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

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Short title: Tetrazolium salts and formazans in Cell Biology

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.

Keywords:
Cytotoxicity; fluorescence imaging; formazan localization; MTT; tetrazolium salts; viability assays

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).

2. Tetrazolium salts and formazans

The structures of the tetrazolium ring and the corresponding formazan are shown in Fig. 1 A 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 Figs. 1 C 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 Merloo 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 reticulum (Berridge et al., 1996, 2005), see Section 4.

Therefore, cellular MTT reduction should be rather viewed as a measure of the rate of glycolytic NAD(P)H production.

Exemplar sulfonated tetrazolium salts — MTS (Dunigan et al., 1995), XTT (Roehm et al., 1991) and WST-1 (Tan and Berridge, 2000) — are shown in Fig. 2. The cellular uptake of these second-generation tetrazolium salts is limited, as their hydrophilic character turns them largely cell-impermeable, see Table 1. Consequently, they are not as readily reduced as MTT. In addition, the reduction mechanisms of sulfonated tetrazolium salts (WSTs) such as WST-1 is significantly different from MTT reduction. Note here that WST-1 is, unlike MTT, predicted to accumulate in the plasma membrane due to its amphiphilic character, see Table 1. All sulfonated tetrazolium salts use intermediate electron acceptors such as PMS or menadione and produce water-soluble sulfonated formazans (Marshall et al., 1995). PMS has been used as an electron-transfer agent in place of flavin coenzymes and in the histochemical detection of dehydrogenases by tetrazolium salts (Farber and Buelding, 1956), but possible dangerous effects and mutagenic potential on living cells cannot be ruled out (Venitt and Crofton-Sleigh, 1979), which are disadvantageous for its use.

Compared to MTT, MTS has the advantage that the viability assay can be carried out sequentially several times for kinetic studies, and no extracellular formazan crystals are formed. MTS is used on cell cultures in conjunction with PMS for cytotoxicity, proliferation and viability assays. The amount of sulfonated formazans delivered into the culture medium and colorimetrically detected is directly proportional to the number of live cells in culture (Cory et al., 1991; Roehm et al., 1991). Interestingly, the histochemical reagent zincon (Fig. 2, see Section 5) is also a sulfonated formazan derivative (Lillie, 1977, p. 234–235).

In the presence of electron acceptors, both WST-1 and XTT are rapidly reduced by NAD(P)H and several reducing agents in the absence of cells and dehydrogenases (Berridge et al., 1996). Consequently, caution should be exercised in using sulfonated tetrazolium salts in viability assays. With whole cells, XTT and WST-1 reduction appears to be extracellular or associated with the plasma membrane, as well as related to the superoxide anion (Berridge et al., 1996). In this context, it may be significant that WST-1 will accumulate in the plasma membrane, see Table 1. It has been previously shown that superoxide is involved in the reduction of NBT and INT (Liochev and Fridovich, 1995). Problems have been also reported when using XTT and WST-8 for *Candida* assays (Kuhn et al., 2003).

In addition to its use on mammalian cell cultures, the colorimetric MTT assay is employed for testing the viability of protozoan (Dias et al., 1999) and sperm cells of several species (Aziz, 2006; Van der Berg, 2015). Microbiological viability assay applications of tetrazolium salts are also well known and widely used (Thom et al., 1993; Bhupathirajua et al., 1999; Wang et al., 2014; Villegas-Mendoza et al., 2015; Grela et al., 2015), mainly for biofilms (Pérez et al., 2010; Trafny et al., 2013). MTT, CTC, XTT, and INT are the most widely used tetrazolium salts for assessment of microbial viability. In the case of INT, toxicity has been reported for prokaryote cells (Villegas-Mendoza et al., 2015).

4. Localization of formazans

Although in early studies Koenig (1965) claimed that lysosomes and mast cell granules were stained *in vivo* by tetrazolium salts (NTC, NBT), this seems unlikely. In the case of MTT, Bernas and Dobrucki (2002) have shown that most MTT formazan deposits imaged using backscattered light under confocal microscopy are not coincident with the mitochondria, but the endoplasmic reticulum, cytosol, and plasma membranes seem to participate as the reduction sites of MTT in mammalian cells. Regarding the mechanisms and localization of several tetrazolium salts undergoing reduction, previous studies had suggested that different cell organelles such as the plasma membrane, mitochondria, endosomes and lysosomes could be involved in the process (Liu et al., 1997; Bernas and Dobrucki, 1999, 2000; Rich et al., 2001).

