Environmentally Robust Rhodamine Reporters for Probe-based Cellular Detection of the Cancer-linked Oxidoreductase hNQO1

Quinn A. Best, Amanda E. Johnson, Bijeta Prasai, Alexandra Rouillere, and Robin L. McCarley*

Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803-1804, United States

ABSTRACT: We successfully synthesized a fluorescent probe capable of detecting the cancer-associated NAD(P)H:quinoneoxidoreductase isozyme-1 within human cells, based on results from an investigation of the stability of various rhodamines and seminaphthorhodamines toward the biological reductant NADH, present at ~100–200 μM within cells. While rhodamines are generally known for their chemical stability, we observe that NADH causes significant and sometimes rapid modification of numerous rhodamine analogues, including those often times used in imaging applications. Results from mechanistic studies lead us to rule out a radical-based reduction pathway, suggesting rhodamine reduction by NADH proceeds by a hydride transfer process to yield the reduced leuco form of the rhodamine and oxidized NAD+. A relationship between the structural features of the rhodamines and their reactivity with NADH is observed. Rhodamines with increased alkylation on the N3- and N6-nitrogens, as well as the xanthene core, react the least with NADH; whereas, non-alkylated variants or analogues with electron-withdrawing substituents have the fastest rates of reaction. These outcomes allowed us to judiciously construct a seminaphthorhodamine-based, turn-on fluorescent probe that is capable of selectively detecting the cancer-associated, NADH-dependent enzyme NAD(P)H:quinoneoxidoreductase isozyme-1 in human cancer cells, without the issue of NADH-induced deactivation of the seminaphthorhodamine reporter.

Fluorescent molecules are widely used in the study of biological systems. The characteristics of the light emitted by these molecules has allowed researchers to visualize the intricate structural features of tissue, individual cells, and their cellular compartments upon their “staining” by fluorescent species. More recently, fluorophores have been given specific modifications that endow them with the ability to probe biological systems so as to report on the presence, activity, and concentration of various target analytes and environmental features, e.g., polarity and charge.

Although fluorescence-based techniques are routinely used by researchers, potential intrinsic instabilities of a fluorophore can be problematic during a respective application. For example, many fluorophores are known to undergo degradation as a result of photo-bleaching and/or chemical reaction with species found in the biological environment. These degradation processes result in a loss of or a change in probe or reporter fluorescence, which may compromise measurement of the intended analyte or the ability to yield high fidelity images. Therefore, fluorophores and their derivatives possessing exceptional photo-chemical stability for the application at hand are of the utmost importance.

A prominent family of fluorophores in the field of biological imaging is that based on rhodamines. Typically, rhodamines consist of a xanthene scaffold with differing degrees of alkylation on either of the two nitrogen atoms at the 3- and 6-positions or the xanthene ring itself (Figure 1).

Such alkylations affect the quantum yield, molar extinction coefficient, and absorption and fluorescence maxima of the fluorescent reporter. Rhodamines are well known to have desirable photophysical properties, e.g., high quantum yield (Φ) and high molar absorptivity (ε), but it is their high photostability that makes them exceptional in biological studies. Moreover, responsive-probe-based and traditional reporter applications that employ rhodamines have routinely demonstrated the chemical stability of the rhodamine fluorophore, with several reports showing their stability toward pH, metal ions, anions, thiols, and reactive-oxygen species. Thus, rhodamines have been a popular choice in cell imaging and fluorescent probe-based applications.

Interestingly, very little has been reported regarding the stability of rhodamines toward ubiquitous biological reductants, specifically, reduced nicotinamide adenine dinucleotide (NADH). A variety of well-known enzymes catalyze the reducing ability of NADH to modify a wide range of substrates. High concentrations of this enzyme cofactor exist in healthy human cells, and NADH has been
shown to be present at roughly twice the normal value in
cancerous cells (~200 μM).¹² There are a few detailed
reports of NADH reacting with dye molecules, for example
resorufin¹³ and methylene blue.¹⁴ However, based on our
review of the literature, the stability of rhodamines in the
presence of NADH has not been investigated in a detailed
fashion. There is one report on the fluorescence of
rhodamine 110 (R110) being quenched in the presence of
NADH, but very little is said about the nature of this process
or if this scenario is generalizable to other rhodamines.¹⁵

