Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Efficacious fluorescence turn-on probe for high-contrast imaging of human cells overexpressing quinone reductase activity

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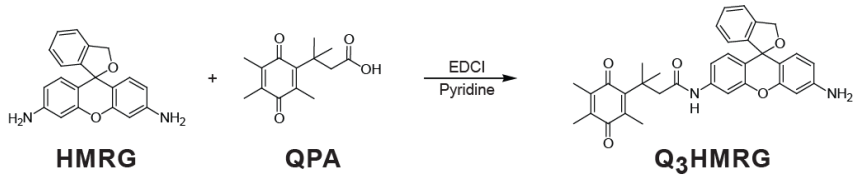
1. Synthetic material and methods

All chemicals were purchased from Sigma-Aldrich or Fisher Scientific and were used as received. Column chromatography was performed using silica gel columns. Thin-layer chromatography was performed on aluminum-backed 60 F_{254} silica plates from EMD Chemicals. ^{1}H and ^{13}C NMR spectra were collected in $CDCl_3$ at room temperature on Bruker AVIII-400 or Bruker AVIII-500 spectrometers. All chemical shifts are reported in the standard δ 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 doublet of doublets (dd), and coupling constants (*J*) are reported in hertz (Hz). Mass spectral analyses were carried out using an Agilent 6210 ESI-TOF mass spectrometer.

2. Synthesis

Protocols found in the literature were used in synthesizing **HMRG**[1] and QPA.[2] In our hands, the synthesis of **HMRG** yielded two forms (spiro- and open-). The open-form was converted to the spiro-form upon the former's dissolution in 4:1 DCM:MeOH with subsequent washing of the organic layer with 1M NaOH. The spiro-form was used in the synthesis of **Q_3HMRG**.



Q_3HMRG
In an oven-dried 10 mL round-bottom flask, 30.6 mg of HMRG was dissolved in 1 mL of DMF and 0.2 mL of pyridine under an N_2 atmosphere, and the solution was cooled to 0 °C. In a separate flask, 33 mg of QPA and 27 mg of EDCI were dissolved in 1 mL of DMF and 0.2 mL of pyridine, and the solution was taken up into a syringe and then added to the chilled HMRG solution dropwise, over the course of 1.5 h. The solution was allowed to warm to room temperature, and it was stirred for an additional 24 h with intermittent monitoring of the reaction by TLC. Upon product formation, the solvent was removed in vacuo, and the crude mixture was purified by column chromatography yielding **Q_3HMRG** as an off-white yellowish solid. Yield = 14.3 mg (27%) R_F = 0.3 in 35% ethyl acetate/hexanes. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45–7.33 (m, 4H), 7.28–7.20 (m, 1H), 6.96–6.86 (m, 2H), 6.84 (d, J = 8.5 Hz, 1H), 6.74 (d, J = 8.4 Hz, 1H), 6.46 (d, J = 2.3 Hz, 1H), 6.37 (dd, J = 8.4, 2.3 Hz, 1H), 5.29 (d, J = 2.9 Hz, 2H), 3.80 (s, 2H), 2.97 (d, J = 2.8 Hz, 2H), 2.17 (s, 3H), 1.97 (s, 6H), 1.48 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 191.54, 187.53, 170.29, 152.91, 151.55, 150.92, 150.90, 147.64, 144.84, 143.31, 139.15, 138.34, 138.19, 129.85, 129.35, 128.30, 127.99, 123.94, 120.60, 114.83, 114.68, 114.59, 111.46, 107.44, 101.44, 83.62, 83.58, 71.89, 50.34, 38.42, 29.10, 29.07, 14.20, 12.75, 12.18. ESI-MS: for $C_{34}H_{32}N_2O_5$: expected *m/z* = 549.2384 $[M+H]^+$; found *m/z* = 549.2398 $[M+H]^+$; 2.6 ppm error.