Although the microscopic appearance of the intracellular formazan deposits is generally granular, it has been claimed that they are structurally crystalline (Bernas and Dobrucki, 2004). Following MTT incubation, and in addition to the cytoplasmic granules, a variable quantity of extracellular needle-shaped formazan crystals can be observed. The origin of this phenomenon is not clear. There are several suggested causes, one being that following intracellular MTT reduction, formazan deposits are extruded to give rise to the extracellular needles (Liu et al., 1997; Diaz et al., 2007). However, it has also been said that intracellular crystal deposits are not released by exocytosis, but pierce the plasma membrane and produce cell death (Bernas and Dobrucki, 2004). In addition, MTT can be reduced by the cell culture medium alone to form extracellular formazan (Young et al., 2005), and the plasma membrane-bound NADPH oxidase could also produce formazan (Honoré et al., 2003). These mechanisms might be involved in the appearance of the extracellular MTT formazan crystals.

Following current MTT assay protocols, normal cell cultures (controls, without any experimental treatment) show intracellular round formazan granules, and very few or not at all extracellular needle-shaped crystals, although sometimes, variable amounts of extracellular crystals are found in control cell cultures. This feature remains basically unexplained at present and should be further investigated. The occurrence of extracellular formazan deposits could introduce a serious error when assessing cell viability, giving rise to false positive values. Regarding formazan solubility, the consequences of the lipophilic character of many formazans (see Section 1) are however undisputed. Localization artefacts resulting from this lipophilicity effect arising in dehydrogenase histochemistry have been discussed (Horobin, 1982).

As it will usually be the case that cell uptake and accumulation of MTT precede its reduction, localization of MTT tetrazolium salt and formazan has been investigated using a quantitative structure-activity relationship (QSAR) modeling approach (Stockert et al., 2012). In this context note that it has been long been known that probes for the endoplasmic reticulum first label mitochondria (Terasaki, 1994). A QSAR analysis showed that this correlated with a probe being both lipophilic and amphiphilic (Horobin et al., 2006, Horobin et al., 2015). Of current significance, the QSAR parameters of MTT (see Table 1) indicate that it also is both cationic and amphiphilic (Horobin and Stockert, 2011). Noting that such vital probes label both mitochondria and endoplasmic reticulum, and that treatment with MTT typically involves long incubation times and high tetrazolium salt concentrations, and considering the oxidizing power of mitochondria, it is likely that the selective reduction of MTT occurs at the endoplasmic reticulum and not at the mitochondria.

Within cells, MTT formazan does not appear as crystalline needles but as granules that correspond to lipid droplets (Diaz et al., 2007; Stockert et al., 2012; Stockert and Blázquez-Castro, 2017, pp. 467-469). Given the hydrophobicity of MTT formazan molecules, it comes as no surprise that once formed intracellularly they will diffuse by partitioning and accumulate at hydrophobic structures: membranous and lipid storage organelles. Again, the QSAR analysis supports the view that the stain has accumulated in lipid droplets, see Table 1.

MTT formazan deposits and fluorescent signals from specific organelles subjected to image processing and analysis allowed comparison of the localization of MTT formazan product with those of mitochondria and lysosomes, showing that these organelles do not accumulate the formazan product (Stockert et al., 2012). Fig. 3 shows that the localization of mitochondria (based on the autofluorescent NAD(P)H and FAD signals) and MTT formazan signal are not coincident.

To confirm the localization of MTT formazan in lipophilic structures, lipid droplets can be easily induced within cultured cells by incubation for several hours with a 10:1 (v/v) Dulbecco's modified Eagle's medium–sunflower oil emulsion (Stockert et al., 2010; Horobin and Stockert, 2011; Stockert and Blázquez-Castro, 2017, p. 344). After incubation with an oil emulsion, MTT induces a strong accumulation of formazan in the lipid droplets (Fig. 4). In this case, the amount of formazan is clearly greater than that of the controls, which suggests an increase of the reducing power in cells treated with oil emulsions. This point deserves further investigation because of the

possible influence of lipid uptake and lipogenic processes on the results of MTT viability assays. Interestingly, it has been recently reported that on account of their lipidic nature, empty-liposomes interfere with MTT assays (Angius and Floris, 2015).

