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Rational Design of a Red Fluorescent Sensor for ALDH1A1 Displaying Enhanced Cellular Uptake and Reactivity

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Abstract

High aldehyde dehydrogenase 1A1 (ALDH1A1) activity has emerged as a reliable marker for the identification of both normal and cancer stem cells. To facilitate the detection, molecular imaging, and sorting of stem cells, a green fluorescent probe based on the xanthene dye scaffold was recently developed. However, green dyes are less amenable to multicolor imaging because most commercial reagents are also green. Overcoming this limitation will enable the simultaneous tracking of multiple stem cell markers. Herein, we report the development of a red congener, red-AIDeSense. Through chemical tuning we were able to achieve excellent isoform selectivity and chemostability, a good turn on response, and enhanced cellular uptake and reactivity. Importantly, red-AIDeSense represents one of only a few turn-on sensors in the red region that use the d-PeT quenching mechanism. By employing red-AIDeSense and a green anti-CD44 antibody, we were able to demonstrate staining of these two stem cell markers is independent of one another in A549 lung adenocarcinoma cells.

Graphical Abstract

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Author Contributions

All authors have given approval to the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](https://doi.org/10.1021/acs.bioconjugchem.9b00723) at DOI: 10.1021/acs.bioconjugchem.9b00723. Experimental procedures, additional figures, syntheses, compound characterization, and spectral data (PDF)

The authors declare the following competing financial interest(s): T.E.B. and J.C. hold a patent for AIDeSense and are in the process of licensing the technology.

Safety statement: No unexpected or unusually high safety hazards were encountered.

Along with other members of the aldehyde dehydrogenase (ALDH) family (19 in total), ALDH1A1 is an important cytosolic enzyme that serves to detoxify endogenous and xenobiotic aldehydes through oxidation to their corresponding carboxylic acid products.¹ Although the precise reasons are not well understood, ALDH1A1 is overexpressed in many normal and cancer stem cell types, where it is used as a well-established stem cell marker.² Patient sample analyses using immunohistochemistry and PCR-based methods have revealed that ALDH1A1 levels are commonly elevated in breast,³ lung,^{4,5} ovarian,⁶ and prostate cancer,⁷ as well as in leukemia⁸ and lymphoma.⁹ These results often correlate with poor prognosis and patient survival.

Noninvasive detection of ALDH1A1 in live samples, as opposed to the destructive approaches mentioned above, can enable real-time monitoring and longitudinal tracking of stem cell properties. We recently reported the development of AIDeSense, an activity-based sensor that permitted the first studies of stem cell plasticity (via ALDH1A1 activity) in tumorsphere and animal models (Figure 1).¹⁰ Owing to donor-photoinduced electron transfer (d-PeT) quenching from the benzaldehyde substrate, this sensor is weakly fluorescent prior to activation. ALDH1A1-catalyzed oxidation to the carboxylic acid product is accompanied by a robust fluorescence turn-on response. Despite the major advance this approach represents, we now seek to improve two properties to broaden its general utility. First, AIDeSense is not cell permeable unless it is chemically modified with capping groups (i.e., acetoxymethyl ether) to mask the intrinsic negative charge character on the phenolic alcohol ($pK_a = 4.81$). Consequently, intracellular esterases are required for full activation (Figure 1). This process generates byproducts, namely acetate and formaldehyde, which are released upon uncapping. Second, the absorbance and emission profiles of AIDeSense overlaps with that of FITC and GFP, small-molecule and protein handles, respectively, that are commonly used to visualize biological processes via molecular imaging.