Because of their importance in cell staining/imaging and
probe applications, we set out to systematically study the
chemical stability of a series of rhodamines—both
synthesized in house and commercially available—toward
the potential biological reductant NADH. A trend was
established between the structure of the rhodamine and its
propensity to react with NADH. Based on these results, we
selected a rhodamine, possessing a suitable stability toward
NADH, in the construction of a fluorescent probe for the
detection of the NADH-dependent enzyme, NAD(P)H:
quinone oxidoreductase isozyme-1 (NQO1), an enzyme with
multiple known links to numerous cancers.¹⁶,¹⁷

Figure 2. Photophysical properties and NADH reaction kinetics of rhodamines. (A) The structures of custom asymmetric
rhodamines and seminaphthorhodamines, and those of commercially available rhodamines. (B) Photophysical properties of
rhodamines measured in aqueous PBS at pH 7.4, and reaction kinetics characteristics obtained from rhodamine incubation with
1 × 10⁻³ M NADH at 37 °C. Quantum yields were measured using rhodamine B (RB) in EtOH¹⁸ or cresyl violet in MeOH¹⁹ as a
standard. * Reported from Ref 20 (Lavis et alia) in PBS at pH 7.4. ° Reported from Ref 18 (Casey et alia) in EtOH. (C) The
proposed reaction mechanism for the reduction of rhodamines by NADH involving a hydride transfer. (D) Reaction kinetic plots
of MorphR110 with 1 × 10⁻³ M NADH in pH 7.4 PBS buffer solution incubated at 37 °C in the absence and presence of the
radical inhibitor di-tert-butylcatechol (DTBC); a control study employing only MorphR110 in PBS was performed under similar
conditions.
RESULTS AND DISCUSSION

NADH Reduction of Rhodamines. Our curiosity about the chemical stability of rhodamines toward NADH originated while working with an analogue of rhodamine 110 (R110) that has a morphilino urea moiety on one of the R110 nitrogens. This rhodamine analogue, dubbed MorphR110, was reported to possess an exceptional ability to form a cell passive spiro-lactone structure, while maintaining single-hit enzyme kinetics.20,21 This attractive feature inspired us to utilize MorphR110 in the development of a fluorescent probe for the turn-on detection of the cytosolic enzyme NAD(P)H:quinone oxidoreductase isozyme-1 (NQO1),23 which is capable of probe activation by NQO1- positive cells was unsuccessful,23 instigating the current investigation. During the work at hand, we noticed an unexpected and remarkable decrease in the fluorescence and absorbance of the reporter (MorphR110) after probe activation by NQO1. Upon examining the MorphR110 reporter with the same concentrations of NADH used in the NQO1 experiments, but without the NQO1 enzyme, we observed the same drastic decrease in color; we concluded a reaction readily occurs between NADH and MorphR110 reporter.

These observations prompted us to examine the literature for examples of rhodamines or other fluorophores that react with NADH. The mechanistic details of the reaction of NADH with the oxazine dyes, resorufin and methylene blue (MB), have been reported. Resorufin reacts with NADH via a radical mechanism; when in the presence of oxygen, the resorufin radical intermediate is converted back to resorufin (oxygen oxidation) via consumption of NADH.13 On the contrary, methylene blue reacts with NADH via hydride transfer from NADH to MB, resulting in reduced MB and NAD+.14 Only one report mentions alterations in the behavior of R110 by NADH; few details were given, thereby preventing any conclusions to be drawn about the nature of this reaction and whether other rhodamines may also react.15

Considering the importance of rhodamine fluorophores in chemical biology, and the ubiquity of NADH in the biological environment, we investigated the reaction between NADH and a series of rhodamine compounds (Figure 2A). We posited that there would exist a relationship between rhodamine structure and rate of their NADH reduction. In addition, we conducted a select number of mechanistic studies so as to learn about possible generalizations that can be made regarding the rhodamine-NADH reactions, based on known systems,14 including those used in hundreds of commercial applications.

We chose to study the reduction of rhodamines by NADH under a standard set of conditions that resemble those of physiological environments. Solutions of rhodamine (5 μM) were incubated in 0.1 M phosphate-buffered saline (0.1 M KCl, PBS) at 37 °C. The reaction was initiated upon addition of NADH to the solution, giving a final concentration of 1 mM NADH; the change in absorbance was monitored periodically. For several of the rhodamines, we observed an exponential decay in the color (absorbance) that correlated well with pseudo-first-order kinetics. The determined pseudo-first-order rate constant (kobs) and the half-life (t1/2) values were found to have an interesting relationship with the structure of the rhodamine (Figure 2B).