5. Fluorescence imaging

It is currently claimed that MTT formazan is non-fluorescent (Bernas and Dobrucki, 2004; Ladyman et al., 2016). However, CTC formazan shows red fluorescence (λexc: 380-440 nm, λem: 625 nm) in the solid state (Severin et al., 1985; Stellmach and Severin, 1987). This feature has found uses in flow cytometry and fluorescent viability assays of aerobic and anaerobic bacteria in cultures, biofilms, marine bacterioplankton, and environmental samples (Severin et al., 1992; Rodriguez et al., 1992; Sieracki et al., 1999; Bhupathirajua et al., 1999). Unfortunately, CTC cannot be used in mammalian cells as it does not cross the eukaryotic cell membrane (Frederiks et al., 2006), perhaps due to its being retained in the plasma membrane due to its strongly amphiphilic character, see Table 1. Although CTC is used as an indicator of both extracellular reduction and bacterial viability (Gruden et al., 2003), several studies have raised questions about the accuracy of the assay as different species give different responses (Ladyman et al., 2016).

As compared to MTT formazan with three aromatic rings, the more efficient fluorescence emission of CTC formazan is probably related to the lower rotational freedom of its two aromatic rings, compare Figs. 1 C and 1 D (Stockert and Blázquez-Castro, 2017, pp. 467-469). It has also been postulated that the two methyl groups in the phenyl rings of CTC formazan are essential for the fluorescence, as the compound lacking them is non-fluorescent (Ladyman et al., 2016).

Although MTT formazan shows no fluorescence in some organic solvents *in silico*, considerable emission is apparent in viscous media, and needle-like crystals also show a bright red microscopical fluorescence under green (546 nm) excitation (Stockert and Blázquez-Castro 2017, p. 469). Uptake of the lipophilic MTT formazan into lipid droplets and the Golgi apparatus of living HeLa cells treated with sunflower oil emulsion followed by the MTT viability assay (50 μg/mL MTT in culture medium) confirms the accumulation of this formazan in lipid structures, which is illustrated in Fig. 5 as bright-field, fluorescence and pseudocolored images.

Fluorescence offers more sensitivity compared to colorimetric assays, and fluorescent assays would enable flow cytometry and confocal fluorescence to be applied for cytotoxicity analysis. Accordingly, new tetrazolium derivatives were recently synthesized by incorporation of known coumarin-, fluorescein- and rhodol-based fluorophores with disruption of their conjugated system, preventing or reducing fluorescence of the tetrazolium derivatives (Ladyman et al., 2016). These compounds were successfully reduced to the fluorescent formazans, thus allowing the development of fluorescent cytotoxicity assays, in which the fluorescent MTT formazan could be also incorporated.

Other linear and flexible compounds whose structures are analogous to formazans are also candidates for fluorescence applications in more rigid environments. An interesting example is a tetra-azapentamethine cyanine dye, generated by reaction of MBTH (3-methyl-2-benzothiazolinone hydrazone HCl) with aldehydes (Lillie, 1977, p. 234). Such formazans or structurally analogous derivatives, either alone in rigid environments or complexed with suitable metals, might show useful emission properties. In this context, note that spectroscopic analysis of formazans should be made in the absence of metal cations, to avoid the formation of complexes with different spectral features.

Regarding metal derivatives, it is well known that formazans can easily form chelates (e.g., with Co^{2+}, Cu^{2+} and Ni^{2+} ions) with useful histochemical applications (Pearse, 1957; Lillie, 1977, p. 227–228; Horobin and Kiernan, 2002, pp. 158, 162–163 and 168). In this context, the water soluble zincon formazan (Fig. 2) is a known chelating reagent for the histochemical demonstration of Zn^{2+}, Mg^{2+}, and Cu^{2+} ions (Lillie, 1977, p. 228). Unfortunately, fluorescence studies on diamagnetic metal complexes with zincon or other formazans have been scarcely reported.

