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Fluorescent Formazans and Tetrazolium Salts – Towards Fluorescent Cytotoxicity Assays

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Abstract: Formazan-based colorimetric cytotoxicity assays, such as the MTT assay, are typically used to assess cell viability with only metabolically active cells reducing tetrazolium salts into the formazans, which is then quantified by absorbance. Fluorescence offers several advantages compared to colorimetric assays and would enable techniques such as flow cytometry and confocal microscopy to be used for analysis. Here, fluorescent formazans and corresponding tetrazolium salts were developed, including 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol)-1,3-benzene disulfonate salt (WST-1) 7 (Figure 1B) [11], and other WSTs similar to 7 have become available for use in cytotoxicity assays. Although the tetrazolium salt is reduced to a highly water-soluble formazan, the mechanism of reduction is significantly different to the reduction of MTT, with reduction by membrane-based enzymes at the cell surface. Additionally, these WSTs may be reduced by other species in the media such as glutathione [12].

Fluorescence offers several advantages compared to colorimetric assays, such as sensitivity. A fluorescent cytotoxicity assay would enable techniques such as flow cytometry and confocal microscopy to be used for analysis. The most frequently used fluorescent assay is the Alamar blue (or resazurin) assay [13], which has been used to test for bacterial contamination in milk since the 1950’s. In the Alamar blue assay, resazurin is reduced by oxidoreductases to generate the strongly fluorescent resorufin (\(\lambda_{ex}/\lambda_{em}\) 570/590 nm); however, resorufin can be further reduced to a non-fluorescent dihydroresorufin, which in cytotoxicity assays can significantly underestimate cell viability. Given the utility of the MTT assay and the benefits of fluorescence-based detection it is surprising that there is not currently a fluorescent formazan-based cytotoxicity assay for mammalian cells. 3-Cyano-1,5-ditolyl tetrazolium chloride 8, which upon reduction gives a fluorescent formazan \(\lambda_{ex}/\lambda_{em}\) 488/630 nm (Figure 1C), and has been used as a viability assay for anaerobic bacteria [14] but cannot be used for mammalian cells. The addition of triphenyltetrazolium chloride 1 to cells resulted in reduction of the pale yellow 1 to the deeply coloured triphenylformazan 2 (Figure 1A) [3]. This discovery resulted in the development of the formazan-based colorimetric cytotoxicity assay (typically called an MTT assay) using 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 3 with the dye only reduced by cells that are metabolically active to give 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenylformazan 4 (Figure 1A) [4]. The mechanism of MTT reduction is still debated, but it is clear that it is reduced intracellularly. MTT was originally thought to be up-taken by endocytosis and reduced in perinuclear vesicles, such as lysosomes and endosomes [5]; however, MTT has since been used to measure membrane potentials, which is only possible if the tetrazolium salt can permeate the cell membrane [6]. Inside cells, the tetrazolium salt 3 is reduced (in an NADH/NADPH dependent manner) to give the strongly coloured formazan 4, which is readily quantified by absorbance and can be used as a cellular viability marker [7]. However, the reduction product 4 is not soluble in aqueous media, requiring a solubilisation process, which complicates the assay and introduces error.

Sulphonated tetrazolium salts, including 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide 5 and 3-(4,5-dimethylthiazolyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium salt 6, were developed to increase the aqueous solubility of the formazans, eliminating the need for a solubilisation step in the assay (Figure 1B) [8]. However, their cellular uptake is limited and the reduction is not as efficient as for MTT 2 and requires the use of an intermediate electron acceptor (such as phenazine methosulfate) to promote reduction [9, 10]. Based on these, another class of water soluble tetrazolium salts (WSTs) was developed, including 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol)-1,3-benzene disulfonate salt (WST-1) 7 (Figure 1B) [11], and other WSTs similar to 7 have become available for use in cytotoxicity assays.

