, M.; Dominietto, M.; Keist, R.; Kotevic, I.; Wollenick, K.; Ametamey, S.; Wenger, R. H.; Rudin, M. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 14004–14009.

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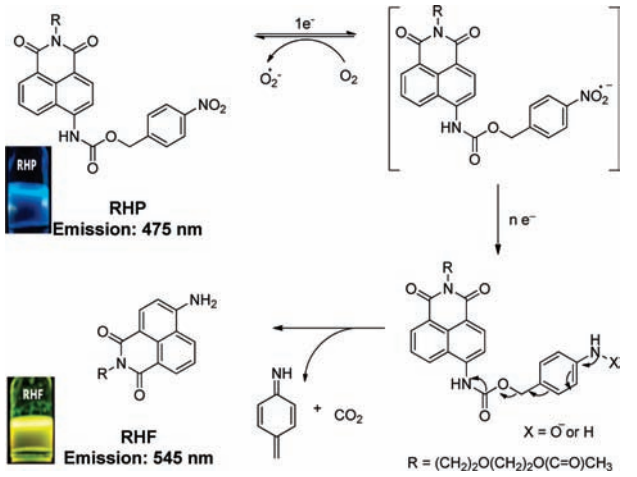
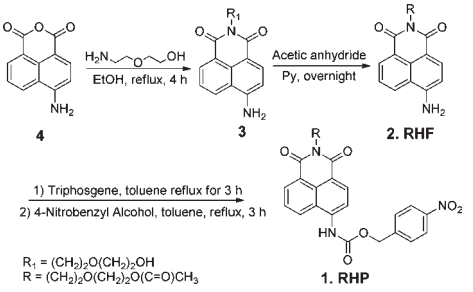


Figure 1. Proposed detection mechanism of **RHP**.

Various methods for hypoxia detection are available.[5] However, their practical applications are restricted due to the invasiveness. Fluorescence based techniques have some obvious advantages since they are noninvasive, display high sensitivity, and provide high spatiotemporal resolution. They have also been viewed as a powerful and versatile toolbox in the field of life sciences, environmental monitoring, and disease diagnosis.[6] Based on nitro groups, which can be reduced to amino groups under hypoxic conditions, several fluorescent probes for hypoxia were reported.[7] Fraser et al. reported an elegant sensing platform that emits both fluorescence and phosphorescence for hypoxia imaging.[8] Probes with dual emission are particularly viable because they enable a built-in calibration for environmental effects.[9] Herein, we describe a novel prodrug-derived ratiometric fluorescent probe **RHP** in aqueous solution based on an intramolecular charge transfer (ICT) mechanism.[10]

Scheme 1. Synthesis of the Probe **RHF** and **RHP**



The *p*-nitrobenzyl moiety is used frequently in the development of prodrugs for hypoxia.[11] Therefore, we designed our hypoxia probe **RHP** by connecting a *p*-nitrobenzyl moiety and a signaling moiety **RHF** via a carbamate group. The electron-withdrawing carbamate group weakens the ICT effect and results in a fluorescent emission wavelength blue shift. Upon reduction, the amino group of **RHF** will be released and the fluorescence emission will be restored[12] (Figure 1). Both probe **RHP** and bioreduction product **RHF** were efficiently synthesized (Scheme 1) and well characterized.

Next, we studied their spectral characteristics in chemical, enzymatic, and cell media. Spectroscopic evaluation of **RHP** and **RHF** was carried out under physiological conditions at 37 °C in PBS buffer (pH = 7.0, 0.01 M) with 1% DMSO (Figure S1). The absorption spectra of **RHP** and **RHF** have an intersection at a wavelength of 410 nm implying that they could be equally excited at this wavelength. Thus, we chose 410 nm as the excitation wavelength for ratiometric studies. The maximum emission wavelength of the **RHP** locates at 475 nm. And that of **RHF** is longer by ca.75 nm and falls at 550 nm (Figures S2, S3). Thus, the probe exhibited a favorable ratiometric signal change[13] (Table S1).

We then tested the uncaging sensitivity of the *p*-nitrobenzyl moiety under enzymatic reduction. The nitro group of **RHP** was reduced by an oxygen-sensitive nitroreductase (NTR) expressed in *Escherichia coli*. The fluorescence spectra of **RHP** were collected in the presence of NTR (3.2 unit of NTR), and a time dependent fluorescence change originated from RHP was observed (Figure 2). Within 10 min, the maximal fluorescence intensity at a wavelength of 550 nm was enhanced by more than 4-fold and the ratio (F_{550}/F_{475}) of **RHF** to **RHP** emission upon excitation at 410 nm exhibited

(5) (a) Dasu, A.; Denekamp, J. *Int. J. Radiat. Oncol.* **1999**, *43*, 1083–1094. (b) Stern, S.; Guichard, M. *Radiother. Oncol.* **1996**, *41*, 143–149.