On the other hand, metal to ligand charge transfer complexes (MLTC) of formazans could be also useful on account of their specific emission features. In addition to the well-known luminescence of MLTC complexes of ruthenium(II) with diimine ligands (Bradley et al., 1981; Bertolesi et al., 1995), the similar tridentate MTT formazan displaying pentagonal metal coordination can use the equivalent azo-imine group to form a luminescent Ru^{2+} complex (Stockert and Abasolo, 2011). The luminescence of this complex in ethanol under blue excitation is shown in Fig. 6. No emission is found for aqueous MTT or ethanolic non-complexed MTT formazan under the same excitation. The Ru-chelated MTT formazan could be applied in luminescent analysis and labeling methods.

6. Pharmacological and labeling perspectives

In addition to the use of tetrazolium–formazan systems as markers of cell viability, there is an increasing interest in other biological effects of these compounds. Thus, the versatile pharmacological activity of functionalized formazans as analgesic, antibacterial, anticonvulsant, antifungal, anti-helminthic, anti-inflammatory, anti-oxidant, antiparkinsonian, antitubercular, antiviral, and cardiovascular therapeutic agents has been recently reviewed (Shawalin and Samy, 2015). Perhaps the most relevant effect of the investigated formazan derivatives is their antibacterial, anticonvulsant and antifungal properties. Predating such investigations, the antiviral activity of some tetrazolium salts and formazans has also been reported (Misra and Dahr, 1980).

Regarding labeling, reduction of tetrazolium salts to insoluble formazans could be useful for *in vivo* labelling of tissues, organs and whole organisms. As an example, MTT and XTT have been applied in studies on filarial worms (Comley et al., 1989; Comley and Turner, 1990) and *Saccharomyces* cultures (Berlowska et al., 2006). Growing plants, *Drosophila* larvae, and *Caenorhabditis* worms would be suitable to test the uptake and reduction of tetrazolium salts *in vivo*.

Warburg's observation that tumors exhibit a high rate of glycolysis even in the presence of oxygen (aerobic glycolysis) led to the postulate that cancer cells and normal differentiated cells may be metabolically distinct (Warburg, 1930; Potter, 1951; Greenstein, 1954). Although the role of glycolysis in both cell types remains controversial, the increased glycolysis would facilitate the synthesis and uptake of nutrients to support anabolic reactions in proliferating cells (Vander Heiden and Cantley, 2009; Lunt and Vander Heiden, 2011).

On account of this metabolic feature of tumor cells, attempts to detect a higher reducing power compared with normal tissues were carried out using tetrazolium salts. Intravenous injection of 25 mg/kg triphenyl tetrazolium chloride (TTC) into rabbits showed no accumulation in tumors (Straus et al., 1948), and higher intravenous doses (100 mg/kg) were lethal, turning the blood serum ruby red. However, fresh or frozen tumor sections incubated in TTC *in vitro* strongly reduced the reagent to the red formazan (Straus et al., 1948). INT (2-(4-radioiodophenyl)-3-(4-nitrophenyl)-5-(phenyl) tetrazolium chloride) administered intravenously to mice at a 0.1mg/kg dose, and evaluated through ^{131}I radioactivity, was non-lethal, but showed no accumulation of ^{131}I in tumors (Masouredis et al., 1950). Several types of rat tumors similarly showed no reduction of BTC after daily intraperitoneal injections, although the liver and kidneys appeared strongly blue-colored by the BTC formazan (Rutenburg et al., 1950). Given intraperitoneally to adult rats, 2.5 mg of neotetrazolium (NTC) and nitroblue tetrazolium (NBT) were lethal in a few hours (Koenig, 1965). Following intratumoral injection of MTT (10 μg and 100 μg in 0.1 mL of 0.9 g % NaCl saline solution), intradermal LM3 tumors in mice became intensely blue after 3 h and 5 min, respectively (Fig. 7). In contrast, intravenous administration of 1 mg MTT in saline solution was lethal, in agreement with previous observations using other tetrazolium salts.