The compound we initially investigated, MorphR110, reacted the most readily with NADH (kobs = 2.34 × 10−4 s−1 and t1/2 = 0.81 h), while its parent R110 (kobs = 9.99 × 10−6 s−1 and t1/2 = 19.3 h) reacted more slowly with NADH. The propensity of the rhodamine-NADH reaction was decreased for those reporter molecules having increased alkylation on the rhodamine core nitrogen atoms, such as in the dimethylrhodamine (DiMeR) and julolidyl rhodamine (JR) variants. The common rhodamine B (RB) and rhodamine 6G (R6G) were found to have virtually undetectable reaction with NADH. From these results, we concluded the presence of electron-donating groups, such as alkyl functionalities on the nitrogen or the rhodamine core, stabilizes the molecule toward reduction by NADH; we posited this to be caused by decreased electrophilicity of the C9 central carbon.

The reduction of rhodamines by strong reducing agents, such as NaBH4 or LiAlH4, is well documented, and the resulting product is the reduced, or leuco form of rhodamine, having a hydrogen atom at the 9-position (hydride reduced).24,25 Thus, we hypothesized the colorless and non-fluorescent leuco species is likely the product formed during reaction of the rhodamines with NADH; indeed, mass spectrometry evidence supports formation of the leuco-rhodamine for MorphR110, see Supporting Information. However, the reaction pathway—hydride transfer or radical reduction—was unknown.

To that end, we compared the NADH reduction kinetics of MorphR110 in the presence and absence of the known radical inhibitor, di-tert-butylcatechol (DTBC). The reaction kinetics were observed to be unaltered by DTBC presence, indicating the rhodamine reduction reaction does not proceed through a radical mechanism, but rather the reaction is like that of MB.24 In the proposed route, there occurs a concerted hydride transfer from NADH to the C9 carbon of the rhodamine core, resulting in the reduced leuco-rhodamine and NAD+ (Figures 2C & D). Control experiments, without NADH, afforded constant absorbance profiles during the study, indicating NADH is the sole agent responsible for the changes.

Due to our interests and those of others in developing analyte-responsive probes that are turned on to yield a reporter having an emission energy in the near-infrared range, we also examined a series of rhodamines that have sparked interest due to their possessing an extended pi-electronic system, referred to as seminaphthorhodamines (SNRs).26 These compounds have attractive photophysical properties, e.g., long wavelengths of emission and large Stokes shifts. Furthermore, the SNRs can be easily derivatized due to their structure and facile synthesis. A series of SNRs with
varying degrees of alkylation on the SNR nitrogen sites were made (see Supporting Information) and examined under similar NADH reduction conditions. The non-alkylated derivative SNR reacted quickly with NADH ($t_{1/2} = 1.44$ h.), having a rate comparable to that of MorphR110. As expected, increased alkylation—dimethyl-SNR (DiMeSNR) and julolidyl-SNR (JSNR)—resulted in stabilization of the rhodamine core toward reduction by NADH, allowing for larger $t_{1/2}$ values to be achieved. The position of the alkylated nitrogen on the rhodamine core, that is, the benzo-nitrogen or the naphtho-nitrogen, also played a significant role, as a lower $t_{1/2}$ was observed in the case of DiMeSNR compared to DiMeSNR.

Based on these observations, we developed the compounds JSNR and MJSNR, and found the latter to be the most stable of the SNR series. This environmental stability of the MJSNR reporter is evidenced by the slow time for its NADH reduction, indicating significant stabilization toward reduction that results from the presence of the additional methyl group.

From these studies, a clear structure–activity relationship between rhodamines and their propensity to react with NADH has been demonstrated. Overall, alkylation has a stabilizing effect on rhodamines by decreasing the electrophilicity of the C9 central carbon. Previous studies have shown that the lowest unoccupied molecular orbital (LUMO) is associated with the electrophilicity of rhodamines and their intramolecular spirocyclization equilibrium constants.$^{24,27}$ We studied our series of rhodamines using DFT calculations and found that indeed a linear correlation exists between the reaction rate ($t_{1/2}$) and the LUMO energy level (Supporting Information, Figure S4), allowing for a level of predictability when determining the stability of rhodamines with NADH.

