An improved cell-permeable fluorogenic substrate as the basis for a highly sensitive test for NAD(P)H quinone oxidoreductase 1 (NQO1) in living cells.

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Abstract: NAD(P)H:quinone oxidoreductase 1 (NQO1) is a flavoenzyme upregulated in response to oxidative stress and in some cancers. Its upregulation by compounds has been used as an indicator of their potential anti-cancer properties. In this study we have designed, produced and tested a fluorogenic coumarin conjugate which selectively releases highly fluorescent 4-methylumbelliferone (4-MU) in the presence of NQO1. It was found that measuring 4-MU release rapidly and specifically quantitated NQO1 levels in vitro and in live cells. Both the substrate and its products freely perfused through cell membranes and were non-toxic. The substrate was very specific with low background, and the assay itself could be done in less than 10 minutes. This is the first assay to allow the quantitation of NQO1 in live cells which can then be retained for further experiments.

Abbreviations: 4-MU: 4-methylumbelliferone; NQO1: NAD(P)H:quinone oxidoreductase 1

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1. Introduction

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase [1], vitamin K reductase [2], menadione reductase [3]) is a flavoenzyme predominantly present in the cytoplasm, with a small amount associated with mitotic spindles in the nucleus [4]. It is a homodimer which binds quinones with the co-factor NADH or NADPH, leading to reduction of the quinones by a ping-pong mechanism of catalysis [5]. The enzyme has wide ranging roles within the cell. It is primarily thought to be an important chemoprotective enzyme which is capable of reducing various quinones and aromatic nitro-compounds [6], reducing cellular quinone levels and minimizing reactive oxygen intermediates. It is further capable of regenerating ubiquinone and vitamin E [7,8] into anti-oxidants thus forming another important part of the cell’s defense system against oxidative stress. NQO1 can also activate anti-cancer agents such as mitomycin C and β-lapachone using the same 2-electron reduction that inactivates cell-damaging quinone moieties [9–13]. NQO1 also has a complementary activity to its catalytic function in that it can bind the 20S proteasome [14], preventing the proteasome from binding and degrading other proteins.

NQO1 activity is clearly of interest in a number of fields. It is upregulated in many solid tumours [15], and is a potential tumour marker [16] as well as an activator of anti-cancer chemotherapeutics [9]. The complementary use of NQO1 as a primary screen for natural anticarcinogens such as sulforaphane and chlorogenic acid from broccoli sprouts and green coffee beans respectively is also well documented [17,18]. However, the current most generally applicable method by which NQO1 is assayed, the Prochaska assay, requires that cells are lysed and a cell extract is made to release NQO1 [19], limiting further investigations, particularly in situations such as the use of unique, limited patient samples. More recent assays have fluorescent products which are retained inside the cell [16,20]. In the current work we describe a cell permeable substrate for NQO1 which contains 4-methylumbelliferone (4-MU) in a quenched fluorescent state. Cleavage of the quenching moiety by active NQO1 within live cells releases the fluorescent 4-MU, which is cell permeable. The amount of 4-MU present in the surrounding medium can be measured in a fluorimeter to give a quantitative measure of NQO1 activity without fixing or lysing the cells. The non-toxic nature of the substrate and its products uniquely allow further tests such as apoptosis assays or flow cytometric analysis to be performed on the same cells as the quantitation of NQO1.
2. Methods

Chemicals and reagents were obtained from Sigma Aldrich Chemical Co, Dorset UK, Lancaster Synthesis Ltd, Lancashire, UK and VWR International, Leicestershire, UK.

HPLC was performed using a ThermoFisher Accela U-HPLC (Ultra-High Pressure Liquid Chromatography) System equipped with a C18 reverse-phase column (dimensions: 50x2.1 mm; particle size 1.9 micron). The chromatograms were analyzed using a PDA (Photo Diode Array) system or FL-Plus detector with settings as follows: PMT (low), λex= 360 nm; λem = 450 nm and processed using ChromQuest software (version 4.2).

