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Highly Selective Fluorescence off-on Probes for Biothiols and Imaging in Live Cells

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Abstract

Three sulfonyl benzothiazole-based fluorescent probes (**RSHP1**, **RSHP2**, and **RSHP3**) for the detection of biothiols (cysteine, homocysteine, and glutathione) are developed based on thiol-mediated nucleophilic aromatic substitutions. The probes exhibited good selectivity and sensitivity toward biothiols over other analytes. The probes were successfully applied for visualizing endogenous thiols in living cells.

Introduction

Intracellular thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play important roles in biological systems, especially in maintaining biological redox homeostasis through the equilibrium established at a given electrical potential between reduced free thiols (RSH) and oxidized disulfides (RSSR).¹ Abnormal levels of these compounds are associated with many diseases like cancer, AIDS, and cardiovascular diseases.² Therefore, the detection of biothiols in biological and environmental samples consistently attracts a great deal of attention. Among the various detection techniques, fluorescent probes have become a powerful tool because of their high sensitivity and spatiotemporal resolution capability.³

Most of known fluorescent probes recognizing thiols are based on two characteristic reactivities of thiols: 1) the strong nucleophilicity, and 2) high binding affinity to metal ions.⁴ Recently, our laboratory discovered a group of new thiol blocking reagents, sulfonyl benzothiazoles (SBT) (Scheme 1). These compounds showed high selectivity and reactivity to both small molecule and proteinthiols.⁵ We expected that if the SBT group is conjugated to an -OH sensitive fluorophore, the resultant compound **RSHP** would be a specific fluorescent probe for biothiols, as it should selectively react with biothiols to release the fluorophore. This strategy should be useful in the design of novel fluorescent probes for thiols. Herein we report our results.

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Results and Discussion

The syntheses of three SBT-based probes **RSHP1-3** are shown in Scheme 2. Briefly 2-mercaptobenzothiazole (**1**) was first converted to benzothiazole-2-sulfonyl chloride (**2**), and subsequently treated with 7-OH-coumarin and fluorescein derivatives to provide the corresponding probes. Coumarin and fluorescein were selected as the fluorophores because of their readily availability, excellent fluorescence properties, and easy fluorescence quenching by hydroxy group substitutions.⁶ For **RSHP3**, two reaction centers were introduced to the core structure of fluorescein. Upon reaction with RSH, it should produce free fluorescein, a highly fluorescent species. According to previous experience this design usually leads to higher levels of fluorescence turn-on.⁷ With these probes in hand, **RSHP1** was used as the model to study the reaction between the probes and thiols. As expected, the reaction of **RSHP1** with a cysteine derivative **3** went well and the desired product **4** and 7-hydroxycoumarin were obtained in excellent yields (82%). In addition the reaction was found to be fast as it was completed within 30 min at 37 °C. These results confirmed the good reactivity of SBT substrates toward thiols.

We then tested the fluorescence quantum yields of these probes. 7-hydroxycoumarin ($\Phi_f = 0.76$, excited at 330 nm in 0.1 M pH 7.4 sodium phosphate buffer, for **RSHP1**) and fluorescein ($\Phi_f = 0.85$, excited at 490 nm in 0.1 N NaOH, for **RSHP2** and **RSHP3**) were used as the standards. As shown in Table 1, **RSHP1-3** exhibited very weak fluorescence with low quantum yields ($\Phi_f < 0.1$), owing to the sulfonylation of the hydroxy groups of the fluorophores. This low background fluorescence is critical for highly sensitive detection of biothiols.

The fluorescence ‘off-on’ responses of the probes to thiols were next measured. Each probe (10 μ M) was treated with GSH, Cys, and Hcy separately (all at 100 μ M). The measurements were carried out in PBS buffer (containing 10% DMSO) for 15 min. As shown in Figure 1, all samples gave strong fluorescence increases. These results demonstrated that the probes were sensitive to all biothiols. Among these probes, **RSHP3** appeared to be most reactive as it gave the highest fluorescence enhancements. Therefore **RSHP3** was selected for the following studies to further understand the probe's properties.

Figure 2 illustrated the time-dependent fluorescence enhancements of **RSHP3** in the presence of GSH, Cys, and Hcy. The fluorescence intensity (at 518 nm) increased dramatically when RSH was present in the solution. In all cases, the fluorescence signals were able to reach a steady state in about 15 min.