**Fluorescent Probe for hNQO1.** Having in hand reporters possessing excellent photophysical properties and outstanding stabilities in the presence of NADH levels that surpass those within a variety of human cells, we sought to develop a rhodamine-based fluorescent probe for detection of the NADH-dependent enzyme, human NQO1 (hNQO1). The determination of this enzyme in biological systems is of great interest. hNQO1 is intimately associated with cancer$^{16}$ and is overexpressed 15–1300 fold in the cytosol of numerous human tumor cells (e.g., colorectal, breast, pancreas, ovary, and non-small cell lung), including those that are chemotherapy resistant.$^{17,28-32}$ In addition, hNQO1 has been reported to exist at high levels in certain normal epithelial and endothelial tissues but not others, as noted by immunohistochemical staining.$^{31}$ bringing into question the use of NQO1 as a diagnostic target. Also, its activity levels are influenced by cellular environment and history.$^{33,35}$ Furthermore, with respect to design of turn-on probes capable of sensing hNQO1, it is well known that hNQO1 catalyzes the 2-electron reduction of quinones to hydroquinones using NADH as a hydride donor. With these considerations in mind, we pursued construction of a probe capable of sensing hNQO1.

Among the rhodamines we made and examined, MJSNR was selected as the probe’s fluorescent reporter unit, because of its superior chemical stability ($t_{1/2}$ approximately 39 h.) and attractive photophysical properties, e.g., a large Stokes shift, long emission wavelength, and high brightness ($\phi \sim 10^4$ M$^\text{-}1$ cm$^3$. The NQO1-sensitive probe Q3MJSNR (Figure 3) was synthesized by typical amide coupling conjugation of MJSNR to a trimethyl-locked quinone propionic acid (Q3PA) trigger group. The activatable Q3PA group was selected due to its rapid$^{38}$ and selective (biological milieu and NQH:quinone oxidoreductase 2)$^{23,37}$ reduction by human NQO1 and subsequent fast elimination of the reduced trigger group.$^{38}$

![Figure 3. Reaction scheme for synthesis of the hNQO1 probe Q3MJSNR.](image)

The addition of the Q3PA moiety resulted in a dramatic reduction in the quantum yield ($\Phi = 0.025$) and the molar absorptivity ($\varepsilon = 4400$ M$^\text{-}1$cm$^3$) of the Q3MJSNR probe relative to its free reporter, allowing for a quenched or “turned-off” type probe. We found it quite intriguing that the probe has such a characteristically low quantum yield, considering the fact that rhodamines having one amidated nitrogen typically display only somewhat decreased fluorescence vs their non-amidated variants.$^{27}$ Therefore, we thought additional quenching of probe fluorescence may occur, due to presence of the quinone (Q3PA) moiety. We synthesized and examined an acetylated analogue AcMJSNR (Supporting Information) that has a larger quantum yield ($\Phi = 0.10$) relative to that of the Q3MJSNR probe ($\Phi = 0.025$). Considering there are several examples of Q3PA-fluorophore conjugates that exhibit efficient Q3PA-based quenching of various fluorophores, we concluded the highly quenched state of the Q3MJSNR probe was likely caused by a combination of effects; namely, decreased rhodamine conjugation due to presence of the amidic moiety, and favorable photo-induced electron transfer (PeT) of the MJSNR fluorophore and the covalently attached Q3PA moiety.$^{39,37}$ As a result of the large differences (10-fold) in quantum yield between the Q3MJSNR probe (0.025) and its reporter version (0.25), the Q3MJSNR probe has the potential to provide highly selective and sensitive detection of NQO1.

Next, we investigated the “turn-on” speed and selectivity of Q3MJSNR. Sodium dithionite (NaS$_2$O$_4$) is a strong reducing agent capable of reducing the Q3PA moiety.$^{38}$ Upon addition of dithionite to an aqueous solution containing Q3MJSNR, a rapid increase in the fluorescence is observed, because the quinone is reduced to its hydroquinone form, which is then shed by a subsequent intramolecular cyclization reaction to yield a lactone species$^{38}$ and the highly fluorescent reporter MJSNR (Figure 4A). Additional reducing agents—glutathione (GSH), dithiothreitol (DTT), and ascorbic acid (AA)—were investigated to test the selectivity of the probe; these potential interferents were found incapable of activating the probe (Figure 4B). We have already shown that NADH is incapable of rapidly reducing the Q3PA amidic bond. Therefore, we
We then examined probe response to hNQO1 by adding NADH to a solution containing 5 μM Q₃MJSNR and 0.75 μg hNQO1 (130 U). Importantly, probe activation was achieved in a matter of minutes (Figure 4C), as noted by a 30-fold increase in fluorescence intensity and a dramatic change in the absorption spectrum (Figures 4D & E). In the absence of NADH, the trigger group is not activated by the hNQO1, and there is no discernible difference in spectral properties of the probe over time.

