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Preparation of a conjugation-ready thiol responsive molecular switch

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ABSTRACT

In this work we synthesize molecular switches that are responsive to cysteine, homocysteine, and glutathione; three redox systems that make up the majority of the body's antioxidant defenses. Synthesized spiropyran isomers with conjugation-ready linkages showed good selectivity of response to these major antioxidant thiols over nucleophilic amino acids; however the position of the linking group can affect selectivity and reversibility of the switching response. An isomer with selectivity for cysteine against GSH and Hcy was identified.

Introduction and background of thiols

Oxidative stress has been shown to precede and contribute to the progression of diseases including cancer, cardiovascular disease, ulcerative colitis, and neurodegenerative disorders.[1,2] Under normal physiological conditions, the production of reactive oxygen species (ROS) is important for a host of cellular processes including cell growth and differentiation. Sustained high levels of ROS, however, can lead to deleterious cellular events such as lipid peroxidation, protein and DNA modification, and eventually impaired cellular function or apoptosis. In a healthy individual the production of ROS is highly regulated by a number of antioxidant systems. Imbalances in these important antioxidant systems can leave the body vulnerable to oxidative stress which can lead to development of disease.[1]

Intracellular thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are important antioxidants that scavenge ROS. Antioxidants are important interceptors of ROS and alterations in antioxidant levels that accompany oxidative stress could serve as a biomarker for disease, or as an endpoint for monitoring therapeutic efficacy.[3] Several studies have illustrated that local administration of antioxidants can provide neuronal protection in patients with traumatic brain injury, prevent bone loss related to osteoporosis, and improve the immune response of aged mice.[4–6] Intracellular thiol concentrations are dominated by glutathione, with concentrations ranging from 1 to 10 mM. Glutathione is also the major thiol antioxidant extracellularly with concentrations ranging from 0.5 to 1 mM. At 0.1 mM cysteine concentrations are lower than glutathione but still ten to twenty fold greater than homocysteine concentrations. Glutathione, cysteine, and homocysteine are important antioxidants for cellular defense and the depletion of these antioxidant thiol levels has been linked to a number of diseases.[7–14] For example, patients with Parkinson's disease had 30% lower GSH concentrations in the rostral and caudal regions of the brain than neurologically healthy control patients.[15] The ability to monitor thiol levels in tissues could provide a useful marker for understanding disease pathogenesis. Most methods for thiol quantification require destructive analysis of tissues.[16] Alternatively, noninvasive imaging modalities capable of identifying regions of oxidative stress and depleted antioxidant defenses would be highly desirable.

Current methods for in vivo quantification of thiols and other antioxidants include magnetic resonance spectroscopy (MRS) and electron paramagnetic resonance (EPR). However, both suffer from poor signal to noise ratios, require analyte concentrations in the high millimolar range, and have low resolution (cm^3), limiting these techniques to more advanced diseases with larger volumes.[17] A clear need exists for better non-invasive sensors of this important thiol redox system and its role in disease progression.

Magnetic Resonance Imaging (MRI) is an excellent method for visualizing antioxidant levels in vivo because of its ability to non-invasively obtain high resolution ($0.5\ mm^3$) anatomical images.[18] However, MRI requires a biosensor to translate antioxidant levels into an MR signal. We have previously shown that spiropyran switches can be converted to redox active imaging

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agents through conjugation to a gadolinium chelate MRI contrast agent. In the presence of NADH in cells these probes produced contrast enhancement greater than 20%, opening up an attractive new class of imaging agents capable of characterizing the concentrations of redox active species in vivo.[19,20] In this work we address the issue of developing spiropyran molecular switches that demonstrate thiol response with a functional handle for future use with MRI contrast agents.

Li and co-workers have demonstrated that glutathione responsiveness requires two nitro groups on the chromene ring of a spiropyran switch,[21] and in our previous work the linker to the gadolinium chelate occupies one of these positions. In this study we investigated two other positions for functional modification of spiropyrans and characterize their effect on switching by determining the response, selectivity, and reversibility toward thiol sensing of these spiropyrans. We found that adding functionality to the indoline portion of a dinitro spiropyran had little effect on the thiol response of these spiropyrans. This work demonstrates thiol responsive spiropyran isomers ready for conjugation and overcomes the difficult task of adding functionality without significantly altering thiol response.[21,22]

Results and discussion

Spiropyrans are a well known class of molecular switching compounds that can be converted between the closed spiropyran form (SP) or the open merocyanine form (MC) in response to external stimuli (Scheme 2).[23] In this work we synthesized switches for antioxidant sensing, the presence of thiols induces ring opening; this can be reverted back to the closed form using visible light. This conversion can be easily observed by UV/Vis spectroscopy with the MC form absorbing strongly in the visible region while the SP form absorbs strongly in the UV region. Measurement of the MC absorbance peak was performed to monitor switching from SP to MC form induced by response to various amino acids.

