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Coumarin-based fluorescent probe for the detection of glutathione and nitroreductase

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ABSTRACT

With this research we set out to develop a coumarin-based novel fluorescent probe **NTR-AHC** for the detection of biological thiols and nitroreductase (NTR). Probe **NTR-AHC** was constructed by attaching the NTR trigger moiety (*p*-nitrobenzyl) and biothiol acceptor (maleic acid monoamide), to the core fluorophore **AHC**. In the presence of both glutathione (GSH) and NTR, probe **NTR-AHC** produced a >8-fold increase in fluorescence intensity at 463 nm.

1. Introduction

Biothiols play many crucial roles in intracellular antioxidant defense systems.[1] Glutathione (GSH), for example, the most prevalent intracellular non-protein thiol ranges in concentration from 1 to 15 mM depending on the cell type.[2-5] It is a natural tripeptide (γ-L-glutamyl-cysteinyl-glycine) that exists in the thiol reduced form (GSH) and disulphide-oxidised (GSSG) form.[6] GSH serves many cellular functions, including the protection of cellular components against oxidative stress, redox homeostasis, and intracellular signal transduction.[1,7-9] However, an imbalance of GSH is observed for a wide range of pathologies, including cancer, Parkinson's disease, HIV and aging.[10-12] Therefore, significant effort has been devoted towards the development of efficient fluorescent probes for the detection of GSH.[13-15] While, many of these fluorescent probes have focused on the detection of a single biomarker (GSH), many processes associated with GSH often involve more than one biochemical species. For example, while hypoxia is reported to decrease cellular GSH stores,[16] maternal hypoxia has been reported to facilitate fetal liver GSH production.[21] Hypoxia is caused by an insufficient supply of oxygen[9] and can result in increased reductive stress; a consequence of the upregulation of various reductive enzymes such as nitroreductase (NTR).[17] Indeed, upregulation of NTR has been associated with various health problems, including inflammatory disease, solid tumors, and stroke.[18-20] Hence, the development of methods that help explore the relationships between GSH and NTR are of great importance in terms of pathological analysis and early diagnostics.

As a useful analytical tool, fluorescence spectroscopy is of broad interest in bioanalysis.[22] Fluorescence-based small-molecule probes are able to selectively sense and image analytes through the utilization various chemical reactions, furthermore, fluorescent probes are often less invasive and more convenient than many other methods for the detection of biologically-relevant cellular targets.[23,24] Recently, a number of fluorescence-based probes for dual or multi-analyte detection have been developed.[25-27] These types of fluorescent probes have been used for the construction of molecular logic gates and/or for medical diagnostics.[28] AND logic-based fluorescent probes require both analytes to be simultaneously present or work in tandem in order to elicit a fluorescence response, and provide different information to that available from serial measurements of two analytes within the same biological system.

In our group, we have recently focused on developing dual analyte activated probes.[29-33] With this design principle in mind, we identified a suitable fluorophore based on coumarin —3-amino-7-hydroxy-2*H*-chromen-2-one (**AHC**). We anticipated that its free phenol and amine functionalities could provide an excellent opportunity for differential derivatization (Scheme 1).