Mammalian cells can potentially contain several other reductase enzymes capable of 1- or 2-electron reduction of various substrates. For example, NRH:quinone oxidoreductase 2 (NQO2) exists in some mammalian cells and has been shown to reduce substrates similar to those reduced by hNQO1; although ubiquinone-like species are reduced well by both NQO2 and NQO1, the simple 1,4-benzoquinone is unreactive toward NQO2, while it is reduced by NQO1 at near diffusion-controlled rates. In addition, there are several isomers of carbonyl reductase; however, the only isomer found in humans that acts on 1,4-benzoquinones to any extent is carbonyl reductase 1 (CBR1). With a reported activity of 0.87 μmol min⁻¹ mg CBR1⁻¹ for reduction of 2,6-dimethyl-1,4-benzoquinone, CBR1 demonstrates selectivity toward NADH compared to reduced by NQO1 at near diffusion-controlled rates.42

Finally, cytochrome P450 reductase (CPR) is well known for its single-electron reduction of cytochrome P450 enzymes, as well as select exogenous species; one-electron reduction of 2,3,5,6-tetramethyl-1,4-benzoquinone by CPR is very limited, with no suggestion of a two-electron reduced hydroquinone product.46

With the concern that some of these enzymes may also activate the quinone moiety of the probe, which would lead to a non-selective increase in fluorescence (reporter release), we decided to screen them under their respective standard in vitro assay conditions using an established synthetic cofactor (NQO2) for NQO1 activity, and there is no discernible difference in spectral properties of the probe over time.

For example, the ratio of CPR activity:hNQO1 activity in HT29 human colorectal tumor xenografts has been reported to be ~0.04,79 while the ratio used to assess probe selectivity here is 73 times that value (2.9). Similarly, the ratio of 2.5 for NQO2 activity:hNQO1 activity that we used dwarfs the observed value of ~0.002 in various human tumor cell lines. To our knowledge, there is no report that directly compares CBR1 activity to hNQO1 activity in human cells, but the CBR1 activity in human liver and placenta tissues in comparison to that of murine NQO1 activity is approximately 0.06, when using the same quinone substrate (menadione). The value used here is ~0.01, due to limitations on accessible amounts of available human CBR1. In summary, activation of the Q₃MJSNR probe by hNQO1 to yield the fluorescent MJSNR reporter is quite selective upon in vitro examination of the probe with some of the most commonly tissue-expressed reductases.

To evaluate the hNQO1-dependent kinetics for release of the reporter species upon probe activation, we utilized the fluorescent product formation technique. Solutions containing ~1–30 μM Q₃MJSNR were incubated with hNQO1 (0.75 μg, 130 U), and the reaction was initiated by adding NADH (100 μM). A steady increase in fluorescence was measured, and the initial rate of MJSNR reporter formation, V (μmol min⁻¹ mg hNQO1⁻¹), was calculated and

**Figure 4.** (A) Reaction scheme for the release of the fluorescent reporter MJSNR upon Q₃MJSNR probe reacting with hNQO1 or dithionite, S₂O₄²⁻. (B) Activation of probe (5 μM) by 1.6 mM dithionite in contrast to lack thereof by 1 mM other reductants. (C) Activation of 5 μM probe by hNQO1, in contrast to lack thereof by other reductase enzymes (human). (D–E) Activation of 5 μM probe by 0.75 μg (130 U) hNQO1 and 100 μM NADH in pH 7.4, 0.1 M PBS for 30 min. T = 25 °C.

**Figure 5.** Kinetics of hNQO1 (0.75 μg or 130 U) with Q₃MJSNR (1.25–30 μM) in pH 7.4, 0.1 M PBS. Values reported are the average (n = 3) with error bars of ±1 sample standard deviation. Curve is the best fit to the data. T = 25 °C.
then plotted as a function of \( \text{Q7MJSNR} \) concentration (Figure 5). Apparent kinetic parameters were obtained by fitting the data in Figure 5 to Michaelis–Menten kinetics, namely, the Michaelis constant \( (K_m) = 13.9 \pm 3.0 \, \mu\text{M} \), maximum velocity \( (V_{\text{max}}) = 3.14 \pm 0.29 \, \mu\text{mol min}^{-1} \) mg hNQO1\(^{-1}\), catalytic constant \( (k_{\text{cat}}) = 96.8 \pm 9.0 \, \text{min}^{-1} \), and catalytic efficiency \( (k_{\text{cat}}/K_m) = 6.96 \pm 0.36 \times 10^3 \, \text{M}^{-1} \text{min}^{-1} \).