Keywords: Fluorescence, MTT, cytotoxicity assay, formazan, tetrazolium salt, synthesis

1. INTRODUCTION

Formazans were first described at the end of the 19th century but were largely ignored until the 1940’s when their potential as localisation stains in living systems was reported [1,2]. Interest in formazans increased after the discovery that the addition of triphenyltetrazolium chloride 1 to cells resulted in reduction of the pale yellow 1 to the deeply coloured triphenylformazan 2 (Figure 1A) [3]. This discovery resulted in the development of the formazan-based colorimetric cytotoxicity assay (typically called an MTT assay) using 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 3 with the dye only reduced by cells that are metabolically active to give 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenylformazan 4 (Figure 1A) [4]. The mechanism of MTT reduction is still debated, but it is clear that it is reduced intracellularly. MTT was originally thought to be up-taken by endocytosis and reduced in perinuclear vesicles, such as lysosomes and endosomes [5]; however, MTT has since been used to measure membrane potentials, which is only possible if the tetrazolium salt can permeate the cell membrane [6]. Inside cells, the tetrazolium salt 3 is reduced (in an NADH/NADPH dependent manner) to give the strongly coloured formazan 4, which is readily quantified by absorbance and can be used as a cellular viability marker [7]. However, the reduction product 4 is not soluble in aqueous media, requiring a solubilisation process, which complicates the assay and introduces error.

Sulphonated tetrazolium salts, including 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide 5 and 3-(4,5-dimethylthiazolyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium salt 6, were developed to increase the aqueous solubility of the formazans, eliminating the need for a solubilisation step in the assay (Figure 1B) [8]. However, their cellular uptake is limited and the reduction is not as efficient as for MTT 2 and requires the use of an intermediate electron acceptor (such as phenazine methosulfate) to promote reduction [9, 10]. Based on these, another class of water soluble tetrazolium salts (WSTs) was developed, including 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol)-1,3-benzene disulfonate salt (WST-1) 7 (Figure 1B) [11], and other WSTs similar to 7 have become available for use in cytotoxicity assays. Although the tetrazolium salt is reduced to a highly water-soluble formazan, the mechanism of reduction is significantly different to the reduction of MTT, with reduction by membrane-based enzymes at the cell surface. Additionally, these WSTs may be reduced by other species in the media such as glutathione [12].

Fluorescence offers several advantages compared to colorimetric assays, such as sensitivity. A fluorescent cytotoxicity assay would enable techniques such as flow cytometry and confocal microscopy to be used for analysis. The most frequently used fluorescent assay is the Alamar blue (or resazurin) assay [13], which has been used to test for bacterial contamination in milk since the 1950’s. In the Alamar blue assay, resazurin is reduced by oxidoreductases to generate the strongly fluorescent resorufin (\(\lambda_{ex}/\lambda_{em}\) 570/590 nm); however, resorufin can be further reduced to a non-fluorescent dihydroresorufin, which in cytotoxicity assays can significantly underestimate cell viability. Given the utility of the MTT assay and the benefits of fluorescence-based detection it is surprising that there is not currently a fluorescent formazan-based cytotoxicity assay for mammalian cells. 3-Cyano-1,5-ditolyl tetrazolium chloride 8, which upon reduction gives a fluorescent formazan 9 (\(\lambda_{ex}/\lambda_{em}\) 488/630 nm) (Figure 1C), and has been used as a viability assay for anaerobic bacteria [14] but cannot be used...
with eukaryotic cells as it cannot cross the eukaryotic cell membrane [15]. Although 9 is commonly used as an indicator of bacterial viability, studies have raised questions about the accuracy of the assay as different species give different responses and 8 may also be reduced by chemical additives in the culture media [16, 17]. Here, we present the design and synthesis of fluorescent formazans and their corresponding tetrazolium salts.