To evaluate the potential applications of **RSHP3** in different biological environments, we studied pH effects on fluorescence ‘turn-on’ of the probe (Figure 3). **RSHP3** appeared to be stable in pH range 4~9 as almost no fluorescence changes were observed for the probe itself. In the presence of thiols, the probe gave strong fluorescence responses under physiological or weak basic pH (7~9). In contrast the probe did not show significant signals under basic pH (4~6), indicating the reactivity of thiols is diminished under this pH range.

We next examined the selectivity of **RSHP3** for other reactive sulfur species and some common biological oxidants (Figure 4A). Reactive sulfur species used in this study include oxidized glutathione (GSSG), thiosulfate ($S_2O_3^{2-}$), and sulfite (SO_3^{2-}). These species did not give any significant fluorescence enhancement. Oxidants such as hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), nitrite (NO_2^-), and potassium superoxide (KO_2) were found to be non-reactive either. In comparison, thiols showed very significant fluorescent signals. To evaluate the selectivity of **RSHP3** for RSH over other natural amino acids, we measured the fluorescence intensity of **RSHP3** in the presence of various amino acids (100 μ M) in the absence and presence of GSH (100 μ M) (Figure 4B). The results revealed that **RSHP3** possessed high selectivity toward biothiols.

To demonstrate the efficiency of **RSHP3** in determining RSH concentrations, a series of different concentrations of GSH and Cys were treated with **RSHP3** (10 μ M), respectively (Figure 5 and Figure S1). We found that the intensities increased almost linearly in the range of 1 ~ 30 μ M for GSH and Cys. The detection limits⁸ were calculated to be 313 nM for GSH and 356 nM for Cys, suggesting the probe is reasonably sensitive.

Fluorescence imaging in living cells

It is well accepted that living cells maintain high concentrations of biothiols.⁹ To test the capability of **RSHP3** in monitoring RSH in living cells, we applied **RSHP3** to freshly cultured HeLa cells for thiol detection. As shown in Figure 6a, **RSHP3** was able to penetrate cell membrane and react with intracellular thiols, resulting in strong fluorescence signals. In one control experiment, HeLa cells were pre-treated with 500 μ M N-methylmaleimide (NMM) for 20 min to deplete intracellular thiols. Then **RSHP3** was applied to cells and a distinct decrease of fluorescence was observed (Figure 6b), further confirming the specific reaction of **RSHP3** with biothiols. In another control experiment HeLa cells were pre-treated with Cys (150 μ M) for 20 min, leading to an increased cellular thiol concentration. When **RSHP3** was incubated with these cells a dramatic enhancement of fluorescence intensity was observed (Figure 6c). These results clearly demonstrated that **RSHP3** is suitable for fluorescent imaging of biothiols in living cells.

Conclusions

In summary, we reported herein the development of three sulfonyl benzothiazole (SBT)-based fluorescence off-on probes for biothiols (Cys, Hcy and GSH). These probes exhibited good selectivity and sensitivity toward biothiols over other analytes. Moreover, **RSHP3** was successfully applied for bioimaging of endogenous thiols in living cells.

Experimental Section

Materials and instruments

All solvents were reagent grade. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040-0.062 mm). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Proton and carbon-13 NMR spectra were recorded on a 300 MHz spectrometer. The solvent

for NMR measurements were CDCl₃ or DMSO-d₆ using TMS as internal reference. Absorption spectra were recorded on a Lambda 20 UV/VIS spectrophotometer using 1 cm quartz cells. Fluorescence excitation and emission spectra were measured on Cary Eclipse fluorescence spectrophotometer.

Synthesis of RSHP1-RSHP3

Benzothiazole-2-sulfonyl chloride (**2**) was prepared from 2-mercaptobenzothiazole (**1**) following a known procedure.¹⁰

