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COMMUNICATION

A Hemicyanine-based Fluorescent Probe for Hydrazine Detection in Aqueous Solution and Its Application in Real Time Bioimaging of Hydrazine as a Metabolite in Mice

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Tao Li,[a] Jian Liu,[a] Linjiang Song,[b] Zicheng Li,[a] Qingrong Qi*[c] and Wencai Huang*[a]

A hemicyanine-based fluorescent probe Hcy-Ac is developed for the specific detection of hydrazine. With good water solubility and high sensitivity, Hcy-Ac has been successfully applied for the monitoring of *in situ* hydrazine release during the metabolism of isoniazid in mice.

Hydrazine (N_2H_4) is widely used in many fields owning to its flammable and detonable characteristics, high reducibility and alkalinity.[1-2] Meanwhile, it is also applied as a building block to synthesize various of chemical products such as pharmaceuticals, pesticides, antioxidants, plant growth regulators, textile dyes and polymers.[3] On the other hand, when absorbed by skin, respiratory or digestive tract, hydrazine can cause serious damages to the liver, lungs, kidneys and central nervous system of the human body.[4-5] In 1999, the U.S. Environmental Protection Agency (EPA) classified hydrazine as a suspected carcinogen and the threshold limit in drinking water was proposed to be 10 ppb.[6] To avoid serious environmental pollution during its production, transportation, utilization and disposal, it is of great significance to detect hydrazine sensitively and effectively.

In comparison to the traditional methods for hydrazine detection such as chromatography,[7] spectrophotometry,[8] coulometry,[9] potentiometry[10] and titrimetry,[11] fluorometric method has attracted increasing attention in recent years because of its high sensitivity and selectivity, easy implementation, noninvasiveness, and good compatibility for biosamples.[12] To date, fluorescent probes based on different mechanisms have been developed for the detection of hydrazine, among which reaction-based small-molecule fluorescent probes are extremely attractive. Notably, different fluorescent molecules could be obtained to meet different analytical requirements because the structure of probe is devisable. Generally, the strategy applied for the design of such a fluorescent probe is to anchor a hydrazine nucleophilic reaction site to a specific fluorophore. Typical recognition units which have been applied successfully include acetyl,[13-15] 4-bromobutanoate,[16-17] levulinate,[18] 4-bromobenzoate,[19] aldehyde,[20-21] 1,3-diketones,[22-23] 1-(2-hydroxy-2*H*-pyran-3-yl)ethan-1-one,[24] malononitrile,[25] ethyl cyano-acetate[26] and phthalimide.[27-29]

In the past decade, great advances have been achieved in the construction and use of fluorescent probes for hydrazine detection in environment protection, water treatment and safety inspection. By contrast, their applications in biosystems, especially in living animals such as mice,[13,14,17,30] zebrafish[31] and fruit-fly larvae[22] were comparatively limited. It is well known that high percentage of organic cosolvent is unfavourable to living cells or animals. However, the fluorescent probes for hydrazine with good water solubility and the potential to be applied in living animals have been scarce.[32] As a consequence, new fluorescent probes with these favourable properties are highly desirable for biological application.

On the other hand, hydrazine presents in the animal or human body may result from not only absorption from the environment but also the metabolism of some drugs such as isoniazid (INH) and pasiniazide. Up to now, almost all the bioimaging of hydrazine in living systems involved in cells, zebrafish, fruit flies and mice treated with exogenous hydrazine. Recently, some experiments were performed on HepG2 cells incubated with INH and the enzymatic release of hydrazine as a metabolite was detected *in situ* with fluorescent probes,[27,33] but Triton X 100 or 30% of DMSO must be added to improve the solubility of the probes in buffer. These shortcomings may restrict their applications in living animals such as mice. Herein, we describe a novel fluorescent probe **Hcy-Ac** with an acetyl group as the recognition unit of hydrazine. With hemicyanine as the key fluorophore, the probe exhibited excellent water solubility and high stability. It responded to hydrazine sensitively and selectively in aqueous solution at physiological pH via an intramolecular charge transfer (ICT) progress, thus, the bioimaging of hydrazine in both living cells and mice was