2. EXPERIMENTAL

All chemicals were purchased from commercial suppliers and used as received. Microwave assisted heating was done in a Biotage Initiator at 2.45 GHz at a fixed temperature. 1H and 13C NMR spectroscopy were recorded on an automated Bruker AVA400 (400 and 100 MHz, respectively) or Bruker AVA500 (500 and 126 MHz, respectively) spectrometer and the chemical shifts are quoted in relative to the solvent. ESI-MS analysis was recorded on an Agilent 1100 system with mass spectra acquired on a VG platform single Quadrupole MS-electrospray positive or negative mode. HRMS were acquired using a Bruker MicroToF II in positive or negative mode, or using a Finnigan MAT 900 XLP high-resolution double-focusing mass spectrometer. FT-IR spectra were recorded on a Bruker Tensor 27 with a golden gate accessory for solid samples. Melting points were measured on a Gallenkamp melting point apparatus.

UV absorbance analysis was conducted on an Agilent 8453 spectrophotometer using 100% solvent as blank. Fluorescence emission was measured using a Jobin Yvon SPEX Fluoro Max fluorometer with Data Max version 2.20 software in EtOH unless otherwise indicated. Quantum yields were calculated by comparison to either fluorescein (in 0.1 M NaOH) or harmaline (0.005 M H2SO4) standard.

Absorbance and fluorescence measurements for quantum yields (in EtOH) were acquired with Synergy plate reader loaded with Gen5 (1.1) software using Costar 96-well, flat-bottomed clear plates (see ESI).

2.1. Synthesis

7-Amino-4-methylcoumarin diazonium (14)

7-Amino-4-methyl coumarin (0.2 g, 1.0 mmol) was suspended in AcOH (10 mL) and cooled to 0 °C. Amberlyst A26 based nitrite resin [91] (0.7 g, 3.0 mmol) was added portion-wise over 20 min, followed by p-toluenesulfonic acid (0.6 g, 3.0 mmol). The mixture was warmed to room temperature and stirred for 1 h. The mixture was filtered and the resulting amber filtrate was poured into cold diethyl ether (100 mL) and the resulting precipitate was collected and washed with cold ether (30 mL) to give 14 as a beige solid (0.2 g, 64%). mp 135–140°C; IR v max (cm–1) 2284 (N=O), 1728 (C=O); 1H NMR (d6-DMSO, 400 MHz) δ 8.77 (1H, d, J = 2.0 Hz), 8.61 (1H, dd, J = 8.8, 2.0 Hz), 8.29 (1H, d, J = 8.7 Hz), 7.46 (2H, d, J = 8.0 Hz), 7.10 (2H, d, J = 7.9 Hz), 6.88 (1H, dd, J = 1.4 Hz), 5.1 (3H, s), 2.29 (3H, s); 13C NMR (100 MHz, d6-DMSO) δ 157.7, 151.7, 151.2, 145.5, 137.5, 129.5, 128.2, 128.0, 127.4, 125.4, 120.4, 120.2, 116.9, 22.7, 17.9; MS (ESI) m/z 381.1 [M+Na]+.

3-(7-Amino-4-methylcoumarin)-2,5-phenyl formazan (10)

Benzaldehyde phenylhydrazone 15 (78 mg, 0.4 mmol) in DMF (0.5 mL) and pyridine (0.1 mL), was cooled to 0 °C, and 7-amino-4-methylcoumarin diazonium salt 14 (144 mg, 0.4 mmol) in cold 2M HCl (0.2 mL) was added drop-wise. The mixture was stirred at room temperature for 16 h. Upon

Figure 1. (A) Triphenyltetrazolium chloride 1 and MTT 3 are reduced in vitro to coloured formazans 2 and 4, respectively. (B) Tetrazolium salts 5, 6, and 7 are used in the 2nd generation cytotoxicity assays, which result in the formation of water-soluble formazans. (C) Non-fluorescent 3-cyano-1,5-ditolyl tetrazolium chloride 8 is reduced by anaerobic bacteria into fluorescent 3-cyano-1,5-ditolyformazan 9 (λmax,λem 488/630 nm) [14]. The two methyl groups in the phenyl rings of 9 are essential for the fluorescence as the structurally similar 3-cyano-1,5-diphenylformazan is non-fluorescent.