3. Enzyme Kinetics

The reaction between hNQO1 and **Q_3HMRG** probe was monitored by fluorescence (λ_{ex} = 480 nm, λ_{em} = 520 nm) at room temperature, using probe solutions in pH 7.4, 0.1 M PBS/0.1 M KCl/0.007% BSA. Enzyme stock solutions containing 100 μM β-nicotinamide adenine dinucleotide (Sigma Aldrich) were made with the PBS buffer. Solutions consisting of 3 μM to 36 μM **Q_3HMRG** were made using the enzyme kinetic stock solution. A 1 μg mL^{-1} stock solution of recombinant hNQO1 (SigmaAldrich) was prepared using the same buffer solution as above, so as to give 0.5 μg, 87 U (one unit (U) will reduce 1 nmol of cytochrome C per min in the presence of menadione substrate at 37 °C) per assay. Each assay was performed in a 1.5-mL quartz fluorescence cuvette containing 0.75 mL of **Q_3HMRG** solution and was initiated by the addition of 0.75 mL of hNQO1 solution. Measurements were collected every 30 s for 5 min. A calibration plot of **HMRG** in the same buffer system was used to convert fluorescence units to concentration of **HMRG**. Plots of velocity versus **Q_3HMRG** concentration were used to obtain K_m and V_{max} values from a nonlinear least-squares analysis employing algorithms developed by Cleland for Michaelis-Menten kinetics.[3] For the LOD and LOQ calculations, the fluorescence responses were recorded for a 10 μM solution of Q_3HMRG with different hNQO1 concentrations in the presence of NADH (100 μM) in pH 7.4, 0.1 M PBS at 25 °C. The fluorescence signal emission at 520 nm (500-nm excitation) was measured 30 s after initiation of the experiment. The LOD and LOQ were calculated from the *slope* and standard deviation $s_{intercept}$ of the intercept of the linear portion of the response versus enzyme concentration curve, with the LOD = 3.3*$s_{intercept}$/*slope* and the LOQ = 10*$s_{intercept}$/*slope*.

4. Cell Culture

HT-29 (human colorectal adenocarcinoma), OVCAR-3 (human ovarian adenocarcinoma), and H596 (human nonsmall cell adenosquamous lung carcinoma) were all purchased from American Type Culture Collection (ATCC; Manassas, VA). HT-29 cells were cultured in McCoy's 5A medium (ATCC) supplemented with 10% fetal bovine serum (FBS; ATCC), 10 IU mL^{-1} penicillin, and 10 μg mL^{-1} streptomycin (Life Technologies). OVCAR-3 and H596 cells were cultured in RPMI-1640 with 10% FBS, 10 IU mL^{-1} of penicillin, and 10 μg mL^{-1} of streptomycin. OVCAR-3 was additionally supplemented with 0.01 mg mL^{-1} bovine insulin. Cells were cultured in 75 cm^2 treated tissue culture flasks at 37 °C under 5% CO_2 in a humidified incubator.

5. Cell Imaging via Fluorescence Microscopy and Cell Quantification

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 μM solution (0.5% DMSO final concentration). Cells were incubated with the probe at 37 °C for 30 min, followed by subsequent treatment with nuclear stain (DRAQ5, 3 μM) for ~2 min. The medium was removed, and the cells were 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. Widefield images were acquired using a Leica DM RXA2 fluorescent microscope equipped with a 100x 1.4NA objective lens and a Cooke SensiCam QE. A yellow fluorescent protein, YFP, filter set (500–520 nm excitation/540–580 emission) was used to visualize **HMRG** (100 milisecond exposure), while a Cy5 filter set (590–650 nm excitation/662–738 nm emission) was used to image the nuclear stain. Images were captured and processed using Slidebook. Specifically, the gray scale histogram of the images from each cell line was normalized. Image analysis and statistical evaluation of cellular intensities from acquired images were performed using Fiji/ImageJ, where individual cells were

traced and the integrated intensity was measured within the region-of-interest (ROI). The background was examined in the images of cells not exposed to probe, but no measurable background signal was obtained in the emission energy range that was examined (590–650 nm excitation/662–738 nm emission).

6. pH Dependency of Q_3HMRG

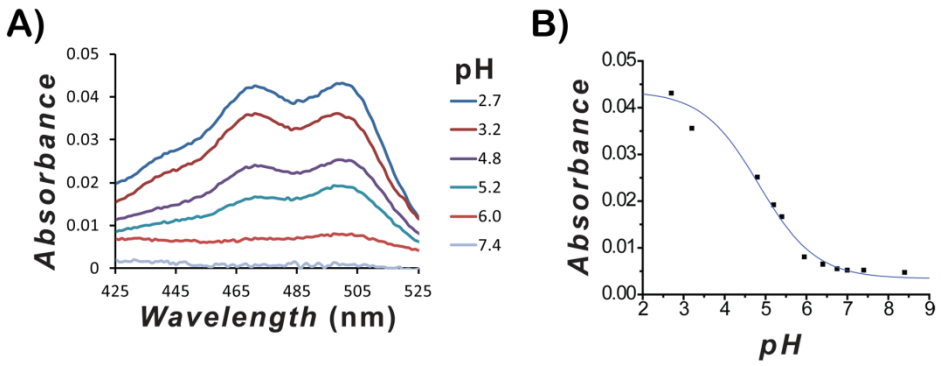


Figure S1. Absorption spectra of **Q_3HMRG** probe as a function of pH (A), with the maximum absorbance at 500 nm being plotted as a function of pH (B). *T* = 25 °C.