[a] School of Chemical Engineering, Sichuan University, Chengdu 610065, China. *E-mail: hwc@scu.edu.cn
[b] School of Medical and Life Sciences, Chengdu University of Traditional Chinese Medicine, Chengdu 610072, China
[c] West China School of Pharmacy, Sichuan University, Chengdu 610041, China. *E-mail: qiqingrong@scu.edu.cn

Electronic Supplementary Information (ESI) available: Experimental procedures, NMR, MS, fluorescence spectra and other materials. See DOI:

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demonstrated. To the best of our knowledge, this is the first report on the real time visualization of *in situ* hydrazine release as a metabolite of drug in animal using a fluorescent probe.

Keeping the requirements of a promising fluorescent probe for the detection of hydrazine in biosystems in mind, a new type of hemicyanine structure with terephthalaldehyde as the linker of 3-(2, 3, 3-trimethyl-3*H*-indol-1-ium-1-yl) propane-1-sulfonate (**1**) and *p*-hydroxyacetophenone was designed. The resulting novel fluorophore (**Hcy-OH**) has a large D–π–A structure and the phenolic hydroxyl could be masked by esterification easily. The probe **Hcy-Ac** was synthesized via a four-step procedure as outlined in Scheme S1 (see ESI†) and the experimental details and structural characterizations are provided in the Electronic Supporting Information.

To our delight, **Hcy-Ac** has a good water solubility and the stock solution of **Hcy-Ac** in DMSO (20 mM, 100 μL) was dissolved in complete aqueous buffer (10 mM PBS, pH=7.4, 200 mL), then, the diluted solution (10 μM) was used throughout the experiments. The absorption spectral properties of **Hcy-Ac** in the absence or presence of hydrazine in the buffer were shown in Fig. 1a. Once 20 equiv of hydrazine was added, the maximum absorption of **Hcy-Ac** at 420 nm decreased remarkably, whereas a new absorption band centered at 320 nm appeared and increased gradually, with a well-defined isosbestic point at 348 nm. At the same time, the color of the solution changed from yellow to colorless, which could be seen with the naked eye (inset of Fig. 1a). On the other hand, addition of hydrazine also resulted in a remarkable fluorescent decrease at 532 nm upon excitation at 430 nm (Fig. 1b).

Then, the fluorescence titration experiments of **Hcy-Ac** (10 μM) with different concentrations of hydrazine (0-200 μM) in buffer solution were performed. As shown in Fig. 2a, the fluorescence intensity at 532 nm decreased steadily and a good linear relationship between fluorescence intensity and concentration of hydrazine with R^2 = 0.9946 was observed (Fig. 2b).

The detection limit (LOD, 3σ/k) of **Hcy-Ac** was thus calculated to be 1.72 ppb, which is lower than the threshold limit value (TLV) of 10 ppb proposed by the U.S. Environmental Protection Agency. And the absolute fluorescent quantum yield of **Hcy-Ac** was measured to be 0.20 in PBS buffer (10 mM, pH=7.4).

To examine the selectivity of probe **Hcy-Ac**, the fluorescence responses of **Hcy-Ac** (10 μM) in the presence of different analytes (40 equiv) in buffer solution were recorded. As shown in Fig. 3, the addition of anions (Cl^-, SO_4^{2-}, NO_2^-, CO_3^{2-}), cations (K^+, Ca^{2+}, Fe^{3+}, Cu^{2+}, Mg^{2+}, NH_4^+), amino acids (Lys, Ile, Pro, Arg,

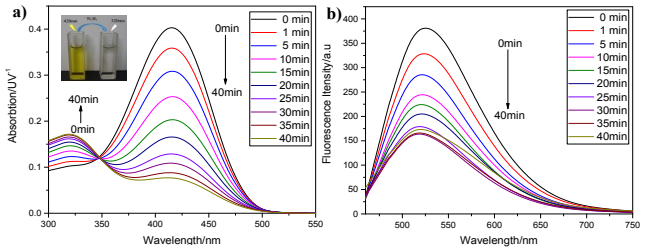

Fig. 1 Absorption spectral (a) and fluorescence spectral (b) changes of probe **Hcy-Ac** (10 μM) in the absence and presence of hydrazine (20 equiv) in buffer solution (10 mM PBS, pH=7.4) with λ_{ex} at 430 nm at 25 °C. Inset: color change of the probe with the addition of hydrazine under natural light.