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3-(7-amin-4-methylcoumarin)-2-phenyl tetrazolium bromide (13)

Formazan 10 (60 mg, 0.16 mmol) was suspended in EtOAc (0.68 mL) and heated to 50 °C. To this N-bromosuccinimide (74 mg, 0.20 mmol) in EtOAc (0.48 mL) was added and the mixture was stirred at 50 °C for 12 hours. After cooling to room temperature, the cream precipitate was collected by filtration and dissolved in DCM (3 mL). The solution was washed with aqueous sodium tetrafluoroborate (5 mL), dried over Na2SO4, and evaporated in vacuo to give 13 as a white powder (42 mg, 69%). No purification was required. mp 234–239 °C; IR vmax (cm\(^{-1}\)) 1721 (C=O), 1607 (N=N), 1507 (C=N), 1386, 1380, 1379 (2 ×), 134.3, 133.7, 131.7, 129.2 (2 ×), 128.3. 1H NMR (400 MHz, d6-DMSO) δ 4.79 (2H, d, J = 10.2 Hz), 7.09 (1H, t, J = 7.6 Hz), 7.63 (1H, t, J = 7.6 Hz), 7.60–7.54 (5H, m), 3.80 (1H, d, J = 12.2 Hz), 2.43 (3H, d, J = 1.1 Hz); 13C NMR (126 MHz, d6-DMSO) δ 161.2, 155.3, 152.4, 175.2, 149.7, 142.4, 136.8, 138.0, 139.0, 129.2 (2 ×), 128.2 (2 ×), 128.0, 125.9 (2 ×), 125.4, 121.5 (2 ×), 116.6, 112.6, 111.9, 102.7, 18.9; ESI-MS [M]+ m/z 381; HRMS (ESI) [M]+ m/z calculated for C29H22O2N4 381.1352 obtained 381.1069; UV/VIS (EtOH) λmax 297 nm; λex/em 350/432 nm; Φ 0.10 (EtOH) and 0.04 (in acidic PBS/MeOH).

3-(Spiroisobenzofuran-1(3H),9'-[9H]xanthen)-3-one,3'-hydroxy-6'-phenyl-4,5-phenyltetrazolium bromide (16)

Fluorescein formazan 16 (11.3 mg, 0.02 mmol) was suspended in EtOAc (0.3 mL) and heated to 50 °C. N-bromosuccinimide (10 mg, 0.05 mmol) in EtOAc (0.3 mL) was added, and the mixture stirred at 50 °C for 12 hours. The precipitate was collected by filtration to give 16 (9.7 mg, 86%). No purification was required. mp 171–175 °C; IR vmax (cm\(^{-1}\)) 3359 (OH), 3034 (CH), 1758 (C=O), 1596 (C≡N); 1H NMR (400 MHz, MeOD) δ 8.39 (2H, d, J = 7.1 Hz), 8.10 (1H, d, J = 7.3 Hz), 8.05 (2H, d, J = 8.6 Hz), 7.88–7.87 (3H, m), 7.85–7.81 (5H, m), 7.77 (2H, d, J = 7.3 Hz), 7.76–7.72 (5H, m), 7.51 (1H, d, J = 8.6 Hz), 7.30 (1H, d, J = 8.0 Hz), 6.98 (1H, d, J = 8.0 Hz), 6.91 (1H, s); 13C NMR (126 MHz, MeOD) δ 161.3, 160.8, 157.9, 156.5, 151.8, 151.5, 145.9, 145.2, 145.1, 141.0, 139.9, 139.8 (2 ×), 138.6, 138.0, 137.9 (2 ×), 137.8, 137.5, 137.5 (2 ×), 135.9 (2 ×), 134.3, 133.7, 131.7, 129.2 (2 ×), 129.1, 128.5, 128.4 (2 ×), 128.3, 127.3, 123.9, 123.8, 122.2, 118.9, 118.8; ESI-MS m/z 613.3 [M]+; UV/VIS (EtOH) λmax 300 nm; λex/em 370/432; Φ 0.01.