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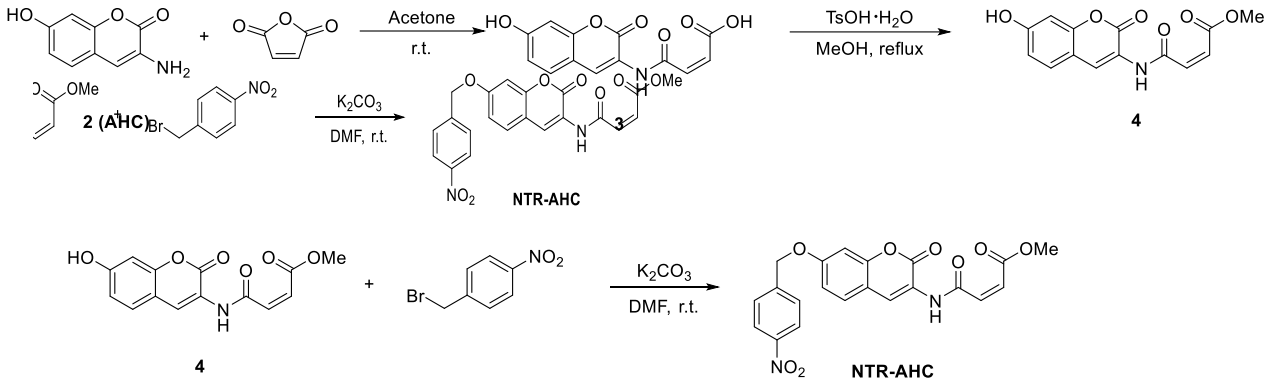
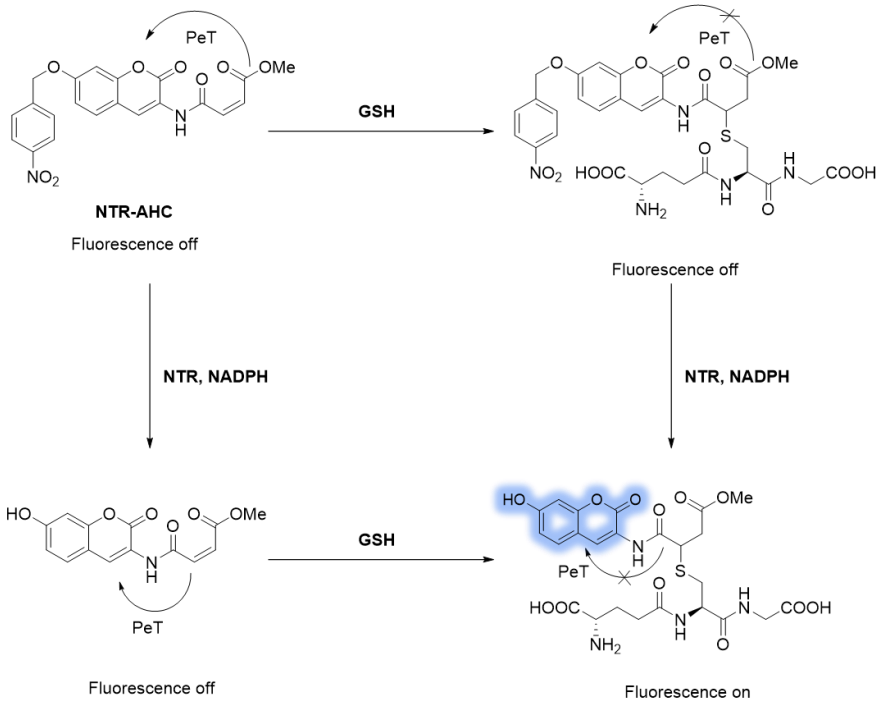
Furthermore, coumarins are an important structural element of various compounds with biological activities including compounds with anticancer,[34] antiproliferative,[35] antioxidant,[36] and antifungal activities.[37] Previous literature reports indicate that the protection of **AHC** with a maleic acid monoamide group results in fluorescence quenching of the coumarin's fluorescence due to a photoinduced electron transfer (PeT) process, the fluorescence is rapidly restored in the presence of biological thiols due to their fast addition to this functional group.[38] Therefore, we envisioned that functionalization of the free phenol of this probe using a *p*-nitrobenzyl should further quench the fluorescence, whilst serving as a selectivity reporter unit toward NTR over other various analytes.[39,40] Consequently, with this research we anticipated that the attachment of a NTR and GSH differentiating motif to **AHC** could produce a selective fluorescent probe **NTR-AHC** for the simultaneous detection of GSH and NTR (Scheme 2).



Scheme 1. Synthesis of target probe **NTR-AHC**.



Scheme 2. Reaction mechanism of **NTR-AHC** with GSH and nitroreducase (NTR) and NADPH.