Docking of the substrate in the active site of human NQO1

The three-dimensional structure of the substrate was constructed using ChemDraw (version 11.0) and the energy minimised. The X-ray crystallographic co-ordinates of human NQO1 (pdb 1H69) were downloaded from the RCSB Protein Data Bank and revised to provide a monomer of the protein with the reduced form of FAD (FADH$_2$) as the bound cofactor. Molegro Virtual Docker (MVD version 3) software was used to predict the binding mode of the substrate in the active site of the enzyme. The docking procedure was randomised and the substrate was docked at 11Å radius around the FAD binding site (active site). The MolDock optimiser algorithms were used with a maximum of 10 runs and 5000 iterations (the docked substrate achieved lowest energy within the set iterations). A maximum of 5 poses were returned for the substrate in which similar poses were clustered. The top ranked pose for the substrate was assigned with regard to its affinity towards the FADH$_2$ (the distance of the quinone carbonyl to the pteridine N5 of the FADH$_2$ substructure (Figure 1)) and substrate-enzyme active site residue hydrogen bonding interactions. The substrate-enzyme interactions were visualised using Molegro Molecular Viewer (version 1.2).

Synthesis of the substrate probe

A mixture of 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dienyl)butanoic acid (800 mg, 3.4 mmol), N,N'-Dicyclohexylcarbodimide (DCC: 830 mg, 4.0 mmol) and 4-(dimethylamino)pyridine (DMAP: 42 mg, 0.4 mmol) were sus-pended in dry DCE (20 ml). The suspension was stirred for 30 minutes. 4-methylumbelliferone (634 mg, 3.6 mmol) was added and the mixture was stirred for a further 24 hours. The resulting suspension was filtered and the filtrate was evaporated. The formed suspension was filtered, evaporated and redisolved in ethyl acetate and filtered. The organic extract was evaporated and recrystallised from ethyl acetate to yield the product as yellow crystals (0.96 g, 69%, mp 143-145ºC uncorr., MS: ESI+ 431.2 (M+Na)). $^1$H NMR (CDCl$_3$): d 7.58 (1H, d, ArH, J=5Hz), 7.02 (1H, d, ArH, J=3Hz), 6.98 (1H, d, ArH, J=3Hz), 6.96 (1H, d, ArH, J=3Hz), 3.29 (2H, s, CH$_2$), 2.41 (3H, s, CH$_3$), 2.18 (3H, s, CH$_3$), 1.94 (3H, s, CH$_3$), 1.53 (6H, s, 2xCH$_3$). $^{13}$C NMR (CDCl$_3$): d 190.8, 187.3 (2x quinone C=O), 170.8 (C=OO), 160.4 (C=O coumarin), 154.1, 152.8, 151.8, 151.5, 142.7, 139.7, 138.8, 125.4,
117.9, 117.8, 114.6, 110.4 (12x ring C), 47.7 (CH$_2$), 38.4 (C(CH$_3$)$_2$), 29.0 (2x gem-CH$_3$), 18.7 (CH$_3$), 14.4, 12.6, 12.1 (3xCH$_3$).

**HPLC analysis of reaction mixtures of substrate and enzyme**

Initial HPLC was carried out on samples containing substrate (0.1 mM), NADH (0.5 mM, Sigma) and 0.5 μg/ml recombinant human NQO1 (Sigma) in phosphate buffered saline (PBS, 10 mM, pH 7) using a 1-99% acetonitrile gradient and a 2 μl injection volume. Temperature was maintained at 37°C. Reduction of the substrate was rapid and an enzyme concentration of 0.2 μg/ml NQO1 was found to be suitable for determining the initial rate of substrate reduction. No significant reduction in the area of the peak was observed in the presence of either substrate alone or with NADH alone. The rate of reduction in substrate concentration was calculated as 5.9 μM/min and when the concentration of the enzyme was halved the rate reduced to 1.5 μM/min. This finding demonstrates that the substrate is efficiently reduced by NQO1. There was an increase in 4-MU peak area when monitored at 325 nm that matched the loss of substrate confirmed by an increase in 4-MU fluorescence peak (data not shown). The cut-off point for the UV spectra was initially set between 220-800 nm but when the lower wave-length was reduced to 180 nm, the lactone, 4-MU and substrate were quite evident above the baseline. The elution times of substrate, lactone and 4-MU were 4.24 min, 3.10 min and 2.16 min, respectively.