In this work, we developed red-AIDeSense, a cell-permeable, red-shifted activity-based sensor for ALDH1A1 based on the TokyoMagenta dye platform (Figure 1).¹¹ Chemical tuning of the substituents on the pendent aryl ring was crucial to maintain excellent isoform selectivity while achieving a good turn-on response upon enzyme-mediated oxidation. To account for nonspecific staining, we designed a nonresponsive control reagent (Ctrl-red-AIDeSense). This tool was used in tandem with red-AIDeSense to identify A549 lung

adenocarcinoma cells exhibiting the highest ALDH1A1 activity via flow cytometry and confocal microscopy. Multicolor imaging of red-AIDeSense with a FITC-labeled anti-CD44 antibody revealed independent staining for ALDH1A1 activity and the non-small cell lung cancer stem cell marker.^{12–14}

We initially proposed to develop a sensor with the requisite properties by simply substituting the endocyclic oxygen with a dimethylsilicon group. Past reports indicated that this modification results in shifts of up to ~100 nm for both excitation and emission maxima.^{11,15–17} However, we found that the resultant sensor (Probe 1) was no longer selective for ALDH1A1 and that it exhibited an insufficiently small 1.7-fold turn-on response (Figure 2). Its relatively large quantum yield (0.32) indicates d-PeT quenching from the benzaldehyde substrate was no longer sufficient. This hypothesis is further supported by the analysis with the Rehm–Weller eq (eq 1).¹⁸

$$\Delta G_{\text{et}} = E(D^+/D) - E(A/A^-) - \Delta E_{00} + w_p \quad (1)$$

The term E_{00} describes the energy difference between the lowest vibrational energy levels of the ground and first electronic energy states. E_{00} can be estimated by the intersecting wavelength of the normalized absorbance and emission profiles. Specifically, AIDeSense has $E_{00} = 2.46$ eV at 503 nm, while TokyoMagenta dyes have $E_{00} \sim 2.07$ eV at 600 nm. Given the ~0.4 eV difference, we hypothesized we could achieve a greater dynamic range by reducing the electron density of the pendant aryl ring. We first explored the effects of introducing a single electron deficient substituent (fluoro, trifluoromethyl, and nitro) by replacing the methyl group at the R1 position. While none of these modifications led to the desired properties, each congener provided useful insights for further chemical tuning. For instance, we found that the fluoro substituent (Probe 2) did not provide sufficient steric bulk to prevent nucleophilic attack (e.g., by water) on the xanthene core which resulted in rapid decomposition of the fluorophore.¹⁹ In contrast, modification with a trifluoromethyl group (Probe 3) displayed slow enzyme kinetics, suggesting that it was too large and therefore not able to effectively bind to the ALDH1A1 active site. Lastly, the nitro moiety was too strong of a d-PeT quencher, as we could not determine by fluorescence kinetics assays whether Probe 4 was not turning over or if its carboxylate product was equally quenched. We then turned our attention to tuning the LUMO energy levels of Probes 1 and 2 by installing fluorines at the R3 and R4 positions, respectively. Relative to the 1.7-fold turn on that was observed for Probe 1, activation of Probe 5 with ALDH1A1 resulted in a 2.6-fold fluorescent enhancement. In contrast to the instability of Probe 2, the second fluoro group was sufficient to prevent water-mediated decomposition of Probe 6. Although this sensor displayed the largest response to ALDH1A1 in the series (3.9-fold), it exhibited cross-reactivity with ALDH1A3. Drawing from our findings, we reasoned we would be able to maintain isoform selectivity and achieve a sufficient turn-on response if we installed an additional fluoro group to Probe 5 at the position R4. The resultant sensor, Probe 7 (named red-AIDeSense), was selective for ALDH1A1 over ALDH1A3 and displayed a 3.2-fold turn-on response when treated with ALDH1A1. Finally, we prepared the constitutional isomer of red-AIDeSense (Probe 8) to probe the impact of different fluoro substitution

patterns. We found the ALDH1A1-to-ALDH1A3 selectivity was eroded when the fluoro group at R3 was moved to the R2 position.