2. Results and discussion

We evaluated the ability of **NTR-AHC** to detect GSH 'AND' NTR in PBS buffer solution (10 mM, pH 7.40, containing 1% DMSO). The maximum absorption of **NTR-AHC** at 344 nm shifted to 340 nm upon addition of GSH with a significant increase at 340 nm following the addition of NTR (Fig. S1). As shown in Fig. 1, probe **NTR-AHC** was initially non-fluorescent, and exhibited a small fluorescence increase upon addition of GSH (5 μM) (Fig. S2), however, the successive addition of NTR (10 μg mL^{-1}) and NADPH (400 μM) led to a significant and time dependent increase in fluorescence intensity (>8-fold). These results illustrated the requirement for both GSH 'AND' NTR to obtain a significant turn 'on' fluorescence response. We then evaluated the kinetic behavior of **NTR-AHC** with both GSH and NTR (Fig. 2 and 3) and determined that **NTR-AHC** exhibits a dose dependent fluorescence increase in response of both GSH and NTR.

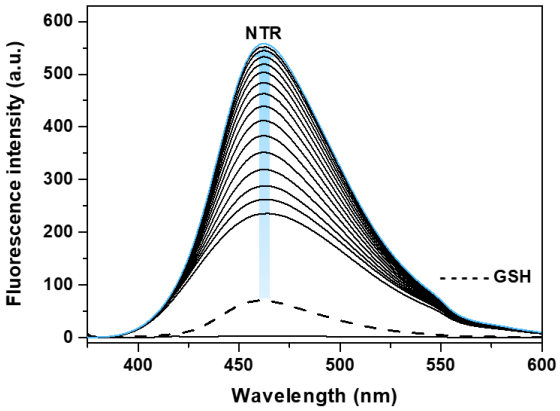


Fig. 1 Fluorescence spectra of **NTR-AHC** (10.0 μM) with initial addition of GSH (5 μM) incubated for 20 min, followed by the addition of NADPH (400 μM) and NTR (10 μg mL^{-1}) monitored for a further 90 minutes in PBS buffer solution (pH=7.4, 10 mM, containing 1% DMSO). λ_{ex} = 365 nm. Ex slit: 5 nm and em slit: 5 nm. Dashed line represents **NTR-AHC** and GSH addition only. Blue line represents highest intensity after addition of NTR.

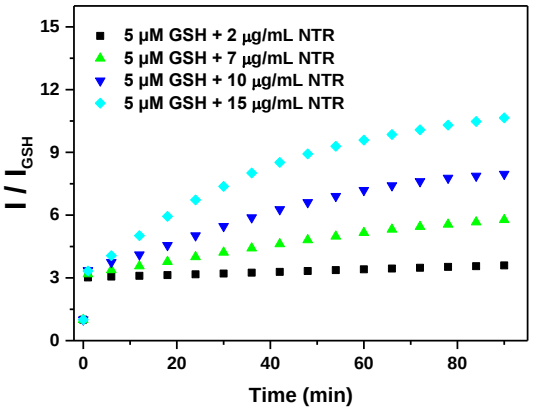


Fig. 2 Fluorescence intensity changes at 463 nm of **NTR-AHC** (10.0 μM) with initial addition of GSH (5.0 μM) incubated for 20 min, followed by the addition of NADPH (400 μM) and NTR (2.0 – 15.0 μg mL^{-1}) in PBS buffer (pH=7.4, 10 mM, containing 1% DMSO). λ_{ex} = 365 nm. Ex slit: 5 nm and em slit: 5 nm.