(6) (a) Elfeky, S. A.; Flower, S. E.; Masumoto, N.; D'Hooge, F.; Labarthe, L.; Chen, W.; Len, C.; James, T. D.; Fossey, J. S. *Chem.—Asian J.* **2010**, *5*, 581–588. (b) Jiang, W.; Cao, Y.; Liu, Y.; Wang, W. *Chem. Commun.* **2010**, *46*, 1944–1946. (c) Zhang, X.; Chi, L.; Ji, S.; Wu, Y.; Song, P.; Han, K.; Guo, H.; James, T. D.; Zhao, J. *J. Am. Chem. Soc.* **2009**, *131*, 17452–17463. (d) Lee, Y. H.; Liu, H.; Lee, J. Y.; Kim, S. H.; Kim, S. K.; Sessler, J. L.; Kim, Y.; Kim, J. S. *Chem.—Eur. J.* **2010**, *16*, 5895–5901.

(7) (a) Zhu, W.; Dai, M.; Xu, Y.; Qian, X. *Bioorg. Med. Chem.* **2008**, *16*, 3255–3260. (b) Dai, M.; Zhu, W. P.; Xu, Y. F.; Qian, X. H.; Liu, Y.; Xiao, Y.; You, Y. *J. Fluoresc.* **2008**, *18*, 591–597. (c) Tanabe, K.; Hirata, N.; Harada, H.; Hiraoka, M.; Nishimoto, S. *ChemBioChem* **2008**, *9*, 426–432. (d) Hodgkiss, R. J.; Parrick, J.; Porssa, M.; Stratford, M. R. *J. Med. Chem.* **1994**, *37*, 4352–4356. (e) Kiyose, K.; Hanaoka, K.; Oushiki, D.; Nakamura, T.; Kajimura, M.; Suematsu, M.; Nishimatsu, H.; Yamane, T.; Terai, T.; Hirata, Y.; Nagano, T. *J. Am. Chem. Soc.* **2010**, *132*, 15846–15848.

(8) Zhang, G.; Palmer, G. M.; Dewhirst, M. W.; Fraser, C. L. *Nat. Mater.* **2009**, *8*, 747–751.

(9) Tsien, R. Y.; Poenie, M. *Trends Biochem. Sci.* **1986**, *11*, 450–455.

(10) (a) Srikun, D.; Miller, E. W.; Domaille, D. W.; Chang, C. J. *J. Am. Chem. Soc.* **2008**, *130*, 4596–4597. (b) Cui, L.; Zhong, Y.; Zhu, W.; Xu, Y.; Qian, X. *Chem. Commun.* **2010**, *46*, 7121–7123. (c) Bozdemir, O. A.; Sozmen, F.; Buyukcakir, O.; Guliyev, R.; Cakmak, Y.; Akkaya, E. U. *Org. Lett.* **2010**, *12*, 1400–1403. (d) Ojida, A.; Miyahara, Y.; Wongkongkatep, J.; Tamaru, S.; Sada, K.; Hamachi, I. *Chem.—Asian. J.* **2006**, *1*, 555–563.

(11) (a) Brown, J. M.; Wilson, W. R. *Nat. Rev. Cancer* **2004**, *4*, 437–447. (b) Xu, G.; McLeod, H. L. *Clin. Cancer Res.* **2001**, *7*, 3314–3324. (c) Denny, W. A. *Lancet Oncol.* **2000**, *1*, 25–29. (d) Denny, W. A. *Eur. J. Med. Chem.* **2001**, *36*, 577–595.

(12) Jaffar, M.; Williams, K. J.; Stratford, I. J. *Adv. Drug Delivery Rev.* **2001**, *53*, 217–228.

(13) The quantum yield (Φ_F) is determined by using Fluorescein with 0.1 M NaOH solution ($\Phi_F = 0.95$) as a standard.

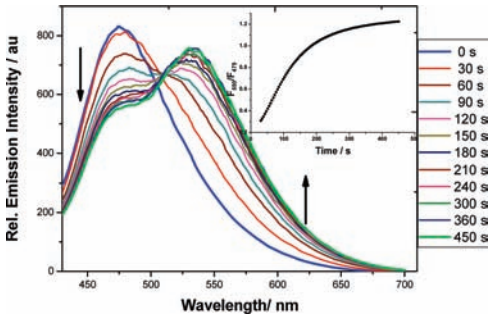


Figure 2. Fluorescent spectra of **RHP** (10 μM) in PBS buffer with 1% DMSO treated with nitroreductase (10 μg/mL) and NADH (500 μM) with excitation at 410 nm. After addition of **RHP** to the nitroreductase buffer, the fluorescent intensity data were collected after certain time intervals as indicated in the figure. Inset: the emission ratio (F_{550}/F_{475}) verse time.