To determine the potential of \( \text{Q7MJSNR} \) for detection of hNQO1 in the cellular environment, we tested probe response in an hNQO1-positive colorectal cancer cell line (HT29) and an hNQO1-negative lung cancer cell line (H596), Figure 6. From confocal fluorescence microscopy images, it is observed there is a clear difference between the two cell lines when they are incubated with 10 \( \mu\text{M} \) \( \text{Q7MJSNR} \) for only 10 min. HT29 cells exhibit a bright red fluorescence in the cytosol where cellular hNQO1 actively converts the probe into the more fluorescent reporter. On the contrary, probe-treated, hNQO1-negative H596 cells are virtually non-fluorescence, as a result of a point mutation in the hNQO1 gene that leads to production of inactive hNQO1 having serine instead of proline at position 187.

To provide further evidence for selective detection of hNQO1 activity inside HT29 cells by the \( \text{Q7MJSNR} \) probe, hNQO1-positive cells were exposed to a known inhibitor of hNQO1—dicoumarol—at a concentration well above the cellular IC\(_{50}\) value of \( \sim 2 \, \mu\text{M} \). We verified dicoumarol inhibition of hNQO1 probe activation, with in vitro experiments that used purified enzyme, wherein was monitored reporter fluorescence (Figure S6). hNQO1-positive HT29 cells pretreated with 10 \( \mu\text{M} \) dicoumarol before probe exposure did not exhibit the same bright fluorescence as did those cells free of dicoumarol, Figure 6. However, dicoumarol has been reported to inhibit other reductases, whose activities inside cells may be different than those we used during the in vitro probe selectivity studies in Figure 4C. To further investigate hNQO1-selective probe activation in the cellular environment, we pretreated hNQO1-positive HT29 cells for 1 h with 10 \( \mu\text{M} \) of cell-permeable, selective inhibitors of NQO2 (quercetin\(^{55}\)) or CBR1 (rutin\(^{56}\)) prior to addition of \( \text{Q7MJSNR} \) probe. There were no apparent differences in the intense fluorescence signal from images of probe-treated HT29 cells with or without these inhibitors (Supporting Information, Figure S7).

Figure 6. Confocal microscopy images of HT29 (positive), HT29 pretreated with 10 \( \mu\text{M} \) dicoumarol, and H596 (negative) cells incubated with 5 \( \mu\text{M} \) \( \text{Q7MJSNR} \) for 10 min at 37 °C. Fluorescence images are in the right column and differential interference contrast (DIC) images in the left. The probe was excited with a 543-nm HeNe laser line (Laser intensity = 48%).

Quantification of the probed response of hNQO1 presence in hNQO1-positive cells vs negative cells (positive-to-negative ratio, PNR) was determined by measuring the fluorescence intensities of 30 HT29 cells and 16 H596 cells in images, such as those in Figure 6. From this, a clear distinction between the two cell lines could be observed, with a PNR of 6.6 from wide-field microscopy images and a PNR of 3.9 computed using confocal microscopy images (see Supporting Information); PNR values are of practical use in cell enumeration and cell/tissue differentiation studies, when the PNR is 1–5 or greater.

Although the PNR ratios obtained here are not as large as in previous probe/reporter systems we have disseminated,\(^{39,37}\) the rapid uptake and turn-on (~10 min), and the lack of potential interferent (NADH, biotiol, ascorbate, various reductases) influence on probe/reporter properties make the \( \text{Q7MJSNR/MJSNR} \) system a valuable asset amongst the select group of enzyme-activated fluorescent probes with possible application in the detection of tiny metastatically disseminated tumors during surgical resection. It is notable that we have demonstrated successful design and implementation in biological systems, a probe whose corresponding reporter fluorescence is not quenched by the presence of potential biological interferents, because such an outcome is a major challenge in development of reporters with longer emission wavelengths.\(^{58,59}\)

**CONCLUSIONS**

We conducted a systematic study on the reduction of rhodamines and seminaphthorhodamines by NADH and determined the structural features governing their stability. Rhodamines lacking alkylation on their respective nitrogen atoms were more prone to being reduced to their non-fluorescent, colorless leuco forms by the ubiquitous cellular component, NADH. This reaction likely occurs through a concerted hydride transfer from NADH to the electron-deficient C9 position of the rhodamine reporter, given that a radical inhibitor had no effect on the NADH reduction rate. These results are vital to the design of probe molecules used...
in the assessment of cellular characteristics. From these studies, we chose the seminaphthorhodamine MJSNR, because of its stability toward NADH and its excellent photophysical properties. Attaching a Q3PA moiety to this fluorophore resulted in a weakly fluorescent probe effective for the "off-on" detection of the NADH-dependent, cancer-linked enzyme hNQO1. The rapid turn-on rate of the probe, i.e. a high $V_{\text{max}}$ and a short incubation time, which results in production of the highly fluorescent MJSNR reporter, was shown to allow for clear discrimination of hNQO1-containing cells from those cells having undetectable amounts of hNQO1. Thus, the probe will find immediate application in cell-based drug screening assays that target hNQO1,60 as well assessment of potential dual therapy strategies for selective killing of cancer cells.32 Furthermore, it will now be possible to evaluate the feasibility of the probe to discriminate various cancer cells of different origin from nearby healthy tissue, particularly disseminated metastases, such as in the case of peritoneally disseminated ovarian and colorectal cancer tumors.28, 61 Favorable outcomes from such studies may allow for future use of the developed probe and its derivatives in fluorescence-guided surgical imaging and resection of small cancer tissues57, 62, 63 upon topical, spray-on application of probe64-66 a fast growing area of interest in selectively activatable probe research endeavors.56