**Kinetic studies**

Kinetic studies were performed in a BMG Clariostar microplate reader. 2ng recombinant human NQO1 enzyme was added to 500 μM NADH and 0 – 25 μM MTL8-252 in a total of 100 μl Hanks balanced salt solution (HBSS, Thermo Scientific). Fluorescence ($\lambda_{ex}$. 360nm, $\lambda_{em}$. 450nm) was read every 15s for 5 minutes, every 30s from 5-10 minutes and every minute from 10-30 minutes from the beginning of the assay. Each assay was performed 3-5 times at 25ºC. Fluorescence readings were standardised to a standard curve of 4-MU of known concentration.

**Cell lines and cell culture**

All cells were grown in media supplemented with 10% FCS, 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine. The human embryonic kidney line 293T was grown in DMEM supplemented as above. The human prostate cancer cell line PC3 was grown in 1:1 Hams:F12. The human bladder cancer cell line RT112 (NQO1*1) [21] and colon adenocarcinoma line BE (NQO1*2) [22] were grown in RPMI1640 and MEM respectively. V79 Chinese Hamster lung epithelial cells transfected with either NQO1 (hDT7 cells) or an empty vector (F179 cells) [23] were grown in DMEM as above additionally supplemented with 10 μg/ml puromycin. All cell culture reagents were sourced from Life Technologies.
**Western blot**

Western blots were performed with 500µg of cell lysate from cell lines described above. All cell lines were subconfluent at harvest. Proteins were separated with standard SDS-PAGE and transferred onto supported nitrocellulose membrane (BioRad). The membrane was blocked with 5% non-fat milk in Tris buffered saline with 0.1% Tween 20 overnight. NQO1 was detected with muIgG clone 4D12 (Abcam, UK) at 1:5,000 and secondary polyclonal anti-muIgG-HRP (Sigma) at 1:20,000. The relevant protein bands were visualised with Pierce Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific).

**Direct fluorescence assay for NQO1**

Cell-free NQO1 was quantitated by resuspending recombinant human NQO1 enzyme with 500 µM NADH and 5 µM MTL8-252 in 240 µl HBSS and incubating at 37°C for 10 minutes. The NADH was then degraded by addition of HCl to 10 mM and further incubation for 5 min at 37°C. The reaction was filtered through a 0.2 µm PTFE filter using a two-piece 1 ml filter vial (Thomson Xtreme Vials, Thomson Instrument Company, CA, USA) and 100 µl of filtrate added to 20 µl of 2 M aqueous Na₂CO₃ in a microcuvette (Promega Ltd.). Fluorescence was then measured using a portable Quantifluor™ fluorimeter (Promega) (365 nm Ex/440-470 nm Em). Cellular NQO1 activity was measured by resuspending the required number of cells in a final volume of 250 µl HBSS and adding the desired concentration of substrate as a solution in 2.5 µl DMSO. After 10min at 37°C, the reaction was filtered as above and 100 µl of filtrate was transferred to a microcuvette. 4-MU fluorescence intensity was maximised by adding 2.5 µl aqueous 2 M Na₂CO₃ prior to fluorescence measurement. The fluorimeter was calibrated using a 4-MU solution of known concentration.