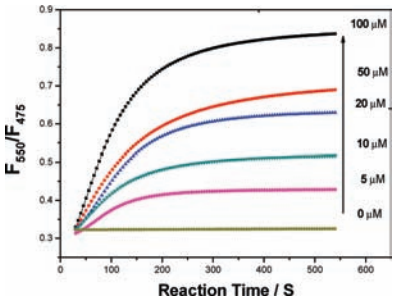


Figure 3. Ratio changes (F_{550}/F_{475}) with the λ_{ex} = 410 nm at 37 °C over an incubation time of 10 min at varied concentrations of **RHP**: 0 (control), 5, 10, 20, 50, and 100 μM. The measurements were performed in PBS buffer with 10 μg/mL nitroreductase and 500 μM of NADH.

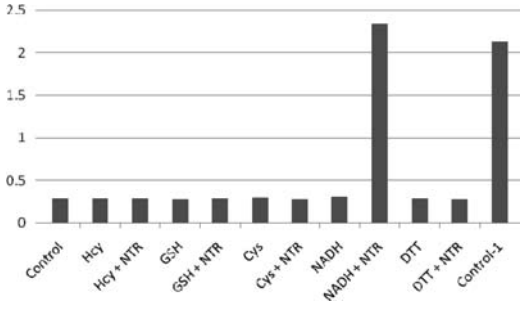


Figure 4. Fluorescence response assays by the biological reductants. Control: no addition compound. Control-1: all of the biological reductants and NTR. Fluorescence responses of **RHP** (10 μM) coincubated with various biological thiol reductants in phosphate buffered saline at pH 7.0. Bars represent the final fluorescence intensity at the wavelength 550 nm after the reaction for 30 min (I_{550}) over final fluorescence intensity at the wavelength 475 nm after reaction for 30 min (I_{475}) at λ_{ex} = 410 nm.

a notable dynamic range from 0.3 to 1.2 (inset of Figure 2). It was calculated, using their photophysical parameters, that around 80% of **RHP** was converted to **RHF**. After the bioreaction for 10 min, the fluorescence intensity of **RHP** was decreased but not fully quenched, as shown in Figure 2; this could be attributed to the uncompleted biological reaction and relatively high fluorescence quantum yield (Table S1) of **RHP**. Fluorescence and naked eye images of **RHP** to NTR also showed a huge color difference before and after reaction (Figure S4). We also certified that the nitroreductase metabolite is **RHF** using HPLC analysis (Figure S5). The above results proved that **RHP** was a successful ratiometric fluorescent probe mediated by nitroreduction.

The kinetics of **RHF** release from **RHP** triggered by nitroreductase in the presence of NADH were measured by adding a known concentration of **RHP** (5−100 μM) to the reaction mixture. The fluorescence change was recorded at a wavelength of 475 and 550 nm, respectively. The rate was calculated using the fluorescence emission intensity ratio at a wavelength of 475 and 550 nm. All assays were performed in triplicate, and the results reported are the average of three experiments (Figure 3).

Fluorescence response assays by the biological reductants were also carried out because biological thiols have been suggested to be the electron providers for reductive activation of various hypoxia sensitive antitumor prodrugs.[14] Therefore, the fluorescence response of **RHP** to other biorelevant thiols, such as dithiothreitol (DTT), glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and β-Nicotinamide adenine dinucleotide (NADH), was investigated. Under normal physiological conditions, the concentration of reduced biological reductants is far lower than 1 mM. As shown in Figure 4, incubation of the probe **RHP** with up to 100 equiv of biological reductants did not induce any noticeable signal modulation. The selectivity was also tested in the mixture of NTR with 100 equiv of other biological reductants, and the changes in fluorescence intensity were also negligible. The competition experiments showed that in the mixture of NTR and NADH with 100 equiv of other biological reductants, no obvious influence was found in comparison to the experiments containing only NTR and NADH (Figure 4). The above results demonstrated that the thiols employed in these experiments exhibited no interferences and our probe **RHP** showed a very high selectivity for NTR. Additionally, this observation could potentially benefit the development of hypoxia targeted prodrugs based on nitroreduction.

To further demonstrate the potential of the probe for practical application, we also applied **RHP** in living cells for ratiometric fluorescence imaging of hypoxia. A549 cells were incubated with **RHP** under hypoxic and normal physiological conditions, respectively, at 37 °C for 7 h and subsequently washed 3 times with PBS buffer to remove the extracellular probe (containing 0.5% DMSO,

(14) (a) Paz, M. M. *Chem. Res. Toxicol.* **2009**, *22*, 1663–1668. (b) Paz, M. M.; Tomasz, M. *Org. Lett.* **2001**, *3*, 2789–2792. (c) Wolkenberg, S. E.; Boger, D. L. *Chem. Rev.* **2002**, *102*, 2477–2495.

