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Review

Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives

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ABSTRACT

For many years various tetrazolium salts and their formazan products have been employed in histochemistry and for assessing cell viability. For the latter application, the most widely used are 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and 5-cyano-2,3-di-(*p*-tolyl)-tetrazolium chloride (CTC) for viability assays of eukaryotic cells and bacteria, respectively. In these cases, the nicotinamide-adenine-dinucleotide (NAD(P)H) coenzyme and dehydrogenases from metabolically active cells reduce tetrazolium salts to strongly colored and lipophilic formazan products, which are then quantified by absorbance (MTT) or fluorescence (CTC). More recently, certain sulfonated tetrazolium, which give rise to water-soluble formazans, have also proved useful for cytotoxicity assays. We describe several aspects of the application of tetrazolium salts and formazans in biomedical cell biology research, mainly regarding formazan-based colorimetric assays, cellular reduction of MTT, and localization and fluorescence of the MTT formazan in lipidic cell structures. In addition, some pharmacological and labeling perspectives of these compounds are also described.

1. Introduction

Cell proliferation and viability assays for cultured cells have been largely based on the reduction of colorless tetrazolium salts to colored formazans. However, other methods such as ^{3}H-thymidine or bromodeoxyuridine uptake, clonogenic assays, staining, and/or redox probes (trypan blue, fluorescein diacetate and derivatives, protein detection by sulforhodamine B, resazurin, *etc*) are also known and widely used (Horobin and Kiernan, 2002; Stoddart, 2011; Stockert and Blázquez-Castro, 2017, pp. 532–539). Several colorimetric procedures for assessing cell viability have been reviewed (Vega-Avila and Pugsley, 2011; Van Tonder et al., 2015). Their principal advantages are avoiding the use of radioisotopes, together with easy and direct quantitative evaluation of viable cells. The applications and comparative results using the tetrazolium salt (MTT) assay and other viability methods (resazurin, neutral red, sulforhodamine B) have been described (Skehan et al., 1990; Van Tonder et al., 2015; Da Luz et al., 2016). In the case of resazurin (a pH and redox indicator of cell viability), the blue oxazone chromophore is easily reduced to the red and fluorescent resorufin (Horobin and Kiernan, 2002). A disadvantage is that further reduction gives a final colorless product, N-hydroresorufin. Resazurin is currently used to detect biochemical activity and cytotoxicity in many different cell types (Visser et al., 1990; White et al., 1996; Nociari et al., 1998).

Colorimetric procedures are based on the extraction of the biologically/biochemically formed water-insoluble formazan by organic solvents, followed by its measurement with spectrophotometers or plate readers (Morgan, 1998; van Meerloo et al., 2011). In this context sulfonated tetrazolium salts are useful reagents because of the water solubility of the colored formazan products. Formazan compounds were first described at the end of the 19th century but were rather overlooked until their potential as localization stains in living systems and redox viability probes were reported much later (Mosmann, 1983; Carmichael et al., 1987).

Our aim with this review is to provide a concise but comprehensive overview on tetrazolium-based viability assays in Cell Biology, considering key historical developments and methods, whilst also noting new applications and current views of the mechanisms of action of tetrazolium salts (mainly MTT). Incidentally, we would also like to celebrate the 60th anniversary (1957–2017) of the first publication describing MTT use in the life sciences (Pearse, 1957).

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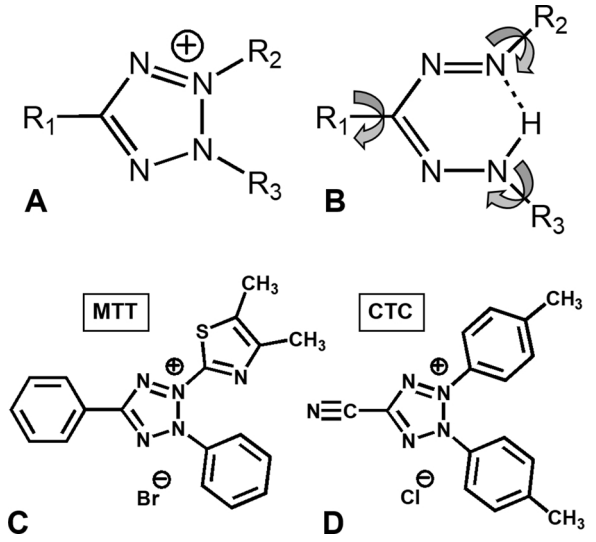

Fig. 1. Chemical structure of the cationic tetrazolium ring (A), the reduced formazan product (B, showing the rotation freedom of aromatic rings (R_1, R_2, R_3) as curved arrows), and the probes MTT (C) and CTC (D). Double bonds and charges are conventionally shown in formal positions.

2. Tetrazolium salts and formazans

The structures of the tetrazolium ring and the corresponding formazan are shown in Fig. 1A and B respectively, and their properties and uses have been extensively described (Altman, 1976; Lillie, 1977, p. 227–228; Horobin, 1982; Seidler, 1992; Horobin and Kiernan, 2002, chapter 13). Different mono- and di-tetrazolium salts have been extensively used in histochemical applications (Horobin and Kiernan, 2002, see chapter 13; Kiernan, 2015) but only mono-tetrazolium compounds are routinely employed for assessing cell viability. The mono-tetrazolium salts 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, thiazolyl blue), and 5-cyano-2,3-di-(*p*-tolyl) tetrazolium chloride (CTC) are represented in Fig. 1C and D. Compared to MTT, which has three aromatic rings, the more efficient fluorescence of CTC is probably related to its more restricted molecular rotational freedom resulting from possessing only two aromatic rings (Stockert and Blázquez-Castro, 2017, pp. 467–469).