7. Q_3HMRG Response to Various Levels of hNQO1

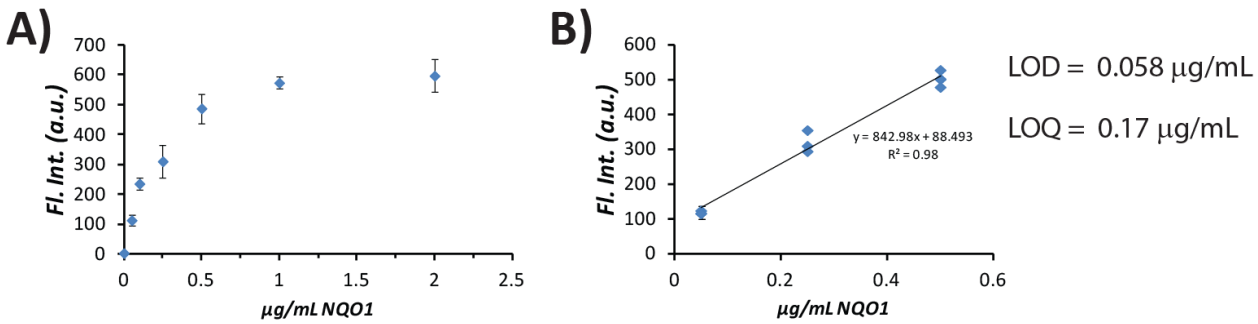


Figure S2. Fluorescence response of a 10 μM solution of **Q_3HMRG** probe with different hNQO1 concentrations in the presence of NADH (100 μM) in pH 7.4, 0.1 M PBS at 25 °C. The fluorescence signal emission at 520 nm (500-nm excitation) was measured 30 s after initiation of the experiment. Each data point is the average ± 1 standard deviation for 3 replicates.

8. Cell Signal Quantification

Data Point#	H596 30 min FL Intensity
1	0
2	0
3	0
4	2.50E-04
5	0
6	0
7	0
8	0
9	0.015
10	0
11	0
12	1.89E-05
13	0
14	2.05E-04
15	6.10E-05
16	0.004
17	0.192
18	0
19	0
20	0
21	0
22	0
23	0
24	0
25	0
26	0
27	0
28	0
29	0
30	0
31	0
32	0
33	0
Average	0.006410154

Data Point#	HT-29 30 min FL Intensity
1	72.00
2	60.23
3	43.35
4	34.90
5	40.74
6	77.38
7	63.44
8	93.29
9	75.73
10	95.08
11	147.71
12	105.66
13	69.43
14	60.95
15	84.70
16	39.32
17	46.11
18	38.58
19	43.56
20	32.92
21	57.11
22	46.74
23	99.59
24	67.30
25	82.57
26	64.81
27	89.87
28	64.51
29	72.50
30	72.53
31	109.46
32	96.87
33	84.50
34	140.05
35	128.68
36	99.95
37	58.46
38	56.89
39	67.20
40	80.80
41	76.28
42	59.04
43	54.38
44	74.42
45	46.30
46	69.01
47	72.43
48	69.49
49	70.22
50	65.03
51	63.09
Average	72.26