RSHP1—To a solution of 7-hydroxycoumarin (81 mg, 0.5 mmol) in dry dichloromethane (5 mL) at 0 °C was added dry Et₃N (89 µL, 0.5 mmol), followed by the addition of compound **2** (116.5 mg, 0.5 mmol). The mixture was stirred for 16 h at room temperature. It was then diluted with dichloromethane (25 mL), washed with saturated NH₄Cl solution (15 mL), brine (15 mL). The organic layer was dried with MgSO₄ and concentrated. The residue was purified by column chromatography (hexane/ethyl acetate = 1:9 to 3:7) to afford **RSHP1** (54 mg, 30%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆, 25 °C, TMS) δ 6.52-6.55 (d, *J* = 9.0 Hz, 1H), 7.19-7.22 (d, *J* = 9.0 Hz, 1H), 7.41 (s, 1H), 7.76-7.81 (t, *J* = 7.5 Hz, 3 H), 8.05-8.08 (d, *J* = 9.0 Hz, 1 H), 8.34-8.38 (q, *J* = 4.0 Hz, 2H); ¹³C NMR (75 MHz, DMSO-d₆, 25 °C, TMS) δ 160.04, 159.91, 154.59, 152.25, 151.11, 144.01, 137.36, 130.85, 129.67, 129.15, 126.11, 124.37, 119.27, 119.07, 117.57, 111.28. MS (ESI) [M + H]⁺ calcd for C₁₆H₁₀NO₅S₂ 360.0000, found [M + H]⁺ 360.0010; M.P. 135 – 138 °C.

RSHP2 was obtained as a white solid (132 mg, 49 % yield). ¹H NMR (300 MHz, CDCl₃, 25 °C, TMS) δ 3.83 (s, 3H), 6.61-6.70 (m, 2H), 6.75-6.78 (m, 2H), 6.84-6.88 (dd, *J* = 3.0 Hz, 1 H) 7.13-7.15 (t, *J* = 3.0 Hz, 1 H), 7.23-7.26 (t, *J* = 3.0 Hz, 1 H), 7.65-7.68 (m, 4 H), 8.00-8.02 (dd, *J* = 3 Hz, 2 H), 8.26-8.29 (m, 1 H); ¹³C NMR (75 MHz, CDCl₃, 25 °C, TMS) δ 161.78, 152.95, 152.27, 152.20, 152.15, 150.55, 135.50, 130.26, 129.93, 129.21, 128.92, 128.26, 126.17, 125.43, 124.16, 122.51, 119.14, 117.62, 112.51, 111.31, 110.77, 101.06, 82.15, 55.86; MS (ESI) [M + H]⁺ calcd for C₂₈H₁₈NO₇S₂ 544.0525, found [M + H]⁺ 544.0515; M.P. 78 – 80 °C.

RSHP3 was obtained as a white solid (130 mg, 38 % yield). ¹H NMR (300 MHz, CDCl₃, 25 °C, TMS) δ 6.77-6.80 (d, *J* = 9 Hz, 2H), 6.90-6.93 (dd, *J* = 4.5 Hz, 2H), 7.11-7.14 (m, 1H), 7.23-7.24 (d, *J* = 3 Hz, 2H), 7.61-7.71 (m, 6H), 7.99-8.03 (m, 3H), 8.25-8.29 (m, 2H); ¹³C NMR (75 MHz, CDCl₃, 25 °C, TMS) δ 168.93, 159.84, 152.56, 152.24, 151.53, 150.78, 137.10, 135.82, 130.64, 129.82, 129.01, 128.33, 126.18, 125.68, 124.12, 122.52, 118.60, 118.37, 111.42, 80.87. MS (ESI) [M + H]⁺ calcd for C₃₄H₁₉N₂O₉S₄ 726.9943, found [M + H]⁺ 726.9924; M.P. 81 – 83 °C.

Fluorescence measurements

All of the measurements were carried out at 37 °C for 15 min in 50 mM PBS buffer (pH 7.4) containing 10% DMSO according to the following procedure: in a test tube, 3.5 mL of 50 mM PBS buffer (pH 7.4) and 380 µL DMSO were mixed. A portion of the stock solution (20 µL) of the probe was then added to the mixture. The resulting solution was well-mixed, followed by the addition of the requisite volume of testing species solution. The final

volume of the solution was adjusted to 4 mL with 50 mM PBS buffer (pH 7.4) containing 10% DMSO. After mixing and standing for 15 min at 37 °C, 3 mL of the solution was transferred into a 1 cm quartz cell and fluorescence signal was recorded.