**METHODS**

Reagents and Synthetic Methods. All chemicals were purchased from Sigma-Aldrich, Life Technologies, TCI America, or Fisher Scientific and were used as received. Enzymes were purchased from Sigma-Aldrich (hNQO1, human NQO2, and human CPR) or Creative Biomart (human CBR1). For details of synthetic procedures and characterization of products, see Supporting Information. Column chromatography was performed with silica gel columns, 60A/40-63 µm, from Sorbtech or on 11-g snap KP-NH Biotage cartridges (an amino-modified silica phase) using a Flashmaster Personal from Biotage. Thin-layer chromatography was performed on aluminum-backed 60 F254 silica plates from EMD Chemicals Incorporated or on glass-backed KP-NH (an amino-modified silica phase) plates from Biotage. ¹H and ¹³C NMR spectra were collected in CDCl₃, DMSO-d₆, acetone-d₆, or methanol-d₆ at room temperature on Bruker AVIII-400 or Bruker AVIII-500 spectrometers. All chemical shifts are reported in the standard d notation of parts per million using tetramethylsilane as an internal reference. Absorption bands in NMR spectra are listed as singlet (s), doublet (d), triplet (t), multiplet (m), or two triplets (2t), and coupling constants (J) are reported in hertz (Hz). Mass spectral analyses were carried out using an Agilent 6210 ESI-TOF mass spectrometer.

**Spectroscopic Methods.** All spectroscopic measurements were performed in pH 7.4, 0.1 M phosphate buffer/0.1 M KCl, solutions. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer, and fluorescence data were collected using a Perkin Elmer LSS5 fluorimeter. Samples for absorption and emission measurements were contained in 1-cm × 1-cm quartz cuvettes (3.5 mL). Fluorescence quantum yields were determined using standards of either rhodamine B in EtOH ($\Phi = 0.49$)¹⁸ or cresyl violet in MeOH ($\Phi = 0.54$).¹⁹

NADH Reduction Studies. Stock solutions (1 mM) of rhodamines and seminaphthorhodamines were prepared in spectroscopic grade DMSO. Aliquots from these stock solutions were subsequently added to test tubes containing 0.1 M PBS for a final concentration of 5 µM (3 trials for each dye), and they were incubated in a water bath maintained at 37 °C. NADH was then added to the solutions, giving a final concentration of 1 mM NADH, and the absorbance was measured at various time intervals.

Enzyme Kinetics. The reaction between hNQO1 and Q3MJSNR was monitored by fluorescence measurements ($\lambda_{ex} = 580$ nm, $\lambda_{em} = 620$ nm) obtained at room temperature, with solutions of pH 7.4, 0.1 M PBS/0.1M KCl/0.007% BSA. Solutions of β-nicotinamide adenine dinucleotide, reduced disodium salt (NADH, Sigma-Aldrich), were made with the PBS buffer so that subsequent solutions possessed a final concentration of 1 × 10⁻⁴ M β-NADH in each assay. Solutions consisting of 2.5 × 10⁻⁶ to 6 × 10⁻⁵ M of Q3MJSNR were made using the NADH stock. A 1.5 µg mL⁻¹ stock solution of recombinant hNQO1 (Sigma-Aldrich) was prepared using the same buffer solution as above, so as to give 0.75 × 10⁻⁶ g hNQO1 (130 U) per assay. Each assay was performed in a 1.5-mL quartz fluorescence cuvette containing 0.75 mL of Q3MJSNR solution and was initiated by the addition of 0.75 mL of hNQO1 solution. Measurements were collected every 2 s for 2 min. Fluorescence units were converted to concentration by relating the signal increase to a calibration plot of MJSNR in the same buffer system. Plots of velocity versus Q3MJSNR concentration were used to obtain $V_{\text{max}}$ and $K_m$ values from a nonlinear least-squares analysis employing algorithms developed by Cleland for Michaelis-Menten kinetics.51