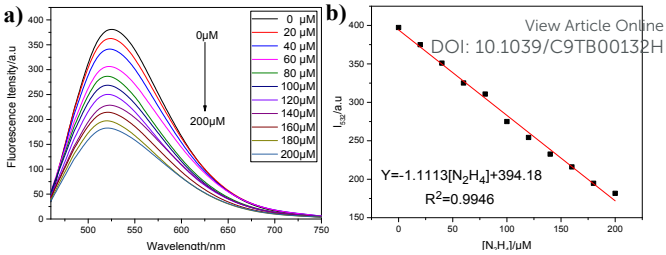

Fig. 2 (a) Fluorescence spectra of **Hcy-Ac** (10 μM) upon the addition of increasing concentrations of hydrazine (0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 μM) when excited at 430 nm at 25 ℃. (b) Fluorescence intensity changes of **Hcy-Ac** at 532 nm as a function of hydrazine concentration.

Phe, His), and biologically relevant amines (aniline, benzylamine, ammonia, thiourea, ethanolamine, cyclohexylamine, diethylamine, ethylenediamine, hydroxylamine) to the solutions of **Hcy-Ac** caused no or slight change in the fluorescence spectra. Notably, INH and acethydrazine which is one of the major metabolites of INH *in vivo* also brought about no obvious change of fluorescence intensity. By contrast, the addition of 20 equiv of hydrazine to **Hcy-Ac** led to a marked fluorescence decrease at 532 nm upon excitation at 430 nm. These results illustrated the good specificity of **Hcy-Ac** for practical hydrazine detection and the potential to be used in biosystems.

Based on the structural and spectral properties of **Hcy-Ac**, we envisioned that the response of **Hcy-Ac** toward hydrazine was attributed to its hydrazinolysis to release **Hcy-OH**, thus triggering the chromogenic and fluorescence changes observed. The proposed sensing mechanism was depicted in Scheme 1 and supported by ^{1}H NMR analysis (Fig. S14, ESI†). Compared with the ^{1}H NMR spectrum of pure **Hcy-Ac**, new proton signals at 8.97 ppm and 4.51 ppm corresponding to the INH in acethydrazine (H_b) and phenolic hydroxyl in **Hcy-OH** (H_c) respectively were observed upon the addition of hydrazine (20 equiv). Meanwhile, the methyl group at 2.39 ppm in **Hcy-Ac** (H_a) was completely disappeared and the methyl proton at 1.58 ppm in acetohydrazide (H_a') was clearly found. Taken together, the differences between the ^{1}H NMR spectra of **Hcy-Ac** in the absence and presence of hydrazine agree with the sensing mechanism depicted in Scheme 1.

Encouraged by the promising results obtained, we carried out a standard MTT assay to investigate the potential toxicity of **Hcy-Ac** against HeLa cells. The results (Fig. S1, ESI†) indicated that HeLa cell

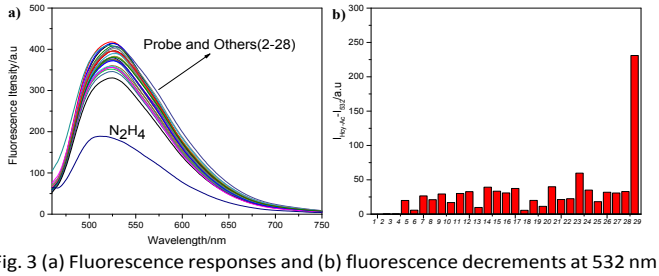

Fig. 3 (a) Fluorescence responses and (b) fluorescence decrements at 532 nm of **Hcy-Ac** (10 μM) upon addition of 20 equiv of hydrazine and 40 equiv of interfering analytes (1, blank; 2, K^+; 3, Ca^{2+}; 4, Fe^{3+}; 5, Cu^{2+}; 6, Mg^{2+}; 7, NH_4^+; 8, Cl^-; 9, SO_4^{2-}; 10, NO_2^-; 11, CO_3^{2-}; 12, Lys; 13, Ile; 14, Pro; 15, Arg; 16, Phe; 17, His; 18, Aniline; 19, Benzylamine; 20, Diethylamine; 21, Cyclohexylamine; 22, Ethylenediamine; 23, Ethanolamine; 24, Hydroxylamine; 25, Ammonia; 26, Thiourea; 27, INH; 28, Acetylhydrazine; 29, Hydrazine) when excited at 430 nm.