Fluorescence imaging of HeLa cells

HeLa cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, Cellgro company) supplemented with 10% fetal bovineserum, 4 mM glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C and with 5% CO₂ for two days. One day before imaging, cells were transferred to 24-well plates. Before use, the adherent cells were washed once with FBS-free DMEM, then treated as described in Figure 6. Fluorescence imaging was performed after washing the cells with PBS buffer. All of the microscopy images were taken on a fluorescence microscope with excitation at 490 nm (green channel).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. (a) Hwang C, Sinskey AJ, Lodish HF. *Science*. 1992; 257:1496. [PubMed: 1523409] (b) Lipton SA, Choi YB, Takahashi H, Zhang DX, Li WZ, Godzik A, Bankston LA. *Trends Neurosci*. 2002; 25:474. [PubMed: 12183209] (c) Zhang SY, Ong CN, Shen HM. *Cancer Lett*. 2004; 208:143. [PubMed: 15142672]
2. (a) Herzenberg LA, De Rosa SC, Dubs JG, Roederer M, Anderson MT, Ela SW, Deresinski SC, Herzenberg LA. *Proc Natl Acad Sci U S A*. 1997; 94:1967. [PubMed: 9050888] (b) Townsend DM, Tew KD, Tapiero H. *Biomed Pharmacother*. 2003; 57:145. [PubMed: 12818476] (c) Nekrasova O, Lawrence NS, Compton RG. *Talanta*. 2003; 60:1085. [PubMed: 18969134]
3. For recent reviews on RSH detection, see: Chen X, Zhou Y, Peng X, Yoon J. *Chem Soc Rev*. 2010; 39:2120. [PubMed: 20502801] Shi W, Ma H. *Chem Commun*. 2012; 48:8732. Yang Y, Zhao Q, Feng W, Li F. *Chem Rev*. 2013; 113:192. [PubMed: 22702347] Santos-Figueroa LE, Moragues ME, Climent E, Agostini A, Martı́nez-Manez R, Sancenon F. *Chem Soc Rev*. 2013; 42:3489. [PubMed: 23400370] Li X, Gao X, Shi W, Ma H. *Chem Rev*. 2014; 114:590. [PubMed: 24024656] Yuan Y, Liang G. *Org Biomol Chem*. 2014; 12:865. [PubMed: 24336835]
4. (a) Guy J, Caron K, Dufresne S, Michnick SW, Skene WG, Keillor JW. *J Am Chem Soc*. 2007; 129:11969. [PubMed: 17850081] (b) Yi L, Li H, Sun L, Liu L, Zhang C, Xi Z. *Angew Chem Int Ed*. 2009; 48:4034. (c) Lu J, Sun C, Chen W, Ma H, Shi W, Li X. *Talanta*. 2011; 83:1050. [PubMed: 21147356] (d) Yang X, Guo Y, Strongin RM. *Angew Chem Int Ed*. 2011; 50:10690. (e) Jung HS, Chen X, Kim JS, Yoon J. *Chem Soc Rev*. 2013; 42:6019. [PubMed: 23689799] (f) Youziel J, Akhbar AR, Aziz Q, Smith MEB, Caddick S, Tinker A, Baker JR. *Org Biomol Chem*. 2014; 12:557. [PubMed: 24297212] (g) Yang XF, Huang Q, Zhong Y, Li Z, Li H, Lowry M, Escobedoc JO, Strongin RM. *Chem Sci*. 2014; 5:2177. [PubMed: 24995124]
5. Zhang D, Devarie-Baez NO, Li Q, Lancaster JR Jr, Xian M. *Org Lett*. 2012; 14:3396. [PubMed: 22681565]
6. (a) Urano Y, Kamiya M, Kanda K, Ueno T, Hirose K, Nagano T. *J Am Chem Soc*. 2005; 127:4888. [PubMed: 15796553] (b) Yuan L, Lin W, Xie Y, Chen B, Zhu S. *J Am Chem Soc*. 2012; 134:1305.