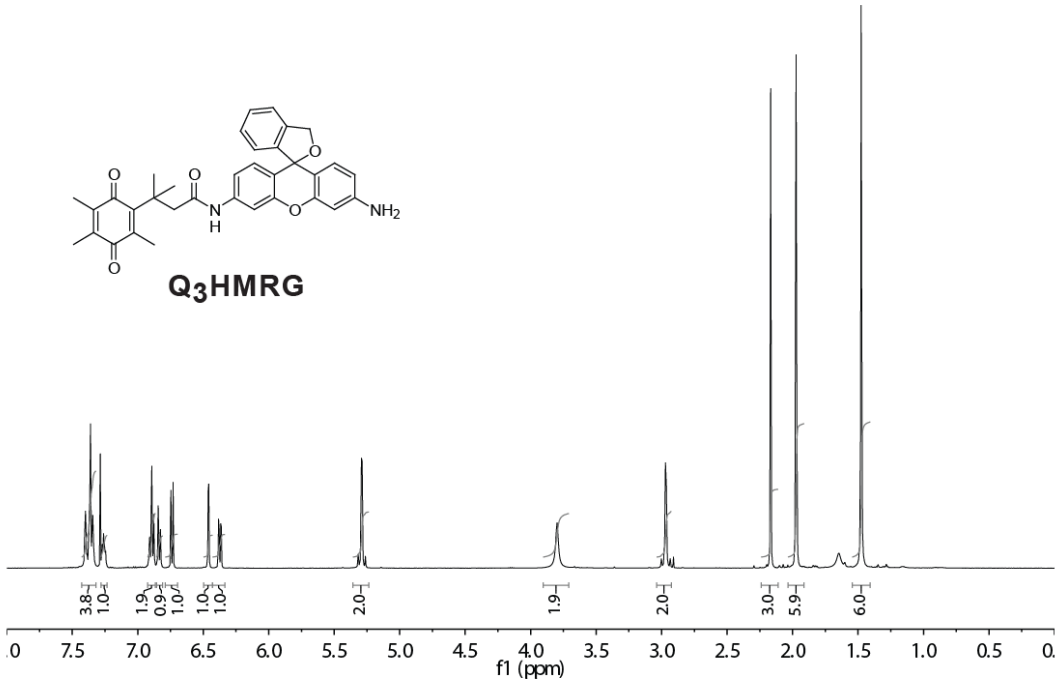
	PNR
HT29/H596	11272

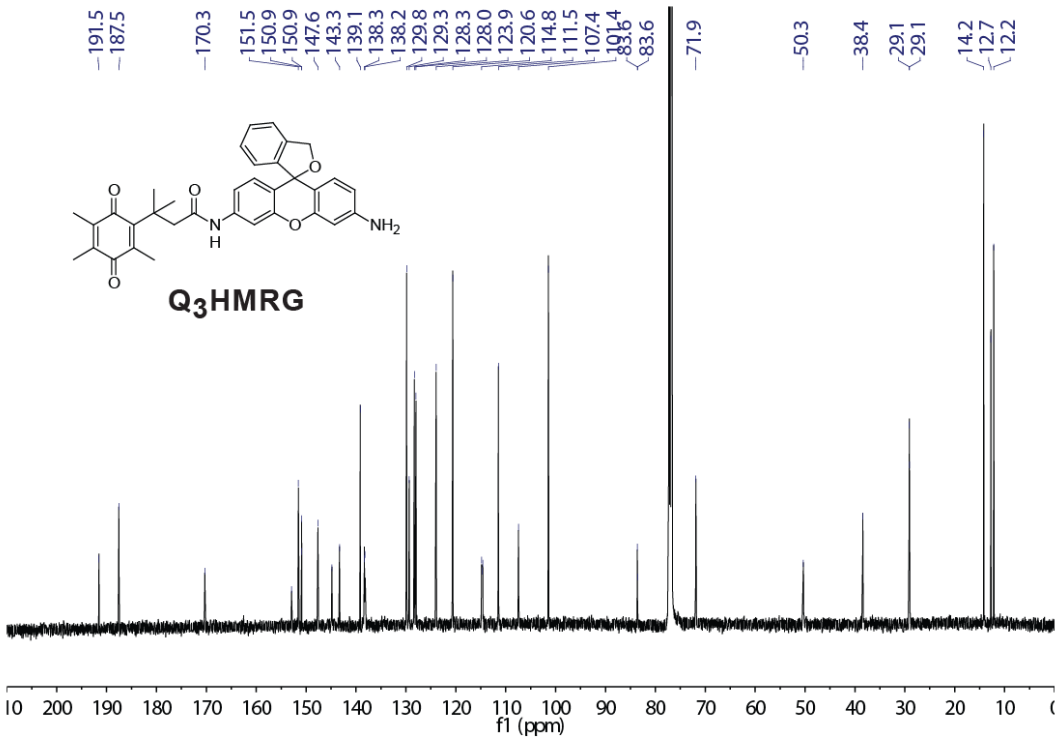
Data Point#	OVCAR 30 min FL Intensity
1	19.03
2	27.99
3	30.24
4	7.24
5	1.57
6	18.28
7	8.67
8	4.59
9	38.25
10	14.75
11	16.24
12	39.62
13	12.25
14	1.07
15	16.51
16	4.40
17	21.62
18	6.80
19	31.48
20	11.59
21	71.12
22	8.35
23	6.46
24	14.35
25	11.28
26	38.39
27	7.84
28	8.71
29	29.24
30	38.19
31	6.49
32	4.95
33	14.16
34	22.46
35	5.05
36	28.82
37	12.43
38	40.35
39	7.05
40	7.05
41	12.34
42	6.31
43	9.25
44	5.61
45	4.85
46	29.88
47	18.95
48	10.92
49	22.19
50	17.02
51	11.07
52	7.78
53	31.83
54	21.43
55	12.98
56	10.49
57	8.98
58	5.18
59	15.33
Average	16.57

	PNR
OVCAR/H596	2584

Table S1. Raw data used for calculation of PNR values.

9. NMR Spectra





10. References

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3 W. W. Cleland, *Methods Enzymol.*, 1979, **63**, 103-138.