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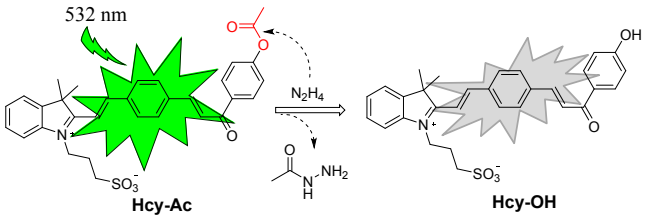

Scheme 1 Proposed sensing mechanism of **Hcy-Ac** toward hydrazine

viability was reduced by about 5% after 48 h incubation at a concentration of 10 μM, and even the concentration was raised up to 100 μM, the cell viability was more than 85%, supporting the ability of **Hcy-Ac** for bioimaging. Then, the confocal fluorescence images of HeLa cells treated with **Hcy-Ac** (10 μM) in the absence or presence of hydrazine (50 μM) were recorded as shown in Fig. 4.

It could be seen that the HeLa cells displayed distinct green fluorescence in the presence of **Hcy-Ac** (Fig. 4b). When the **Hcy-Ac**-pretreated HeLa cells were further incubated with hydrazine for 0.5 h, the green fluorescence markedly decreased (Fig. 4c). These results demonstrated the practicality of this probe to visualize hydrazine in living cells.

INH is a first-line drug in the prevention and treatment of tuberculosis for more than 40 years, but high rates of severe side effects including liver injury and neurotoxicity hindered its safe and wide application in clinic.[34] It is well known that hydrazine is a major toxic metabolite of INH,[35] so, **Hcy-Ac** was employed for imaging hydrazine injected or produced *in situ* in three groups of mice (Fig. S2, ESI†). Owing to the good water solubility of **Hcy-Ac** and hydrazine, tail-vein injection instead of skin-pop injection or intraperitoneal injection was adopted for **Hcy-Ac** and hydrazine, and INH in PBS buffer was administrated by intragastric injection which is similar to the commonly-used administration manner of INH for human beings. In the first group, the Kunming mouse was only given a tail-vein injection of **Hcy-Ac** and then imaged with a small animal living imager at 520 nm (Fig. 5a). In the second group, the mouse was injected with **Hcy-Ac** followed by hydrazine by tail-vein (Fig. 5b). In the last group, the mouse was injected with **Hcy-Ac** by tail-vein and then administrated with INH by intragastric injection (Fig. 5c). It could be seen from Fig. 5a that **Hcy-Ac** was mainly distributed to the kidney after the injection and the decrease of fluorescence intensity was slight (0-120 min), suggesting the stability of **Hcy-Ac** in mice. When hydrazine was administrated followed by **Hcy-Ac**, the fluorescence intensity at 520 nm decreased dramatically, indicating the presence of hydrazine in kidney (Fig. 5b). Notably, a similar decreasing fluorescence intensity was also observed in kidney after INH was administrated by intragastric injection, which illustrated that hydrazine was produced *in situ* as a metabolite of INH (Fig. 5c). With the above results, it was verified that hydrazine was produced in kidney as a metabolite of INH orally administrated. In short, the experimental results revealed that **Hcy-Ac** could be used to monitor the hydrazine injected or produced *in situ* in living mice.

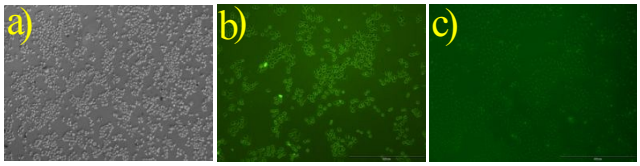

Fig. 4 Confocal fluorescence images of HeLa cells treated with **Hcy-Ac** (10 μM) in the absence (b) or presence (c) of hydrazine (50 μM). a: white light channel, b, c: green fluorescent channel (λ_{ex} = 430 nm, λ_{em} = 480-590 nm), scale bar: 500 μm.