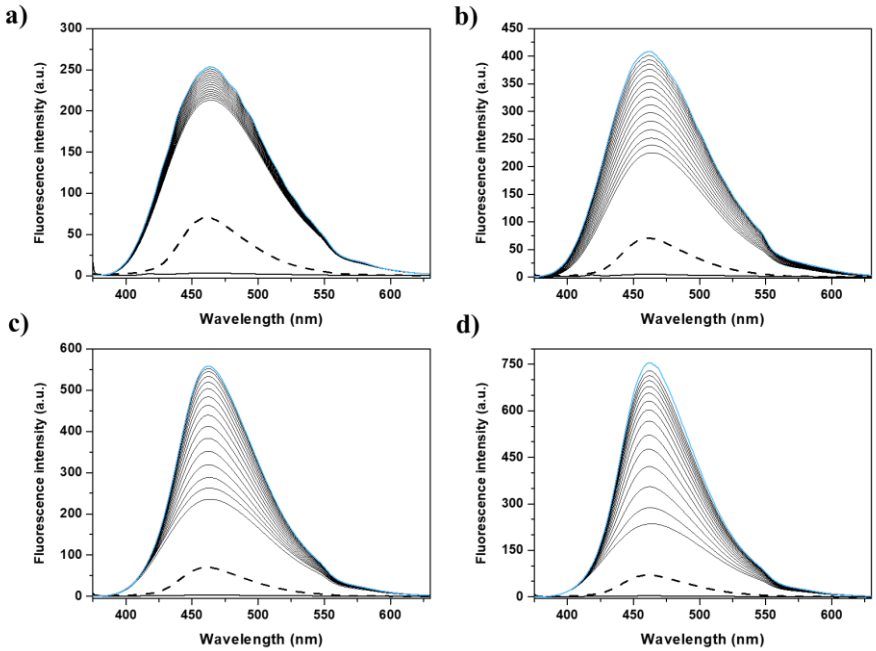


Fig. 3 Fluorescence spectra of **NTR-AHC** (10.0 μM) with initial addition of GSH (5.0 μM) incubated for 20 min (dashed line), followed by the addition of NADPH (400 μM) and NTR ((a) 2 μg mL^{-1}, (b) 7 μg mL^{-1}, (c) 10 μg mL^{-1}, (d) 15 μg mL^{-1}) monitored for a further 90 minutes in PBS buffer (pH=7.4, 10 mM, containing 1% DMSO). λ_{ex} = 365 nm. Ex slit: 5 nm and em slit: 5 nm.

To ensure both analytes were required, the same fluorescence experiments were then carried out in reverse order, NTR and NADPH was kept constant (10 μg mL^{-1} and 400 μM, respectively) resulting in a large fluorescence increase (Fig. 4), attributed to the background fluorescence of NADPH.[41] Subsequent addition of GSH (200 μM) led to a 1.2-fold increase in fluorescence intensity.

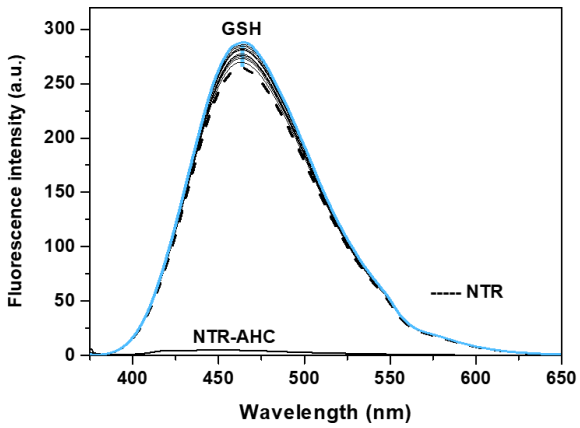


Fig. 4 Fluorescence spectra of **NTR-AHC** (10.0 μM) with initial addition of NADPH (400 μM) and NTR (10 μg mL^{-1}) incubated for 90 min, followed by the addition of GSH (200 μM) monitored for a further 40 minutes in PBS buffer (pH=7.4, 10 mM, containing 1% DMSO). λ_{ex} = 365 nm. Ex slit: 5 nm and em slit: 5 nm. Dashed line represents **NTR-AHC** and NTR addition. Blue line represents highest intensity after addition of GSH.

3. Conclusions

We have developed a novel coumarin-based fluorescent probe **NTR-AHC** for the detection of biological thiols and NTR, respectively, however, an optimum response was only obtained if GSH was added first followed by NADPH and NTR, while, the reverse sequence does not show significant fluorescence changes, which we attribute to a large initial increase to the background fluorescence by NADPH and potential oxidation of the GSH by $NADP^+$ [42,43] generated during the NTR/NADH catalyzed reduction of **NTR-AHC**.[44] In summary, **AHC** is a useful core unit for the development of coumarin-based dual-analyte 'AND' logic fluorescent probes.