**The Prochaska assay**

To determine NQO1 levels by Prochaska assay, confluent RT112 cells were harvested and resuspended in HBSS at a density of 10⁷ cells/ml. Cells were then serially diluted in HBSS and either used for a live cell assay as described above or frozen on dry ice for the Prochaska assay to retain NQO1 activity and disrupt membranes. The Prochaska master mix was made of 25 mM Tris pH 7.4, 66.6 µg/ml BSA, 0.01% Tween 20, 1 mM glucose-6-phosphate, 30 µM NADP, 2 U/ml glucose-6-phosphate dehydrogenase, 5 µM FAD and 300 µg/ml MTT, with 3 µl of 50 mM menadione added per 5 ml master mix just before beginning the assay. After vortexing, the assay was performed at 25°C with 240 µl of master mix to 10 µl cell extract. After 5 minutes the assay was placed on ice and stopped with 100 µM dicoumarol and the absorbance of the assay mixture measured immediately at 600 nm on a Thermo Labsystems Multiskan Spectrum.
Quantitation of NQO1 by NADH consumption.

Kinetic analysis of menadione and quantitation of NQO1 was performed by quantitating the reduction in NADH fluorescence over time. 50ng NQO1 was added to HBSS containing 200 µM NADH and 5 µM FAD. For comparison of MTL8-252 and menadione, serial dilutions of menadione were then titrated into the mixture and NADH fluorescence (λex. 340nm, λem. 440nm) was monitored at 15s intervals for 5 minutes, then at 1minute intervals for 5 minutes. Final readings were taken at 10 minutes. Similarly, titration of the NADH assay was performed by serially diluting NQO1 enzyme and adding master mix to a final concentration of 200 µM NADH, 5 µM FAD and 50 µM menadione in HBSS. Fluorescence readings were taken as described above.

Determination of toxicity

To determine toxicity, 24 well plates were seeded with 5 x 10^5 RT112 cells in phenol-red free DMEM containing 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine and 10% FCS. Following overnight incubation, cells were treated with 10 µl DMSO containing the standard concentration of MTL8-252 (5 µM), ten times the standard concentration (50 µM), or no MTL8-252. Cells were incubated for the times indicated and viability measured using the CellTiterGlo assay (Promega) according to the manufacturer’s instructions.

Cell retention of MTL8-252 and 4-MU

To examine cell retention of MTL8-252, 10^5 F179 or hDT7 cells were suspended in 100µl HBSS containing 20µM MTL8-252 and incubated at 37°C for 5 minutes. The cells were removed by centrifugation (5 min, 500rcf) and the supernatant added to 2.5µl of 2M Na₂CO₃ for reading of 4-MU fluorescence. Cells were rinsed once in 1ml HBSS and collected by centrifugation as bove. They were then resuspended in 100µl fresh HBSS and incubated for 5 minutes before centrifuging as previously and fluorescence readings made of the supernatant. The process was repeated twice. After the final centrifugation the cell pellet was lysed by 3 freeze-thaw cycles in which the pellet was frozen on dry ice then thawed rapidly at 37°C. The cell pellet was incubated with an excess of NQO1 and NADH (10µg/ml and 200µM respectively) and then acidified by adding HCl to 50mM. After 10 minutes, 20 µl of 2M Na₂CO₃ was added and fluorescence of the solution determined in the Quantifluor fluorimeter.
Results and Discussion