1-(O-Methylrhodol)-3,5-diphenylformazan (12)

Sodium nitrate (12 mg, 0.17 mmol) in water (300 μL) was added to O-methylrhodol 22 (50 mg, 0.15 mmol) in 2M HCl (0.75 mL) at 0 °C and stirred for 60 minutes. This solution was added to a solution of diphenyldihydrazine 15 (30 mg, 0.15 mmol) in cold DMF (1.75 mL) and pyridine (0.35 mL), and the reaction brought to room temperature. The reaction was stirred for 22 hours and then poured into water (10 mL). 2M HCl was added until the solution became acidic (pH 1), the resultant crystals were collected by filtration and washed with water (20 mL). The solid was dried in a vacuum oven at 40 °C to give dark red crystals (20 mg, 25%). No purification required. mp 201–204 °C; IR vmax (cm\(^{-1}\)) 3061 (NH), 1760 (C=O), 1614 (C≡N), 1505 (NH), 1218 (CN); 1H NMR (500 MHz, CDCl3) δ 15.15 (1H, s), 8.16 (2H, d, J = 7.3 Hz), 8.07 (1H, d, J = 7.6 Hz), 7.86 (2H, d, J = 7.6 Hz), 7.71 (1H, t, J = 6.5), 7.66 (1H, t, J = 7.4 Hz), 7.59–7.52 (3H, m), 7.51–7.43 (3H, m), 7.41 (1H, t, J = 7.3 Hz), 7.22 (1H, d, J = 7.5 Hz), 7.12 (1H, dd, J = 8.5, 1.9 Hz), 6.86–6.79 (2H, m), 6.73 (1H, d, J = 8.8 Hz), 6.65 (1H, dd, J = 8.8, 2.4 Hz), 3.85 (3H, s); 13C NMR (126 MHz, CDCl3) δ 169.5, 161.4, 153.2, 152.5, 152.4, 150.5, 147.1, 141.6, 136.9, 135.1, 130.3, 129.8, 129.5 (2 ×), 129.2, 129.0, 128.4 (2 ×), 128.0, 126.6, 126.1 (2 ×), 125.1, 123.9, 121.1 (2 ×), 115.2, 112.7, 111.8, 111.0, 103.8, 100.9, 83.0, 55.6; HRMS (ESI) m/z [M+H]+ calculated for C36H32O3N4 553.1870 obtained 553.1726; UV/VIS (EtOH) Λmax 298 & 483 nm; Λex/em 350/428 nm; Φ 0.18.
1-(O-Methylrhodol)-3,5-diphenyl tetrazolium salt (24)

Formazan 12 (20 mg, 0.03 mmol) was suspended in EtOAc (0.6 mL) and warmed to 50 °C. To this N-bromosuccinimide (16 mg, 0.09 mmol, 2.5 eq.) in EtOAc (0.4 mL) was added, and the mixture was stirred at 50°C for 16 hours. The precipitate was collected by filtration, to afford a cream powder (15 mg, 83%). No purification was required. mp 142–147 °C; IR νmax (cm−1) 1695 (C=O), 1102 (COC); 1H NMR (500 MHz, CDCl3) δ 8.17 (2H, d, J = 7.4 Hz), 8.04 (1H, d, J = 7.6 Hz), 7.89 (1H, d, J = 2.2 Hz), 7.82 (2H, d, J = 7.9 Hz), 7.75 (1H, t, J = 7.4 Hz) 7.67–7.64 (2H, m), 7.61–7.49 (5H, m), 7.33 (1H, dd, J = 8.6, 2.1 Hz), 7.18 (1H, d, J = 7.6 Hz), 6.92 (1H, d, J = 8.6 Hz, H8), 6.79 (1H, d, J = 1.9 Hz), 6.74–6.66 (2H, m), 3.85 (3H, d, J = 7.2 Hz); 13C NMR (126 MHz, CDC13) δ 176.9, 168.8, 166.0, 161.8, 152.1, 151.7, 151.6, 135.8, 134.1, 134.0, 133.2, 133.0, 130.5 (2 ×), 130.1, 129.4 (2 ×), 128.8, 128.0 (2 ×), 126.2 (2 ×), 125.8, 125.4, 124.9, 124.0, 122.9, 116.0, 113.1, 109.8, 101.0, 81.1, 55.7; HRMS (ES) m/z [M]+ calculated for C34H25N4O4+ 555.1714 obtained 555.3324.