Synthesis of red-AIDeSense began with Fisher esterification of 4-bromo-2,3-difluorobenzoic acid followed by iodination with *N*-iodosuccinimide to afford methyl 4-bromo-2,3-difluoro-5-iodobenzoate **2** in 71.7% yield. Suzuki coupling with methylboronic acid chemoselectively substituted the iodine with a methyl group to yield compound **3** in 61.0% yield. DIBAL-H reduction of the ester and subsequent TBS protection of the resultant benzyl alcohol afforded the desired aryl bromide **5** in 83.3% yield. One pot lithium-halogen exchange with *t*-BuLi, coupling with the TBS-protected Si-xanthone **6**, and global deprotection afforded the penultimate precursor **7** in 64.8% yield. Finally, red-AIDeSense was synthesized by oxidation of the benzyl alcohol with IBX in 45.1% yield (Figure 3a).

The maximum absorbance (λ_{abs}) and emission (λ_{em}) wavelengths of red-AIDeSense were centered at 594 and 614 nm, respectively (Figure 3b). Of note, λ_{abs} aligns perfectly with the HeNe-594 nm laser line, which is common in many instruments. Compared to AIDeSense ($k_{\text{cat}}/K_{\text{m}} = 3.46 \mu\text{M}^{-1} \text{min}^{-1}$), our new sensor responded significantly faster under identical conditions with a measured $k_{\text{cat}}/K_{\text{m}}$ of $7.91 \mu\text{M}^{-1} \text{min}^{-1}$ (Figure 3c). In addition to determining the ALDH1A1-to-ALDH1A3 selectivity ratio, we also tested against ALDH1A2, ALDH2, ALDH3A1, ALDH4A1, and ALDH5A1. In each instance, we found no cross-reactivity (Figure 3d). We also examined the chemostability of red-AIDeSense across a panel of reactive oxygen, nitrogen, and sulfur species and found only ALDH1A1 elicited turnover of the sensor (Figure 3e).

Before proceeding to live cell imaging, we synthesized a control dye (Ctrl-red-AIDeSense). The difference between the two dyes is the replacement of the aldehyde group with a methyl ketone moiety. Since ALDH1A1 relies on hydride transfer to an NAD^+ cofactor for substrate oxidation, this modification renders Ctrl-red-AIDeSense chemically inert to ALDH1A1. On the other hand, this small change leaves the physical properties nearly identical such that the cell permeability should be the same. Therefore, staining with Ctrl-red-AIDeSense can be used to account for background fluorescence from nonspecific staining. Finally, we established that both red-AIDeSense and Ctrl-red-AIDeSense are nontoxic to K562 cells using the trypan blue assay (Figure S1).

We then turned our attention to determining whether red-AIDeSense could be used to identify A549 lung adenocarcinoma cells with high ALDH1A1 activity via flow cytometry. Cells were first stained with the nonresponsive control compound (Ctrl-red-AIDeSense) to establish gating conditions for background fluorescence (Figure 4a). Using red-AIDeSense, we determined that $78.1 \pm 0.3\%$ of A549 cells displayed high ALDH1A1 activity (Figure 4b). We were able to decrease the extent of red-AIDeSense activation to $67.8 \pm 3.4\%$ by applying 4-diethylaminobenzaldehyde (DEAB), a reversible competitive inhibitor of ALDHs (Figure 4c). Compared to A549 cells, K562 chronic myeloid leukemia cells have lower transcriptional levels of ALDH1A1.²⁰ As such, we evaluated whether this would translate into differences in ALDH1A1 activity. Indeed, only $39.0 \pm 8.3\%$ of K562 cells had high ALDH1A1 activity which correlates with transcriptome data. Moreover, DEAB treatment was also able to decrease sensor activation (Figure 4d). Consistent with flow

cytometry analysis, 77.6% of red-AIDeSense stained A549 cells were determined to have high ALDH1A1 activity using confocal microscopy (Figure 4e).

Lastly, we sought to highlight the multicolor imaging capabilities of the new sensor using a green FITC-labeled anti-CD44 antibody. The surface biomarker expression profiles of cancer stem cells sometimes correlate with and other times are independent of ALDH1A1.^{4,10} Thus, we wanted to investigate whether A549 cells with the highest ALDH1A1 activity would correlate with those with the greatest extent of CD44 staining. Using confocal imaging, we found that most cells were CD44⁺ as evidenced by a clear cell membrane staining pattern. However, only a subset of these cells were highly fluorescent on the red-AIDeSense channel (Figure 4f). This suggests CD44 and ALDH1A1 are independent markers (Figure S2).