- [PubMed: 22148503] (c) Zheng H, Zhan X, Bian Q, Zhang X. Chem Commun. 2013; 49:429.(d) Wei C, Zhu Q, Liu W, Chen W, Xi Z, Yi L. Org Biomol Chem. 2014; 12:479. [PubMed: 24276473]
7. (a) Chen W, Liu C, Peng B, Zhao Y, Pacheco A, Xian M. Chem Sci. 2013; 4:2892. [PubMed: 23750317] (b) Peng B, Chen W, Liu C, Rosser EW, Pacheco A, Zhao Y, Aguilar HC, Xian M. Chem Eur J. 2014; 20:1010. [PubMed: 24339269] (c) Wang H, Zhou G, Gaiand H, Chen X. Chem Commun. 2012; 48:8341.(d) Zhang D, Chen W, Miao Z, Ye Y, Zhao Y, King SB, Xian M. Chem Commun. 2014; 50:4806.(e) Liu C, Pan J, Li S, Zhao Y, Wu LY, Berkman CE, Whorton AR, Xian M. Angew Chem Int Ed. 2011; 50:10327.
8. (a) Chen W, Li Z, Shi W, Ma H. Chem Commun. 2012; 48:2809.(b) Li Z, Li X, Gao X, Zhang Y, Shi W, Ma H. Anal Chem. 2013; 85:3926. [PubMed: 23506563]
9. Hong R, Han G, Fernandez JM, Kim BJ, Forbes NS, Rotello VM. J Am Chem Soc. 2006; 128:1078. [PubMed: 16433515]
10. Bornholdt J, Felding J, Clausen RP, Kristensen JL. Chem Eur J. 2010; 16:12474. [PubMed: 20839183]

Fig. 1.

Fluorescence enhancements (F/F_0) of probes (10 μ M) with RSH (100 μ M). The reactions were carried out for 15 min at 37 $^{\circ}$ C in PBS buffer (50 mM, pH 7.4) with 10% DMSO.

RSHP1: $\lambda_{\text{ex/em}}$ = 340/455 nm; **RSHP2:** $\lambda_{\text{ex/em}}$ = 482/516 nm; **RSHP3:** $\lambda_{\text{ex/em}}$ = 490/518 nm.

Fig.2. Time-dependent fluorescence enhancements of **RSHP3** (10 μ M) to RSH (100 μ M). Data were obtained in PBS buffer (50 mM, pH 7.4) with 10% DMSO at 37 $^{\circ}$ C ($\lambda_{\text{ex/em}}$ =490/518 nm).

Fig.3.
pH-dependent fluorescence intensity changes of **RSHP3** (10 μ M) in the presence of RSH (100 μ M).

Fig.4.

(A) Fluorescence enhancements of **RSHP3** (10 μM) to various RSS or common oxidants. (1) 100 μM GSH; (2) 100 μM Cys; (3) 100 μM Hcy; (4) 100 μM GSSG; (5) 100 μM Na₂S₂O₃; (6) 100 μM Na₂SO₃; (7) 100 μM NaNO₂; (8) 100 μM H₂O₂; (9) 100 μM NaClO; (10) 100 μM KO₂; (B) Competitive fluorescence intensity changes of **RSHP3** (10 μM) with GSH (100 μM) in the presence of various amino acids (100 μM). All the reactions were carried out for 15 min in PBS buffer (50 mM, pH = 7.4) with 10% DMSO at 37 °C ($\lambda_{\text{ex/em}}$ = 490/518 nm).

Fig.5.

(A) Fluorescence emission spectra of **RSHP3** (10 μM) with varied concentrations of GSH (0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50, 60, 70, 80, 100 μM for curves 1-15, respectively). The reactions were carried out for 15 min at 37 $^{\circ}\text{C}$ in PBS buffer (50 mM, pH = 7.4) with 10% DMSO. (B) Linear plot of **RSHP3** (10 μM) upon the addition of different amounts (0, 1, 3, 5, 7, 10, 15, 20, 30 μM) of GSH ($\lambda_{\text{ex/em}}$ = 490/518 nm).

Fig.6.

Fluorescence images of biothiols in HeLa cells. a) Cells were incubated with **RSHP3** (10 μ M) for 20 min. b) Cells were pre-incubated with 500 μ M NMM for 20 min, washed, and then treated with **RSHP3** (10 μ M) for 20 min. c) Cells were pre-incubated with 150 μ M Cys for 20 min, washed, and then treated with **RSHP3** (10 μ M) for 20 min. The second row (d-f) shows the corresponding bright-field image for the first row.

Scheme 1. The design of SBT-based probes for biothiols

Scheme 2. Preparation and model reaction of RSHP1

Table 1Fluorescence properties of **RSHP1–RSHP3**.

Probes	$\lambda_{\text{ex}}[\text{nm}]$	$\lambda_{\text{em}}[\text{nm}]/[a]$	$\Phi_f[b]$
RSHP1	340	455	0.022
RSHP2	480	516	0.034
RSHP3	490	518	0.076

[a] The maximal emission of the probes;

[b] The fluorescence quantum yields.