2.1. Reduction of tetrazolium salt in cells

HEK293T cells were cultured in T75 culture flasks in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10 % FBS, 4 mM glutamine, and 100 units/mL of penicillin/streptomycin, and grown to 50–60% confluence. Tetrazolium salts 13, 16, or 24 (0.5 mM in 1 mL DMSO) were added to phenol red free DMEM media (10 mL) to give a final concentration of 50 μM. DMEM cell media was removed and replaced with DMEM with tetrazolium salts and the cells were incubated at 37 °C for 24 hours. The media was removed and the cells washed with PBS three times. The PBS and media were combined and centrifuged (1200 rpm) for 15 minutes. The supernatant was removed leaving the formazan residue, which was dried at 40 °C in a vacuum oven, and then dissolved in EtOH (5 mL) and fluorescence was recorded. The samples were also analysed by mass spectrometry to confirm presence of the formazans 10–12.

3. RESULTS AND DISCUSSION

It is difficult to predict whether or not a molecule will be fluorescent; however, planarity and extended conjugation are common features in fluorescent molecules [18], but not all highly conjugated molecules are fluorescent (for example MTT 3 and the corresponding formazan 4 are not fluorescent). Here, we targeted fluorescent formazans using three known fluorescent cores (coumarin, fluorescein and rhodol). The hypothesis was that a “switch-on” fluorescent assay, similar to the MTT assay, would be possible by introducing a fluorophore into the formazan, with disruption of the conjugated system preventing or reducing fluorescence, i.e., the positive charge of the corresponding tetrazolium salt (Figure 2).

![Design of fluorescent formazans 10, 11 and 12, incorporating coumarin, fluorescein or rhodol-based fluorophores, for cytotoxicity assays.](image-url)

Figure 2. Design of fluorescent formazans 10, 11 and 12, incorporating coumarin, fluorescein or rhodol-based fluorophores, for cytotoxicity assays. The fluorescence was postulated to be quenched (“turned-off”) in the corresponding tetrazolium salts, proving a “switch-on” mechanism for the fluorescence detection upon cellular reduction.

![Synthesis of fluorophore-based formazans and tetrazolium salts](image-url)

Scheme 1. (a) Amberlyst A26 based nitrite resin, AcOH, p-TsOH, 0 °C to rt, 1 h, (64%) (b) 2M HCl, NaNO3, benzaldehyde phenylhydrazone 15, pyridine, DMF, 4 h (62%); (c) NBS, EtOAc, 50 °C, 20 h (69%).

3.1. Synthesis of fluorophore-based formazans and tetrazolium salts

The first target molecule was 10, which can be considered to be a formazan attached to the fluorophore 7-amino-4-methyl coumarin (AMC), which has an available aniline group for incorporation into the formazan core. Coumarins are of particular interest as they are known to have high fluorescence even following large structural changes [19, 20]. The corresponding coumarin-based tetrazolium salt 13 was synthesised in three steps (Scheme 1). 7-AMC [20] was diazotised using a solid-supported nitrite resin [21] and 14 was isolated as the p-toluenesulfonic acid salt. Benzaldehyde phenylhydrazone 15 [22] was added to the diazonium salt 14 in 2M HCl to give the coumarin–formazan 10 in 62% yield. Oxidation with N-bromosuccinimide (NBS) gave the corresponding tetrazolium 13 in 69% yield.
The “fluorescein-based” tetrazolium salt 16 was synthesised in five steps from fluorescein. Fluorescein monotriflate 17 was synthesised by microwave heating fluorescein and N-phenyl-bis(trifluoromethanesulfonimide) (PhN(OTf)2) in the presence of K2CO3 [23], followed by a Suzuki coupling with 4-aminophenyl boronic acid pinacol ester under thermal conditions to give 18 in 71% yield (Scheme 2) [24]. Diazotisation of 18 and subsequent treatment with benzaldehyde phenylhydrazone 15 afforded the desired fluorescein–formazan 11 in 29% yield, with oxidation using NBS giving the tetrazolium salt 16 in 86% yield.