Formation of formazan crystals within blood vessels could be responsible for this lethal effect, as indicated by the red color of blood serum after intravenous TTC (Straus et al., 1948). Lower MTT concentrations (0.1 and 0.5 mg in 0.3 mL) were non-lethal, but no staining of any

tissue (either normal or tumoral) was observed, possibly because of previous MTT reduction by serum proteins before reaching any organ. In keeping with this is the fact that serum albumin, which accounts for much of the reductive capacity of serum, binds amphiphilic compounds such as MTT with avidity (Fasano et al., 2005), and leads to false-positive MTT assays (Funk et al., 2007).

When compared to the easy reduction of tetrazolium salts by tumors *in vitro* or after intratumoral injection, the absence of reduction by tumors *in vivo* after systemic administration is puzzling. It is possible that, when administered intravenously, MTT and other tetrazolium salts must be protected from reduction by blood components. Perhaps encapsulation into liposomes would be a useful strategy for intravenous delivery of tetrazolium salts into living organism to ascertain tissue staining. Since the solubility of MTT in ethanol is higher than in water (Green, 1990), it might remain trapped in the lipophilic phase of liposomes, and therefore protected from reduction, until its uptake into tumor cells.

Conclusions

The MTT assay is, today, one of the most widely employed viability assays in biomedical research. In the present work we have provided a historical framework as a backdrop to recent publications providing new ideas, models and action mechanisms for MTT. In some of these cases previously well-established concepts have been experimentally challenged. Thus there is a marked discrepancy between the subcellular sites formerly proposed for MTT reduction to formazan and recent experimental observations of such reduction. Therefore, we emphasize the need to better assess this significant issue. Also, tetrazolium salts in general, and MTT in particular, display interesting redox behavior, which can be exploited to study redox processes and redox biology in living samples. Thus, MTT use can go beyond a "mere" viability assay, and become an active compound in Cell Biology; as seen, for example, in the experimental work by Pascua-Maestro *et al*. in this present issue of Acta Histochemica. Finally, there is an opportunity at this time to further investigate the fluorescence of MTT formazan not only in cell cultures but also *in vivo* by using whole animal models. Thanks to the increase in detection threshold and resolution in fluorescence detection chambers, it is becoming increasingly possible to do such studies even with low-efficiency luminescent compounds.

Hopefully, this review will spark such interest, and seed new ideas among those researchers working in Cell Biology, Redox Biology and Physiology regarding the uses of MTT and other tetrazolium's salts. Indeed, we anticipate that sooner than later such work will demonstrate that tetrazolium salts have a broad range of applications, going further than "just" viability assays.

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Legends of figures

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.

Fig. 2. Examples of sulfonated derivatives of tetrazolium salts and a formazan reagent. A: MTS. B: WST-1. C: XTT. D: Zincon.

Fig. 3. HeLa cells treated wih 62.5 µg/mL MTT tetrazolium for 1.5 h and observed immediately after treatment. A, B: Merged images from red-pseudocolored MTT formazan granules and blue emission of NAD(P)H (A) or green emission of FAD (B). Observe the low NAD(P)H and high FAD emission of mitochondria immediately after MTT tetrazolium treatment (see Stockert et al., 2012). To detect possible colocalization, bright-field images of MTT formazan deposits were converted to a gray scale, inverted, and transformed to red-pseudocolored images using the public domain ImageJ software (http://rsb.info.nih.gov/ij/). Arrows indicate formazan-containing lipid droplets.

Fig. 4. HeLa cells treated with 50 µg/mL MTT tetrazolium for 1.5 h and observed in bright-field. A: Control cells without sunflower oil treatment. B: Cells subjected to 6 h pre-treatment with a sunflower oil emulsion before MTT tetrazolium. N: cell nuclei.

Fig. 5. HeLa cells subjected to incubation with a sunflower oil emulsion for 16 h and then treated with 50 µg/mL MTT tetrazolium for 1 h. A: Bright-field image. B: Fluorescence image. C: Red gradient from B converted to false color after image processing and analysis with ImageJ software (LookUp Table: red/green). Arrows indicate formazan-containing lipid droplets. G: Golgi apparatus.

Fig. 6. Emission of the MTT formazan complexed with ruthenium(II) (MTTF-Ru(II), emission peak at 535 nm) from an aqueous 50 µg/mL MTT tetrazolium reduced with 0.5 mg/mL ascorbic acid, air dried, dissolved in 90 % ethanol containing 0.5 mg/mL $RuCl_3$, and excited at 420 nm. Under the same excitation, no emission is found for MTT formazan (MTTF, control curve in 90 % ethanol without Ru(II)).