Design of the NQO1 probe

Scheme 1. Reduction of the substrate to yield fluorescent 4-MU

To create the NQO1 probe, a fluorogenic enzyme substrate probe was rationally designed based on the ‘quinone trimethyl lock’ system [24] in which 4-MU was attached to a quinone acid via an ester link. This conjugation reversibly quenches the fluorescence of the 4-MU. Upon redox ‘activation’ by NQO1, the quinone moiety is reduced to its corresponding hydroquinone. The unfavorable steric interaction caused by the presence of the trimethyl lock encourages spontaneous lactonisation thereby releasing the highly fluorescent 4-MU (Scheme 1). In our experiments, the novel substrate was synthesised by coupling 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dienyl)butanoic acid [25] to 4-MU in the presence of DCC and DMAP (Scheme 2).
Computational chemistry suggested that the conjugate should be an effective substrate for NQO1 as it interacts favorably with the bound co-factor FADH$_2$ and is stabilized by some of the active site residues of the NQO1 enzyme (Figure 1). In addition, the position of the substrate in the active site and its close proximity and orientation to the bound co-factor FADH$_2$ suggested that it should be able to undergo a hydride transfer reaction from the co-factor to the quinone oxygen of the substrate, resulting in the formation of the ‘activated’ hydroquinone species.

To determine the efficacy of human NQO1 in cleaving the substrate, the apparent kinetic parameters were measured by measuring release of 4-MU from the quenched substrate in the presence of NQO1 (Figure 2). Kinetics were measured for substrate concentrations between 1 and 50µM. As can be seen in Figure 2a, the reaction was extremely rapid and was >90% complete within 5 minutes with the presence of relatively small amounts of NQO1. In comparison, there was little background MTL8-252 degradation. When fitted to the Michaelis-Menten equation (Figure 2b), $K_m$ was found to be 5.06 ± 1.31 µM, $V_{max}$ to be 101.5 ± 8.3 µmol min$^{-1}$mg hNQO1$^{-1}$ and $k_{cat}$ to be 157.4 ± 12.9 s$^{-1}$. The $k_{cat}/K_m$ of $3.11 \pm 0.16 \times 10^5$ M$^{-1}$s$^{-1}$ indicated that cleavage of MTL8-252 by NQO1 in non-saturated solutions was at reaction rates comparable to other excellent NQO1 substrates reported in the literature [20,26–28].
Figure 1. The binding mode and interaction of substrate in the FAD-binding site of NQO1. The carbonyl quinone moiety and FADH$_2$ are in close proximity (C=O$^1$--N$^5$H, 3.31Å). This indicates that hydride transfer may occur resulting in the reduction of the probe and ultimate release of the fluorophore. The substrate binding is further stabilised by active site residues Trp-105, Phe-106, Gly-149, Gly-150, Met-154, Tyr-155 and His-161.

Figure 2. Kinetics of enzymatic reduction of the substrate. (A) 4-MU production from MTL8-252 by NQO1. Values are the average of duplicate readings from one of five experiments. To a reaction mixture containing substrate (20 µM) and NADH (500 µM) in HBSS was added 20ng purified human NQO1. Fluorescence at 450nm was monitored as described in materials and methods. (B) Kinetic plot of NQO1 with MTL8-252 as substrate. Values represent mean ± SD of 5 experiments.
**MTL8-252 is effective and specific in detecting NQO1 in live cells**

While there are currently efficient ways to detect NQO1 in cell extracts [19] and in cells for microscopy/flow cytometry [16,20], these retain the signal in the cell and/or require cell lysis for NQO1 quantitation. This restricts NQO1 assays to being single measurements and reduces the ability of researchers to use assays with multiple outputs. Since MTL8-252 can easily diffuse across cell membranes, we wanted to determine whether it would be capable of measuring NQO1 activity in live cells, and whether the substrate was subject to non-specific breakdown when exposed to the intracellular environment.

To assess whether the MTL8-252 could be used to detect NQO1 in live cells, we initially measured MTL8-252 conversion in NQO1-negative V79 Chinese hamster lung epithelial cells transfected with either NQO1 (hDT7 cells) or an empty vector (F179 cells) [23]. It was found that there was a clear increase in the yield of the 4-MU product from MTL8-252 in hDT7 cells compared to F179 cells, and that the difference between the two cell lines was evident within 1 minute of starting the reaction. Indeed, there was approximately a 100-fold increase in the initial rate of formation of 4-MU product in the hDT7 cells relative to the F179 cells (Figure 3). While there was an increase in fluorescence intensity over time in the F179 cells, it was substantially lower than that from NQO1-positive hDT7 cells, and was minimal at the 10 minute timepoint. We subsequently used the 10 minute timepoint for all experiments. Inhibition of the reaction with the competitive NQO1 inhibitor dicoumarol [29] and the irreversible NQO1 inhibitor ES936 [30,31] but not the 1-electron flavoenzyme inhibitor DPI showed that the response in hDT7 cells was NQO1-specific (Figure 4).