To allow regioselective incorporation of the formazan moiety into a rhodol-based fluorophore (compound 12), mono O-methyl fluorescein 19 was synthesised [25]. Fluorescein was first treated with methyl iodide (with concomitant methyl ether and methyl ester formation) and subsequent hydrolysis with LiOH gave 19 in 71% yield. Conversion of the phenol moiety in 19 to the corresponding aniline 22 was achieved by the method of Grimm [26], i.e., triflation (compound 20), followed by Buchwald-Hartwig amination with tert-butyl carbamate (compound 21) and deprotection to afford O-methyl rhodol 22 as a TFA salt in 56% overall yield over three steps (Scheme 3). O-Methyl rhodol 22 was treated with sodium nitrite, to generate the diazonium salt 23, which was then treated with diphenylhydrazone 15 to furnish the desired formazan 12 in moderate 25% yield (Scheme 3). NBS oxidation gave the
corresponding tetrazolium salt 24, which precipitated from the reaction mixture and was isolated by filtration in 83% yield.

3.2. Optical properties of the tetrazolium salts and formazans

The optical properties of the fluorophore-based formazans and their corresponding tetrazolium salts were analysed and compared to the optical properties of the “parent fluorophores” (Table 1). The formazans 10, 11 and 12 all showed fluorescence emission between 420 and 470 nm (in EtOH) (Figure 3). The emission wavelength of the formazans was not dependent on the emission wavelength of the parent fluorophore.

Table 1. Optical properties of the synthesised formazans and tetrazolium salts, and their “parent” fluorophores (in EtOH unless otherwise stated).

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ_ex (nm)</th>
<th>λ_em (nm)</th>
<th>Φ</th>
</tr>
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<tbody>
<tr>
<td>AMC</td>
<td>350</td>
<td>426</td>
<td>0.78</td>
</tr>
<tr>
<td>10</td>
<td>370</td>
<td>468</td>
<td>0.11</td>
</tr>
<tr>
<td>13</td>
<td>350</td>
<td>432</td>
<td>0.10</td>
</tr>
<tr>
<td>13 pH 4[b]</td>
<td>390</td>
<td>432</td>
<td>0.04</td>
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<tr>
<td>18</td>
<td>480</td>
<td>520</td>
<td>0.26</td>
</tr>
<tr>
<td>11</td>
<td>370</td>
<td>445</td>
<td>0.19</td>
</tr>
<tr>
<td>16</td>
<td>370</td>
<td>434[b]</td>
<td>0.18</td>
</tr>
<tr>
<td>22</td>
<td>428</td>
<td>523</td>
<td>0.74</td>
</tr>
<tr>
<td>12</td>
<td>350</td>
<td>428</td>
<td>0.18</td>
</tr>
<tr>
<td>24</td>
<td>350</td>
<td>n/f[c]</td>
<td>–</td>
</tr>
</tbody>
</table>

[a] recorded in acidified PBS/MeOH [b] weakly fluorescent, 4.8-fold difference in emission intensity compared to 11 [c] could not be determined [d] non-fluorescent.

Coumarin-based formazan 10 showed a 42 nm red shift compared to AMC, possibly due to the increased conjugation gained from the formazan structure; however, the quantum yield of 10 was lower than that of reported for AMC (Φ 0.11 versus 0.78) [27]. The coumarin-based tetrazolium salt 13 remained fluorescent in EtOH (λ_em 432 nm, Φ 0.10) with a 36 nm blue-shift in fluorescence compared to 10 (4-fold difference between the emission intensities (λ_em 350 nm) of 13 and 10) (ESI, Figure S1). However, under aqueous acidic conditions (1:1 MeOH/PBS with 1% HCl), 13 was almost non-fluorescent whereas formazan 10 retained its fluorescent properties (60-fold difference in emission intensities) (Figure 4A). The difference in fluorescence intensity between 13 and the reduced form 10 could thus be exploited in a cytotoxicity assay where the tetrazolium salt is reduced to a fluorescent formazan and extracted in acidic PBS/MeOH (similar to that used for the MTT assay).