Fig. 7. MTT labeling of mouse tumors. A: BALB/c mice bearing intradermal LM3 tumors (see Stockert et al., 2009). Animals at top: controls (without MTT). Animals at bottom: 3 h after intratumoral injection of MTT tetrazolium (10 µg in 0.1 mL of 0.9 % NaCl solution). B: Processed image of A after removal of the blue color, subtraction of the new image from A, and marking off the red color to define the optimal contrast between red and blue colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

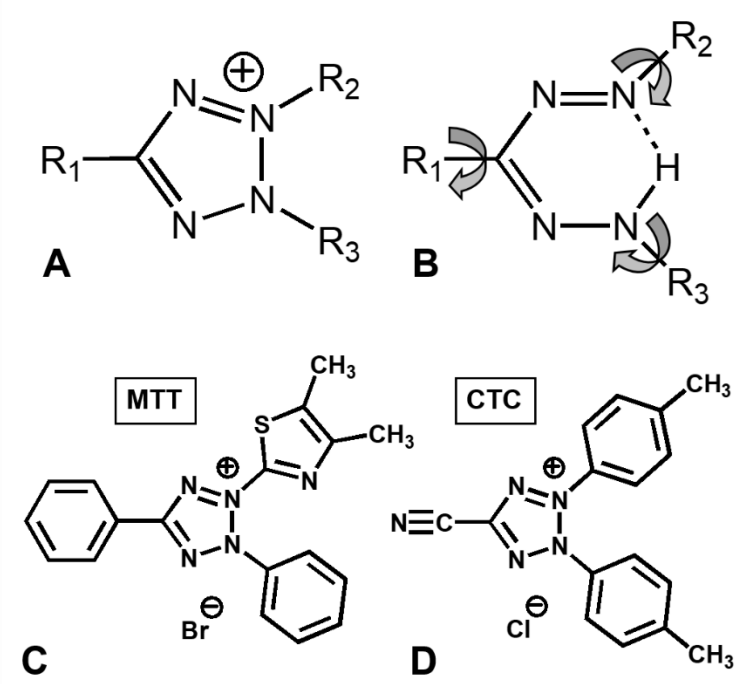

Figure 1.

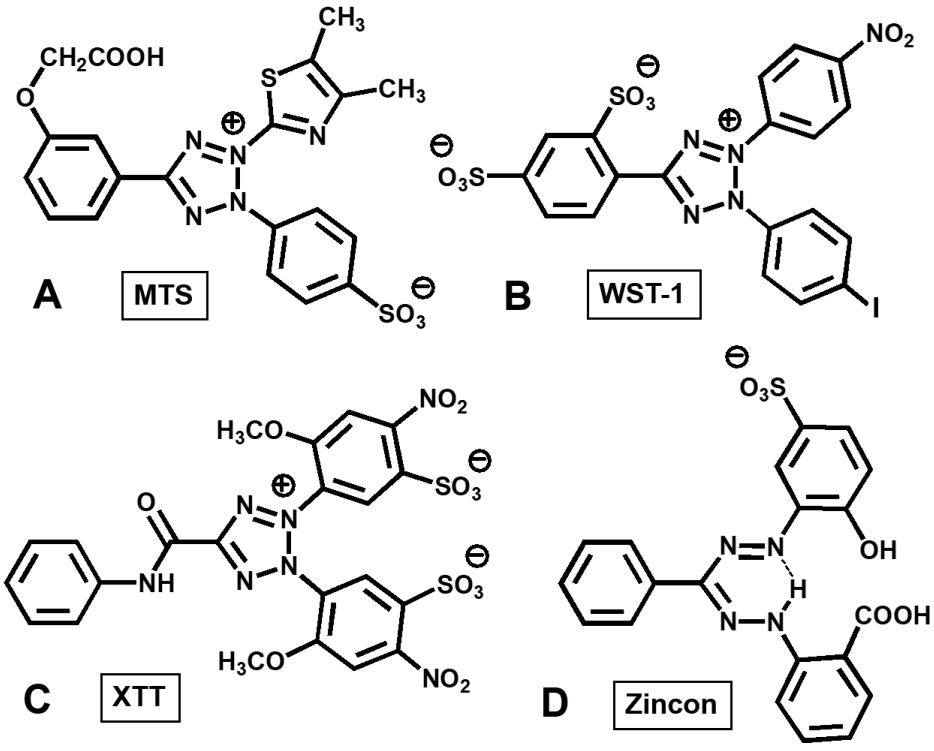

Figure 2.