![Figure 3. Specific detection of NQO1 in live cells.](image-url)

**Figure 3. Specific detection of NQO1 in live cells.** 2 x 10^5 V79 cells transfected with human NQO1 (hDT7) or empty vector (F179) were collected from 90% confluent cultures and suspended in HBSS containing 10 µM MTL8-252. At the indicated time points, samples were filtered through a 0.2 µm Thomsen X-Treme vial and 100 µl of filtrate added to 2.5 µl 2M Na_2CO_3 for reading of 4-MU fluorescence (λex= 360 nm; λem = 450 nm). All samples were tested in duplicate. Results show the mean and range of results for one experiment, and are representative of three independent experiments.
Figure 4. The assay is specific to NQO1. 5 x 10^4 hDT7 (NQO1+) or F179 (NQO1-) cells were incubated with 5 μM MTL8-252 with 20 μM DPI, 50 μM dicoumarol or 40 μM ES936 at 37°C for 10 min. The reaction mix was filtered through a 0.2 μm PTFE filter and 100 μl of filtrate was admixed with 2 M aqueous Na₂CO₃ before reading 4-MU fluorescence in the fluorimeter. All samples were tested in duplicate and data represents mean ± range.

MTL8-252 detects NQO1 in NQO1-expressing cancer cell lines

Having demonstrated that MTL8-252 was capable of detecting NQO1 activity in cell lines genetically manipulated to express the enzyme, we determined whether it could detect differences in naturally-occurring tumoral NQO1 levels. Cancer cell lines with varying levels of NQO1 expression were used: RT112 and PC3 were used as examples of high NQO1-expressing cancers, BE was used as an example of cancers containing the C609T mutation which leads to reduced NQO1 protein levels, and 293T cells were used as an example of NQO1-negative cells. Each was tested in the assay with or without NQO1 inhibition (Figure 5a). It was found that in all cell lines NQO1 status correlated with 4-MU release, and that it was inhibited by the prototypical NQO1 inhibitor dicoumarol. We further investigated the responses of RT112 in the presence of a variety of inhibitors known to affect various stages of the redox pathways: the mechanism-based irreversible NQO1 inhibitor ES936 [26], the NQO2 inhibitor 9aa [32], superoxide dismutase which converts superoxide into hydrogen peroxide [33], capsaicin which inhibits NADH-dependent ubiquinone redox cycling [34] and sodium azide, which inhibits mitochondrial respiration [35]. It was found that only ES936 and dicoumarol inhibited the conversion of MTL8-252, confirming its specificity for NQO1 (Figure 5b).
**Figure 5.** MTL8-252 reliably detects NQO1 in several cancer cell lines. (A) $5 \times 10^4$ cells of the indicated cell lines were incubated with $5 \mu M$ MTL8-252 for 10 minutes either in the presence or absence of $50 \mu M$ dicoumarol, and 4-MU fluorescence subsequently measured. (B) $5 \times 10^4$ RT112 cells were incubated with $5 \mu M$ MTL8-252 for 10 minutes either in the absence or presence respectively of $50 \mu M$ dicoumarol, $40 \mu M$ ES936, $100 \, nM$ 9aa, $1 \, U$ superoxide dismutase (SOD), $200 \, \mu M$ capsaicin or $10 \, mM$ sodium azide (Na Azide). All show mean and range of triplicate readings and are representative of 2 or 3 independent experiments for B and A respectively. Comparisons performed with Student’s t-tests. ** = $p < 0.01$, * = $p < 0.05$. (C) Western blot showing NQO1 expression in each of the cell lines used. Western blot performed as described in materials and methods.