MTT was introduced for the histochemical detection of dehydrogenase activity by Pearse (1957). Neotetrazolium chloride (NTC), nitroblue tetrazolium chloride (NBT) and tetranitroblue tetrazolium chloride (TNBT) are widely used in histochemical detection of dehydrogenase activity, and as redox indicators in combination with indigogenic methods (Horobin and Kiernan, 2002, pp. 164–165 and 166 respectively; Van Noorden, 2010; Kiernan, 2015). Protons generated by the dehydrogenase-catalyzed oxidation of substrates are picked up by the corresponding coenzyme. In the case of some tetrazolium salts, the reduced co-enzyme reduces electron carriers such as phenazine methosulfate (PMS) which, in turn, transfers the electrons to the tetrazolium salt as final electron acceptor producing a water-insoluble formazan.

In addition, NBT is reduced by the metabolites (superoxide radical) produced specifically by the plasma membrane-bound NADPH oxidase, giving a blue di-formazan (Honoré et al., 2003). Blue tetrazolium chloride (BTC) was developed and used for demonstration of enzymes in normal and neoplastic tissues (Rutenburg et al., 1950), and as an indicator for seed germination and nanomolar detection of reducing sugars (Jue and Lipke, 1985).

Formazans derived from CTC, MTT and other non-sulfonated compounds are lipophilic, and only soluble in organic solvents and oils. In contrast, on account of the water solubility of their formazan products, sulfonated derivatives of some tetrazolium salts (Fig. 2) are also used for viability assays (see Section 3). Several reductants, and in particular many thiol-containing biomolecules, mediate in biological redox signaling (Winterbourn and Hampton, 2008), and can reduce MTT and other tetrazolium salts. Ascorbic acid, cysteine, dihydrolipoic acid, glutathione, glutathione S-transferase and tocopherols are known examples (York et al., 1998; Bhupathirajua et al., 1999; Chakrabarti et al., 2000; Stockert et al., 2012).

Although insoluble formazan deposits can be microscopically observed due to their intense color, their direct localization within living cells has been rather overlooked (see Section 4). The TNBT formazan from glucose-6-phosphate dehydrogenase activity in living isolated hepatocytes has been observed to occur in the cytoplasm (Winzer et al., 2001). Early studies showed that formazan deposits are birefringent and can be detected under polarized light (Pfeiffer, 1964; Seidler and Scheuner, 1980). At present, sulfonated tetrazolium salts that are reduced to water soluble formazans using intermediate electron acceptors have found applications for cell viability assays. Whether or not reduction of these reagents is mechanistically similar to that of MTT will be discussed, see Section 3.

3. Viability assays

MTT is one of the most widely used probes for cell viability, proliferation, cytotoxicity, chemo- and radiosensitivity studies *in vitro* (Mosmann, 1983; Carmichael et al., 1987; Merlin et al., 1992). Compared with alternative methods, the MTT viability assay is simpler and less time-consuming, and also allows semi-automated evaluation using multi-well plates and photometric plate readers. It has been suggested that the net positive charge on tetrazolium salts such as MTT and NBT is the predominant factor involved in their uptake by live cells (Berridge et al., 2005). However, it is more likely that the lipophilic character of such salts is the more significant property, as it directly controls membrane permeability, see Table 1. Basic aspects and general applications of the MTT method have been extensively reviewed (Vistica et al., 1991; Thom et al., 1993; Marshall et al., 1995; Morgan, 1998; Horobin and Kiernan, 2002, p. 162; Berridge et al., 2005; van Meerloo et al., 2011; Stockert et al., 2012).

For cell viability assessment, following reduction by dehydrogenases and reducing agents present in metabolically active cells, the yellow MTT is turned into a water-insoluble violet-blue formazan product (absorption peak at 562 nm with shoulders at 512 and 587 nm, when dissolved in sunflower oil). After MTT reduction, the culture medium is currently removed, formazan deposits extracted and colorimetrically assessed. Although Mosmann (1983) originally used acid isopropyl alcohol for extraction, Carmichael et al. (1987) found that this solvent resulted in low optical absorption values and suggested the use of DMSO or mineral oil as alternatives. In strongly acidic media, the presence of the cationic MTT formazan results in a complete disappearance of the absorption at 575 nm (Wang et al., 2014). The acidic pH used in solubilization solvents has two effects: it modifies the absorption spectrum of the cationic formazan and also changes the absorption of any pH indicator if present. Both processes are methodologically misleading and should be avoided. In spite of that, acidified isopropanol keeps on being in use (van Meerloo et al., 2011), but DMSO is a better alternative for formazan solubilization. Other solvents could be also adequate (*i.e.* dioxane, cyclohexane, tetrahydrofuran, dimethylformamide, *etc.*).

It was initially thought that such enzymatic reduction took place in the mitochondria, due to mitochondrial dehydrogenases action. MTT reduction was thus considered a measure of mitochondrial activity, but the process is not dependent on succinate as previously believed (Berridge and Tan, 1993). The origin of this misconception could have some historical rationale, mainly based on early studies by Slater et al. (1963) on succinate-tetrazolium systems, and proposals of MTT for viability assessment by Mosmann (1983) and Carmichael et al. (1987). It is now known that in viability assays, MTT is mainly reduced by the coenzyme NAD(P)H and glycolytic enzymes of the endoplasmic