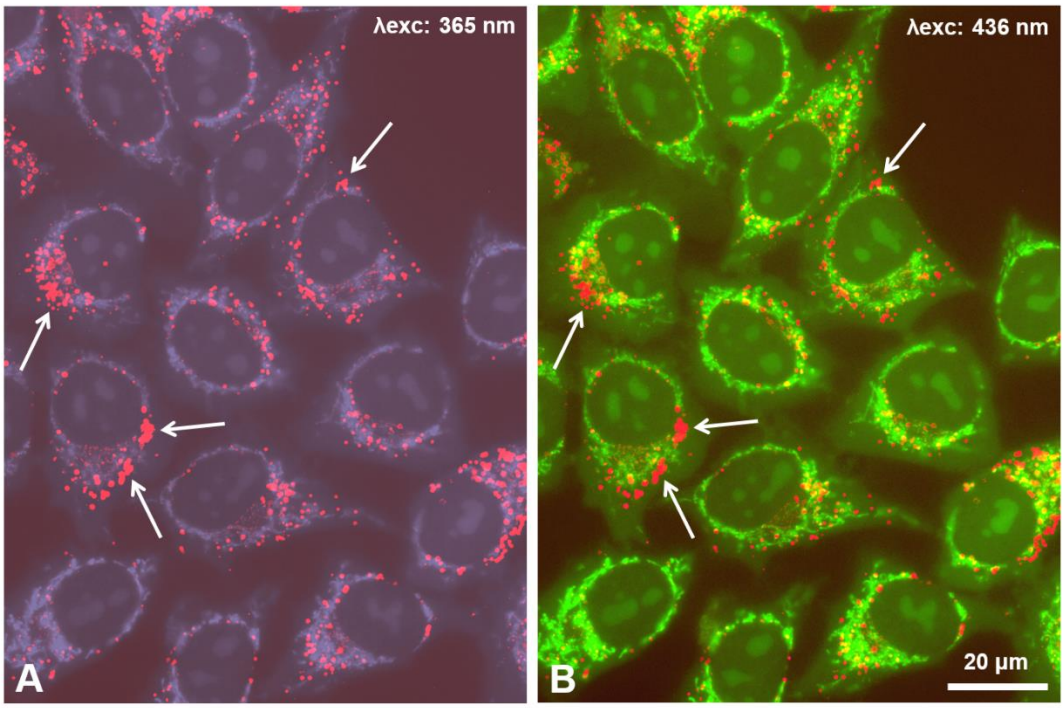

Figure 3.