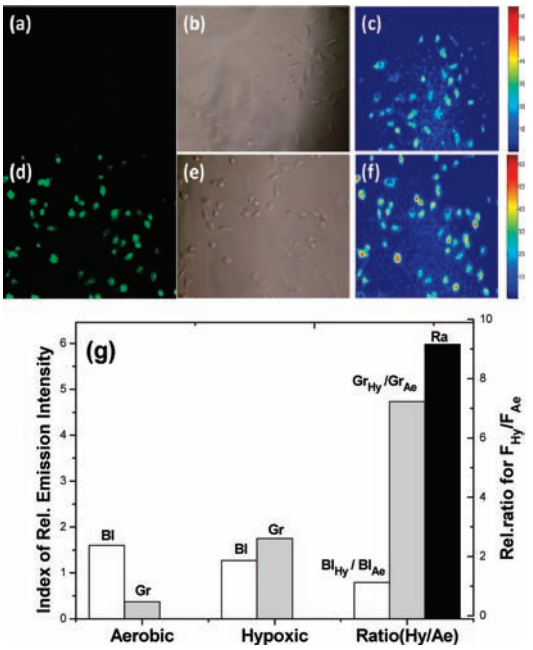


Figure 5. Fluorescence microphotographs of A549 cells incubated with **RHP** (1) at 37 °C in F-12 Nutrient Mixture (Ham). The top row was at aerobic condition (a, b, and c). The bottom row was under hypoxic conditions (d, e, and f). All cells were incubated with 10 μM **RHP** (1) for 7 h. (a) and (d) images were taken in optical windows between 540 and 580 nm (green). (b) and (e) phase contrasts, (c) and (f) displayed in pseudocolor represent the ratio of emission intensities collected in optical windows between 540 and 580 (green) and 430−495 nm (blue), respectively. (g) showed the mean fluorescent photons and relative ratio. ImageJ software gave an average emission value of fluorescent photos. Bl: mean fluorescent intensity (MFI) of single cell between 430 and 495 nm. Gr: MFI of single cell between 540 and 580 nm. Gr_{Hy}/Gr_{Ar} means the green ratio of hypoxic to aerobic; Bl_{Hy}/Bl_{Ar} means the blue ratio of hypoxic to aerobic. Ra (black): the ratio of Gr_{Hy}/Gr_{Ar} to $BlHy/Bl_{Ar}$.

pH = 7.0).[15] The changes in the fluorescence intensity were measured using an inverted fluorescence microscope (Figures 5 and S6). The ratio of fluorescence intensity was collected with a 4 s exposure time from 540 to 580 nm (green) and with a 800 ms exposure time from 430 to 495 nm (blue). The ratio image constructed from 540 to 580 nm (green) and from 430 to 495 nm (blue) fluorescence collection windows using ImageJ software gave an average emission value of the fluorescent photos.

From the images above, we could see that there was a notable increase between 540 to 580 nm (green) (Figure 5a and 5d) of fluorescence under aerobic and hypoxic conditions. The data in Figure 5c and 5f show clear increases in green-to-blue emission ratios under normal conditions (<45) compared to the cells under hypoxic conditions (>45). Finally, we evaluated the cytotoxicity of **RHP** and **RHF**, and the results showed that when the concentrations of **RHP** and **RHF** were less than 1 mM, the death rate of **RHP** and **RHF** were as low as 5% when both of the compounds **RHP** and **RHF** were at the concentration 10 μM (Figure S7). These data have clearly demonstrated that **RHP** is capable of *in vitro* imaging of hypoxia in solid tumors. Moreover, the ratiometric readout provided by this probe allows for detection of a highly imperceptible change of oxygen tension in a solid tumor.

In summary, we have developed a selective ratiometric fluorescent probe (**RHP**) for microenvironment hypoxia detection. **RHP** can be selectively activated by reductive enzymes (NTR) and results in an evident blue to green fluorescent emission wavelength change in both solution phases. It was also successfully applied in *in vitro* imaging of hypoxia in an A549 cell line, displayed no cytotoxicity, and is highly promising for real world applications such as in tumor diagnosis via hypoxia imaging. In addition to monitoring the oxygen environment, other potential applications including monitoring the effect of drug treatment are being investigated.

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Supporting Information Available. Synthesis, experimental details, and additional spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

(15) Grigoryan, R.; Keshelava, N.; Anderson, C.; Reynolds, C. P. *Chemosensitivity* **2005**, *110*, 87–100.