Synthesis of spiropyrans **5** and **6** proceeded from the commercially available 3-aminobenzyl alcohol **2**, which was oxidized to the hydrazine under Sandmeyer conditions. Utilizing continuous extraction over 24 h provided 50% mass recovery, and the crude aryl hydrazine underwent Fischer indole synthesis to generate indoles **3** and **4** as a mixture. The overall yield of both indole isomers was 32% over two steps, a significant improvement on previously reported yields over the same two steps.[24] Indoles **3** and **4** were methylated with iodomethane and the crude enamines were subjected to condensation with 2-hydroxy-3,5-dinitrobenzaldehyde in refluxing ethanol (24 h, 80 °C) to yield spiropyrans **5** and **6** (Scheme 1) in four steps with 2.5% and 1.5% overall yield. Spiropryan **1** (Fig. 1) was prepared as reported by Buback and co-workers[25] (Supporting information).

Spiropyran **1**, featuring two nitro groups in the 6- and 8-positions of the chromene moiety, was reported in the literature to respond to primary thiols such as Cys, Hcy, and GSH, however, no switching to the MC form was reported in the presence GSSG.[21] We used this spiropyran as reference to test for positional effects of the introduction of linkers. Hydroxyl groups, which can readily be converted to the alkyne for conjugation to azide species, were placed in different positions on the indole ring of spiropyran **1** to form spiropyrans **5** and **6**. Spiropyrans **5** and **6** were successfully synthesized and structure identified by accurate mass spectroscopy, proton and carbon NMR spectroscopy (Supporting information).

Following the synthesis, switching ability of spiropyrans **1**, **5**, and **6** in response to Cys, Hcy, GSH and GSSG was investigated by UV/Vis spectroscopy. Representative data from single samples of spiropyrans **1**, **5**, and **6** are shown in Figure 2 to illustrate the relative responses for these spiropyrans in the presence of equimolar amounts of Cys, Hcy, GSH, and GSSG. All three spiropyrans exhibit a response to the sulfhydryl-containing amino acids as illustrated by the strong absorbance increase centered around 500 nm. The disulfide GSSG was used as a control and the lack of response to GSSG supported the necessity of the sulfhydryl moiety for ring opening. Similar to previous literature reports, spiropyran **1** displays a similar response for all three thiols with cysteine and GSH perfectly overlapping, indicating the same extent of switching response for these two primary thiols (0.551 AU, 0.548 AU, at λ_{MC} respectively). The response to homocysteine was slightly lower (0.513 AU at λ_{MC}) and minimal response to GSSG (0.060 AU at λ_{MC}) was observed; these results recapitulate literature observations (Fig. 2A).[21] Spiropyrans **5** and **6** show much greater variability between the three primary thiols. For all three isomers Cys produced the greatest changes in absorbance, resulting in final absorbances of 0.51 AU, 0.71 AU and 0.75 AU at the peak absorbance wavelength of the MC form (λ_{MC} for spiropyrans **1**, **5**, and **6** respectively). Repeating these assays demonstrated that minor fluctuations in absorbance profile in response to analytes could be expected, resulting in no significant selectivity for thiols with spiropyran **6**. The aggregate data is discussed further in the next section. There is also a markedly higher baseline absorbance for spiropyran **5** after 5 min of visible light irradiation compared to the other two spiropyrans, **1** and **6**, suggesting that spiropyran **5** has a greater population of the MC form at equilibrium compared to spiropyrans **1** and **6** (Fig. 2B). Longer visible light irradiation times were tested in an attempt to drive conversion to the closed form, but after 20 min of visible light irradiation this high baseline still persisted (SI, Fig. S1).

Selectivity

Building upon this initial characterization of thiol responsiveness, spiropyrans **1**, **5**, and **6** were evaluated for their thiol selectivity by incubating with GSH, Cys, Hcy, and other nucleophilic amino acids. As expected, all three spiropyran solutions shifted to an orange color in the presence of Cys, GSH, and Hcy indicating a ring opening to the MC form, which is supported by the appearance of a strong absorbance centered around 500 nm (λ_{MC}) (Fig. 3A). Figure 3 represents the ratio of $(A - A_0)/A_0$ for the λ_{MC} for each isomer respectively. Based on these ratios all three isomers showed selectivity for thiols over GSSG and other nucleophilic amino acids without primary thiols (p <0.01, Fig. 3). Spiropyrans **1** and **6** demonstrated poor selectivity among the three primary thiols GSH, Hcy, and Cys (Fig. 3). In contrast, spiropyran **5** exhibits the greatest response to cysteine at the λ_{MC} with a 5.29 fold increase from the baseline prior to incubation with thiol and reduced responsiveness to GSH (4.08 fold, p <0.05), further reduced response to Hcy (3.82 fold, p <0.01) indicating some level of selectivity over primary thiol (Fig. 3B). Like spiropyran **1**, spiropyran **5** had no response to amino acids lacking a primary thiol (Fig. 3B). The addition of the hydroxyl in this position seems to afford some degree of cysteine selectivity over the other sulfhydryl containing amino acids (GSH p <0.05, Hcy p <0.01).

While the values of $A - A_0/A_0$ appear to be smaller for Spiropyran **5**, it is important to note that this ratio may not be reflective of the response of the switch. While final absorbance values between spiropyrans **5** and **6** were similar in magnitude after incubation with Cys (Student t test, $p = 0.1727$) the higher baseline of spiropyran **5** reduced the $A - A_0/A_0$ values. The position of the linker placed on spiropyran **6** had little effect on selectivity, and spiropyran **6** behaved similarly to spiropyran **1** (Fig. 3C).