Aniline modified fluorescein 18 had a fluorescence emission maximum at 520 nm (λ_em 480 nm) and a quantum yield of 0.26. When this moiety was incorporated into formazan 11, the emission was blue-shifted by 75 nm to 445 nm (Φ 0.19) (ESI, Figure S2). The corresponding tetrazolium salt 16 was considerably less fluorescent than the formazan counterpart (Figure 4B). Similarly with O-methyl rhodol-based formazan 12 (λ_em 370/427 nm, Φ 0.18) the emission was blue-shifted by 96 nm compared to parent fluorohore O-methyl rhodol 22 (λ_em 523 nm) (ESI, Figure S3). The tetrazolium salt 24 was non-fluorescent (λ_em 350 nm) showing that as postulated the positive charge of the

![Figure 3. Comparison of fluorescence emission of formazans 10 (λ_ex 370/468 nm), 11 (λ_ex 370/445 nm), and 12 (λ_ex 350/428 nm) in EtOH (recorded at 1 µM).](image)

![Figure 4. (A) Fluorescence emission of coumarin-based formazan 10 (λ_ex/λ_em 370/468 nm) and the corresponding tetrazolium salt 13 (λ_ex 390 nm) in acidic PBS–MeOH (1:1). (B) Fluorescence emission of fluorescein–formazan 11 emission (λ_ex/λ_em 370/445 nm) and its tetrazolium salt 16 emission (λ_ex/λ_em 370/434 nm in EtOH); (C) Fluorescence emission of O-methylrhodol-based formazan 12 emission (λ_ex/λ_em 350/428 nm) compared to its tetrazolium salt 24 (λ_ex 350 nm) recorded in EtOH.](image)
fluorescence of incorporated fluorescein and emission of directly dependent on the parent fluorophores, reduced to the fluorescent formazans with cells. The in vitro large structural changes to the core structure did not affect the were synthesised by incorporation of a known fluorophore. The corresponding tetrazolium salts were solubilised in EtOH, and the fluorescence was measured with the expected emission observed with all the samples (Figure 5C, ESI Figure S4). For the coumarin-based tetrazolium salt 13 the cell-based reduction into 10 was also confirmed by mass spectrometry of both the supernatant and cell lysate, which confirmed that 13 had been taken up by the cells. Coumarin-based 10 and 13 did not show notable cytotoxicity in a MTT assay at 100 µM, whereas with formazans 11 and 12 were non-cytotoxic up to 10 µM (tetrazolium salts 16 and 24 were not toxic at 50 µM) (ESI Figure S5).

CONCLUSION

The fluorescent formazans 10, 11 and 12, and their corresponding tetrazolium salts 13, 16 and 24, respectively, were synthesised by incorporation of a known fluorophore. The large structural changes to the core structure did not affect the in vitro activity of the tetrazolium salts, which were successfully reduced to the fluorescent formazans with cells. The emission wavelengths of the fluorophore conjugated formazans were not directly dependent on the parent fluorophores, i.e., the fluorescence of 10 was red shifted compared to AMC whereas both fluorescein-based 11 and O-methyl rhodol-based 12 were blue shifted to the green region and were lower than the emission of 10. The tetrazolium moiety was able to quench the fluorescence of incorporated fluorescein and O-methyl rhodol (compounds 16 and 24, respectively), whereas with the coumarin-based 13 the fluorescence was only quenched in acidic medium, which is typically used in MTT assay. These new tetrazolium salts and their corresponding formazans offer a step forward in the development of “turn on” fluorescent cytotoxicity assays.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Fluorescence emission of coumarin-based 10 and 13.

Figure S2. Fluorescence emission of 11 compared to 18.

Figure S3. Fluorescence emission of O-methylrhodol 22 and 12.

Figure S4. Fluorescence of recovered 11 and 12 from HeLa cells.

Figure S5. MTT cytotoxicity assays


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