MTL8-252 in live cells is as or more sensitive than the Prochaska assay of cell extracts.

To determine the sensitivity of the assay compared to the current standard Prochaska assay (Figure 6a, b), and the NADH consumption assay (Figure 6c, d), and to test whether substrate was quantitative, the assays were tested in parallel both with isolated NQO1 enzyme and with live cells. When assaying enzyme alone it was found that the MTL8-252 assay was comparable to the Prochaska assay, with consistently slightly higher sensitivity (Figure 6a). Consumption of NADH as shown by a reduction in fluorescence was found to be the most effective method of quantifying NQO1, with sensitivity higher than both other assays (Figure 6c, d). This is in keeping with the very low $K_m$ and high $V_{max}$ of menadione [28] and the sensitivity of direct measurement of NADH by fluorescence. When testing cells, RT112 cells were serially diluted and either frozen in dry ice to produce cell extracts for the Prochaska assay or used directly in the live cell assay. It was found that the Prochaska assay reliably detected down to $2 \times 10^4$ RT112 cells, while the live cell assay could detect down to
$1 \times 10^4$ cells (Figure 6b) and exhibited superior signal-noise ratios at lower cell numbers, an important consideration in applications where cell numbers in analytical samples may be limited. Hence MTL8-252 substrate was suitable for assaying enzyme alone, but it was particularly effective in determining NQO1 levels in live cells.

Figure 6. Comparison between the current assay, the Prochaska assay, and the NADH consumption assay in quantitation of NQO1. (A) NQO1 enzyme was serially diluted and incubated either with 330 µM NADH and the MTL8-252 substrate (left y axis), or with Prochaska assay reagents (right y axis) for 10 min. NADH was degraded with HCl, and 4-MU measured in a UV fluorimeter. Prochaska assay was performed as per materials and methods. (B) RT112 cells were resuspended to $10^7$ cells/ml and serially diluted. Half of the serial dilution was frozen on dry ice for use in the Prochaska assay, as described in the Experimental Section, and half was used in the direct live cell assay. For the live cell assay, cells were incubated with 5 µM MTL8-252 at 37°C for 10 min. The reaction mix was filtered through a 0.2 µm PTFE filter and 100 µl of filtrate was admixed with 2 µl of 2M aqueous Na$_2$CO$_3$ before reading 4-MU fluorescence in the fluorimeter. (C) NQO1 enzyme was serially diluted as previously and incubated with 500 µM NADH and the MTL8-252 substrate. After 10 minutes, 2.5 µl 2M Na$_2$CO$_3$ was added per 100 µl assay fluid and fluorescence read as previously. Dotted lines indicate approximate limits of quantitation. (D) Serial dilutions of NQO1 enzyme were incubated with 50 µM menadione, 5 µM FAD and 200 µM NADH. Reduction of NADH to NAD$^+$ was followed by the quantitating NADH fluorescence ($\lambda_{ex.}$ 340nm, $\lambda_{em.}$ 440nm). All samples were tested in duplicate. Each panel is representative of at least two independent experiments.
MTL8-252 is non-toxic

We next investigated whether the use of MTL8-252 to determine NQO1 activity in live cells would affect their viability. RT112 cells were cultured in DMEM10 lacking phenol red and MTL8-252 was added either at the standard concentration used for assays (5 μM) or at a tenfold higher concentration (50 μM). The standardized assay was performed as described above, with a small amount of medium being drawn off for measurement at intervals for measurement of 4-MU. It was found that up to 1hr after addition of the substrate there were clear differences between samples with or without cells. By 12 h post-addition the substrate was reduced to its constituent 4-MU and lactone group independent of the presence of NQO1 (Figure 7a). Cell growth was followed to confluence (72 hours post-seeding). It was found that MTL8-252 and its products had no detectable effect on cell growth (Figure 7b). Hence MTL8-252 is unique in that it can be used to quantitate NQO1 levels in live cells, and does not appear to affect viability.