Cell Culture. HT29 (human colorectal adenocarcinoma), H596 (human non small-cell lung cancer), cell culture base media, and fetal bovine serum (FBS) were purchased from American Type Cell Culture (ATCC), Manassas, VA. Cell culture was performed as suggested by ATCC. HT29 cells were cultured in McCoy's 5A base medium supplemented with 10% FBS and 10 IU/ml penicillin-streptomycin (purchased from Invitrogen). H596 cells were cultured in RPMI-1640 with 10% FBS and 10 IU/ml penicillin-streptomycin. Cells were incubated at 37 °C in a humidified incubator 5% wt/vol CO₂ atmosphere.

Fixed Cell Imaging via Wide-field and Laser Confocal Microscopy. HT29 and H596 cells were cultured overnight in 22 × 22 mm glass coverslips on a tissue culture treated 6-well plate purchased from Fisher Scientific. Old growth medium was replaced with 2 mL of fresh, phenol-red-free medium and then incubated at 37 °C. Solutions of the probe prepared in 100% DMSO were added to each cell line to give a 5 × 10⁻⁶ M solution of the probe and DMSO concentration of 0.5%. Cells were incubated with the dye at 37 °C for 10 min. To assess the activation of probe by the reductase enzyme, the hNQO1 inhibitor dicoumarol was added to a set of hNQO1-positive cells so as to give a concentration of 1 × 10⁻⁵ M and the cells were incubated for 30 min before addition of the probe. A similar approach was used for the other inhibitors (quercetin, NQO2 inhibitor; rutin, CBR1 inhibitor), but they were incubated with the cells at a concentration of 10 µM for 1 h prior to probe introduction. The medium was removed and cells fixed in 2 mL of 4% paraformaldehyde for 15 min, with
shaking. After fixing, cells were rinsed with Nanopure water, and the coverslips were mounted to glass slides with Immumount (Fisher Scientific). Glass slides were left overnight in the dark to allow the Immumount to dry. Wide-field images were acquired using a Leica DM RXA2 fluorescent microscope equipped with a 100x 1.4NA objective lens and a Cooke SensiCam QE. A Texas Red filter set ($\lambda_{ex} = 540–580$ nm, $\lambda_{em} = 607.5–682.5$ nm) was used to visualize the reporter. Confocal images were acquired using a Leica TCS SP2 spectral confocal microscope equipped with a $40\times$ 1.25NA objective lens. The probe was excited with the 543-nm HeNe laser line (Laser microscope equipped with a 40× 1.25NA objective lens. The acquired using a Leica TCS SP2 spectral confocal microscope equipped with a $40\times$ 1.25NA objective lens. The probe was excited with the 543-nm HeNe laser line (Laser microscope equipped with a 40× 1.25NA objective lens. The acquired using a Leica TCS SP2 spectral confocal microscope equipped with a $40\times$ 1.25NA objective lens. The probe was excited with the 543-nm HeNe laser line (Laser microscope equipped with a 40× 1.25NA objective lens.)

**ASSOCIATED CONTENT**

Supporting Information
Additional information as noted in text. Synthetic schemes, characterization data for all rhodamines (NMR and Mass spectrometry), computation studies, reduction kinetic data and plots, dicoumarol inhibition studies, and PNR imaging statistics. Supporting information is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

Corresponding Author
*Email: tunnel@lsu.edu. Fax: 225-578-3458; Tel: 225-578-3239.

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

Notes
The authors declare no competing financial interest.

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**REFERENCES**


(40) Enzymatic activities for NQO1 and NQO2 are defined as: One unit (U) will reduce 1 nanomole of cytochrome C per min in the presence of menadione substrate at 37 °C. Enzymatic activity for CPR is defined as: One unit will reduce 1 nanomole of cytochrome C per min in the presence of menadione substrate at 25 °C. Enzymatic activity for CBR1 is defined as: One unit will consume 1 nanomole of NADPH per min in the presence of menadione substrate at 25 °C. NQO1 (130 U) was examined in the presence of 100 μM NADH. NQO2 (320 U) was examined using 100 μM 1-carbamoylmethyl-1,4-dihydroroticotiamide as cofactor, while 100 μM NADPH was used for CPR (380 U) and CBR1 (1 U). All reactions were carried out with 5 μM probe in pH 7.4 (0.1 M PBS) at 25 °C.


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