In summary, we have developed a red fluorescent sensor that is selective for ALDH1A1. By utilizing a benzaldehyde-based substrate design we were able to exclude cross-reactivity with a majority of the ALDHs (e.g., ALDH3A1) that feature a narrow channel leading to the active site. Moreover, we were able to achieve selectivity over the 1A2 and 1A3 isoforms through strategic placement of fluoro substituents on the pendant aryl ring that presumably forms crucial hydrophobic contacts with the enzyme. To our knowledge, this is the first example in which a rational design approach was employed to develop a red fluorescent sensor based on the d-PeT mechanism. The major advantage of red-AIDeSense is its cell permeability, which can be attributed to the pK_a value of the phenolic alcohol (6.68). As such, the sensor requires only the action of ALDH1A1 for both accumulation and fluorescence turn on. Moreover, we observed 2.3-fold higher catalytic efficiency (k_{cat}/K_m) for the new sensor. The change to Si-xanthene and modification of the pendant aryl ring substituents resulted in a 100 nm bathochromic shift in absorbance and emission compared to our first-generation sensor (AIDeSense). Bathochromic shifting allowed for multicolor imaging using a green FITC-labeled anti-CD44 antibody. From these studies we showed independent staining for ALDH1A1 activity and CD44 levels in A549 lung adenocarcinoma cells. We believe red-AIDeSense will emerge as a useful tool for studying cells expressing high ALDH1A1 activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Previous Work:

This Work:

Figure 1.

Comparison of the enzymatic requirements for accumulation and fluorescent turn on of AlDeSense AM and red-AlDeSense.

#	Substituents				Quantum	Enzymatic	Enzyme Activity	
	R1	R2	R3	R4	Yield	Turn On	1A1	1A3
1	CH ₃	H	H	H	0.32	1.7	High	Medium
2	F	H	H	H	n.d.	n.d.	n.d.	n.d.
3	CF ₃	H	H	H	0.25	2.7	Medium	Low
4	NO ₂	H	H	H	0.00	n.d.	n.d.	n.d.
5	CH ₃	H	F	H	0.21	2.6	High	Low
6	F	H	H	F	0.13	3.9	High	High
7	CH ₃	H	F	F	0.21	3.2	High	Low
8	CH ₃	F	H	F	0.20	3.1	High	Medium

Figure 2.
Structure and selected properties of Probes 1–8. n.d. = not determined.

Figure 3.

(a) Synthetic scheme for red-AIDeSense. (b) Normalized absorption and emission spectra (upon excitation at 594 nm) of red-AIDeSense. Normalized fluorescence turn on of red-AIDeSense by (c) ALDH1A1, (d) multiple ALDH isoforms, and (e) multiple biologically relevant, reactive species. All assays were performed in 50 mM triethanolamine buffer red-AIDeSense at 2 μM . Enzyme concentrations affording 1 nmol substrate/min were used. Reactive species were tested at 100 μM , except glutathione which was at 1 mM. 5-MT = 5-methoxytryptamine.

Figure 4. Representative plots of the flow cytometry analysis of A549 cells stained for 10 min at 0 °C with (a) Ctrl-red-AIDeSense, (b) red-AIDeSense, and (c) red-AIDeSense plus 20 μ M DEAB. (d) Quantified data of each assay performed in triplicate with A549 and K562 cells. (e) Histogram of the mean fluorescence of cells stained for 10 min at room temperature with Ctrl-red-AIDeSense or red-AIDeSense. (f) Representative confocal images of A549 cells costained with red-AIDeSense (red) and a FITC-labeled anti-CD44 antibody (CD44, green). All assays were performed using 1 μ M Ctrl-red-AIDeSense or red-AIDeSense.