![Figure 7](image)

Figure 7. MTL8-252 is non-toxic and can be used to measure NQO1 activity in live cells. RT112 cells were seeded at 3 x 10^3 cells per well into a 96 well plate and incubated with either 5 μM or 50 μM MTL8-252. (A) 200 μl of medium from wells containing 5 μM MTL8-252 was taken at the indicated intervals from wells with or without cells, and immediately filtered. 4-MU was then measured by fluorescence. (B) Cell viability was measured by CellTiterGlo at 24 h intervals in the presence of either 5 μM or 50 μM MTL8-252. All assays used triplicate wells. Samples compared using Student’s t-test. ** = p < 0.01.

MTL8-252 and its fluorescent products are not retained by live cells.

Our work had so far indicated that MTL8-252 was capable of being catalysed very rapidly by live cells and its fluorescent product measured in the cell medium. This strongly supported MTL8-252 and its cleavage products being able to freely move across cell membranes. To examine whether this was indeed the case or whether the compound was retained in the cell, hDT7 and F179 cells were incubated with 10 μM MTL8-252 for 5 minutes, then centrifuged and the fluid phase removed for reading of NQO1 activity as previously. The cells were washed rapidly to remove any remaining fluid, then washed twice with a 5 minute incubation
and 5 minute centrifugation at each wash, and the wash buffers read for 4-MU. The cells were then lysed and incubated with an excess of NADH and NQO1 enzyme to allow any remaining MTL8-252 to be fully enzymatically cleaved, and fluorescence once again measured. As can be seen in figure 8, the hDT7 cells were strongly positive for NQO1, with the control F179 cells giving a low background reading. 4-MU readings in the wash buffers were negligible for both cell types. This is in keeping with the reported retention time of 4-MU in the cells of $t_{1/2}$ of 2 seconds or less [36]. When the cells were lysed after the third wash and MTL8-252 reacted to completion by the addition of excess NQO1 and NADH, it was found that remaining fluorescence was at background levels, indicating that minimal MTL8-252 was retained in the cells.

![Figure 8](image)

**Figure 8. Minimal MTL8-252 is retained inside of cells.** 5 x 10^4 F179 or hDT7 cells were assayed for NQO1 activity with MTL8-252 for 5 min as described in the materials and methods, and 200µl washes examined for 4-MU by detecting fluorescence as previously. Cells were lysed a total of 30 minutes after the initial incubation and assayed for MTL8-252 as described in the materials and methods. All data represent n=3. Samples compared using Student’s t-test. *** = $p < 0.001$. 

Conclusions

These data show that dicoumerol-inhibitable activation of MTL8-252 can be used as the basis of a method for discriminating between various levels of NQO1 activity in live cells. The kinetic studies illustrate that MTL8-252 is a very effective and rapid substrate for NQO1 which can allow the detection of elevated levels of NQO1 in relatively small numbers of cells within less than 10 minutes. The 4-MU is not entrapped within the cells and so the reaction is conveniently stopped and potentially interfering cellular debris removed in a simple filtration step. This method permits sensitive, quantitative measurement to be made which is particularly important in cases where the level of over-expression of NQO1 is incremental in cells under test relative to “basal” levels. It should be noted that the live cell assay is a measure of NQO1 activity and as such will reflect intracellular changes that will affect NQO1 activity such as intracellular NAD(P)H availability. The test has the advantage of not affecting cell viability, and the high cell membrane permeability means that cells can be assayed for NQO1 activity prior to use in assays such as flow cytometry, which require intact and/or live cells. MTL8-252 forms a unique and important tool in addition to the alternative trimethyl lock probes which are retained in the cells and which are designed for the visualisation of NQO1-high cells [20].

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Keywords: high throughput screening • NQO1 • cancer • natural products • analytical methods

3. References


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