4. Experimental section

4.1. Chemicals and reagents

All starting materials and reagents were purchased from Sigma Aldrich, Alfa Aesar, Fluorochem, or Acros Organics, and used as received without any further purification. Unless otherwise stated, all solvents used were reagent grade and were used without distillation. All water was deionized which is generated by a reverse osmosis (Ultra-Purified Type I, 18.2 Megohm water). Thin-layer chromatography was performed using commercially available Fluorochem aluminum-backed plates coated with a layer of silica gel (60 Å) with fluorescent indicator UV254. These plates were visualized using ultraviolet light with a wavelength of either 254 or 365 nm. Silica gel column chromatography was carried out using Sigma Aldrich 60 Å silica gel (200-400 mesh). All NMR spectra were obtained using an Agilent ProPulse 500 and analyzed using MestreNova. LC-MS analyses were performed using an Agilent QTOF 6545 mass spectrometer, IR spectra were obtained using a Thermo Fisher Nicolet iS5 FTIR spectrometer and analyzed using OMNIC (version 9.7.7).

*4.2. Synthesis of **NTR-AHC***

Compounds **1-4** were synthesized using adapted literature procedures (Scheme S1).[45,38,29] Compound **4** (300 mg, 1.04 mmol) was dissolved in dry DMF (5 mL) and K_2CO_3 (215 mg, 3.15 mmol) was added with stirring. 4-nitrobenzyl bromide (249 mg, 1.157 mmol) was then added and the reaction was stirred for four hours at room temperature under a N_2 atmosphere. The reaction mixture was diluted with EtOAc (90 mL) and washed with brine (90 mL × 3), dried over anhydrous Na_2SO_4 and concentrated in *vacuo*. The crude product obtained was purified by flash chromatography (SiO_2, 30% EtOAc in petroleum ether) to afford the desired yellow solid **NTR-AHC** (198 mg, 48%) (Scheme 1). M.p. 127 – 129 ºC; ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 10.12 (s, 1H), 8.63 (s, 1H), 8.27 (d, J = 8.5 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H), 7.71(d, J = 8.6 Hz, 1H), 7.11 (s, 1H), 7.07 (d, J = 8.6 Hz, 1H), 6.73 (d, J = 11.8 Hz, 1H), 6.51 (d, J = 11.7 Hz, 1H), 5.39 (s, 2H), 3.69 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ_C 167.1, 163.3, 159.5, 157.5, 151.4, 147.1, 144.2, 131.1, 129.4, 129.2, 128.4, 125.7, 123.6, 121.7, 113.4, 113.1, 101.6, 68.6, 51.6. FTIR (ATR, cm^{-1}): 3309 (N-H), 2955 (C-H, alkyl), 1721 (C=O), 1708 (C=O), 1605 (C=C), 1247 (C-N). HRMS (ESI^+): calculated $[M+Na]^+$ 447.0799 *m/z*, found 447.0798 *m/z*, M represents $C_{21}H_{16}N_2O_8$ (chemical formula of **NTR-AHC**).

4.3. Equipment and spectral measurements

All Fluorescence emission spectra were recorded on a Jasco FP-6300 spectrofluorometer. Excitation and emission slit widths were both set at 5.0 nm. The UV–visible absorption spectra were measured at room temperature on a Jasco V-630-Bio Spectrometer. Nuclear Magnetic Resonance (NMR) spectroscopy experiments were performed in deuterated solvent at 298 K on an Agilent ProPulse 500 MHz spectrometer.

4.4. Preparation of samples for analysis

NTR-AHC was dissolved in DMSO and a stock solution (0.1 mM) prepared in PBS buffer (pH =7.4). NTR stock solutions were prepared by dissolving 1 mg of enzyme in 1 mL of PBS buffer solution and individual ampules were stored in the freezer before being thawed and used in each experiment. Stock solutions of NADPH (2 mM) and GSH (0.1 mM) were prepared in PBS buffer (pH =7.4).

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