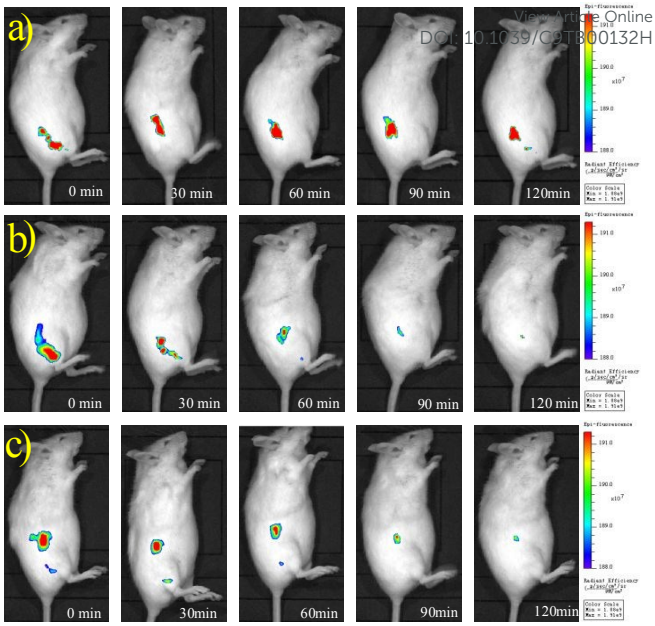

Fig. 5 Representative fluorescence images (pseudocolor) of a Kunming mouse. a: the mouse was given a tail-vein injection of **Hcy-Ac** (25 μL, 50 μM). b: the mouse was given a tail-vein injection of **Hcy-Ac** (25 μL, 50 μM) followed by hydrazine (25 μL, 500 μM). c: the mouse was given a tail-vein injection of **Hcy-Ac** (25 μL, 50 μM) followed by an intragastric administration of INH (5.4 mg) in 0.6 mL PBS buffer (pH = 7.4, 10 mM). The mice were imaged with an excitation filter of 440 nm and an emission filter of 520 nm. Images were taken at 0, 30, 60, 90 and 120 min.

In summary, we developed a novel fluorescent probe **Hcy-Ac** for the specific detection of hydrazine. With hemicyanine as the fluorop--hore and acetyl group as the recognition site respectively, this probe demonstrated a markedly change of fluorescence at 532 nm upon the action of hydrazine in pure aqueous buffer at physiological pH. The detection limit was calculated to be 1.72 ppb which is lower than the TLV proposed by EPA. These features such as good water solubility, high sensitivity and selectivity make it a promising probe for application in biosystems. Thus, the detection of hydrazine in living HeLa cells was performed. More importantly, the monitoring of *in situ* hydrazine release during the metabolism of INH in mice was performed successfully and the results also disclosed some useful information on the metabolic process such as the distribution, concentration change of the toxic hydrazine.

Conflicts of interest

There are no conflicts to declare.

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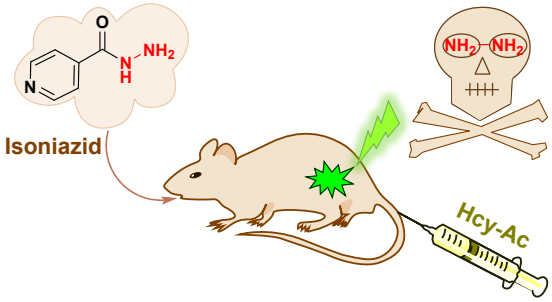
A Hemicyanine-based Fluorescent Probe for Hydrazine Detection in Aqueous Solution and Its Application in Real Time Bioimaging of Hydrazine as a Metabolite in Mice

Tao Li,[a] Jian Liu,[a] Linjiang Song,[b] Zicheng Li,[a] Qingrong Qi*[c] and Wencai Huang*[a]

A fluorescent probe **Hcy-Ac** was developed for the monitoring of *in situ* hydrazine release during the metabolism of isoniazid.



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