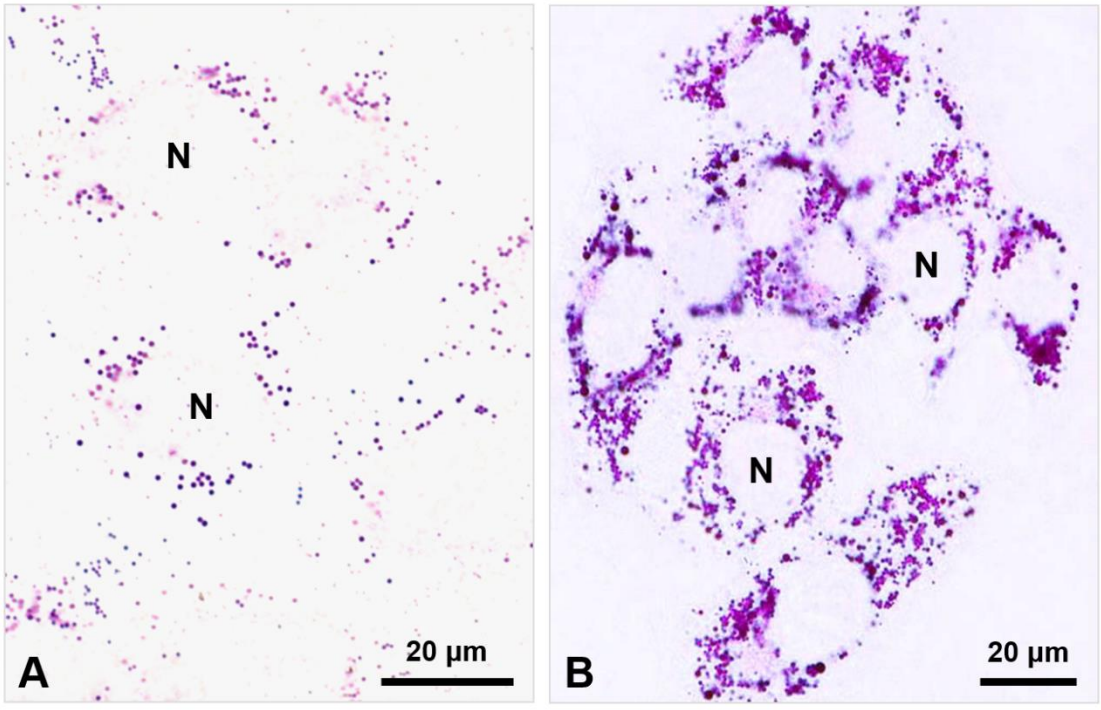

Figure 4.

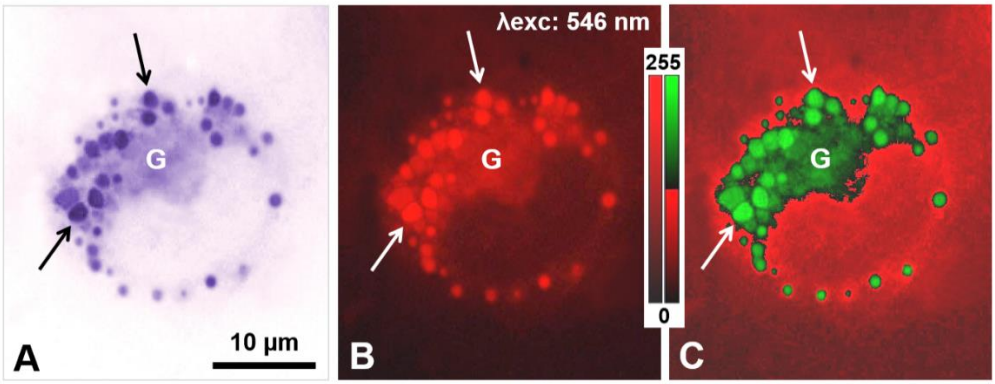

Figure 5.

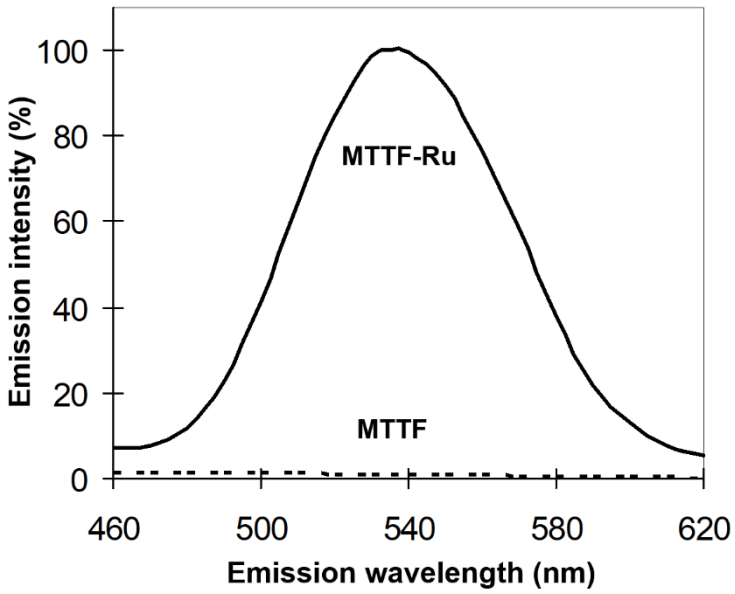

Figure 6.

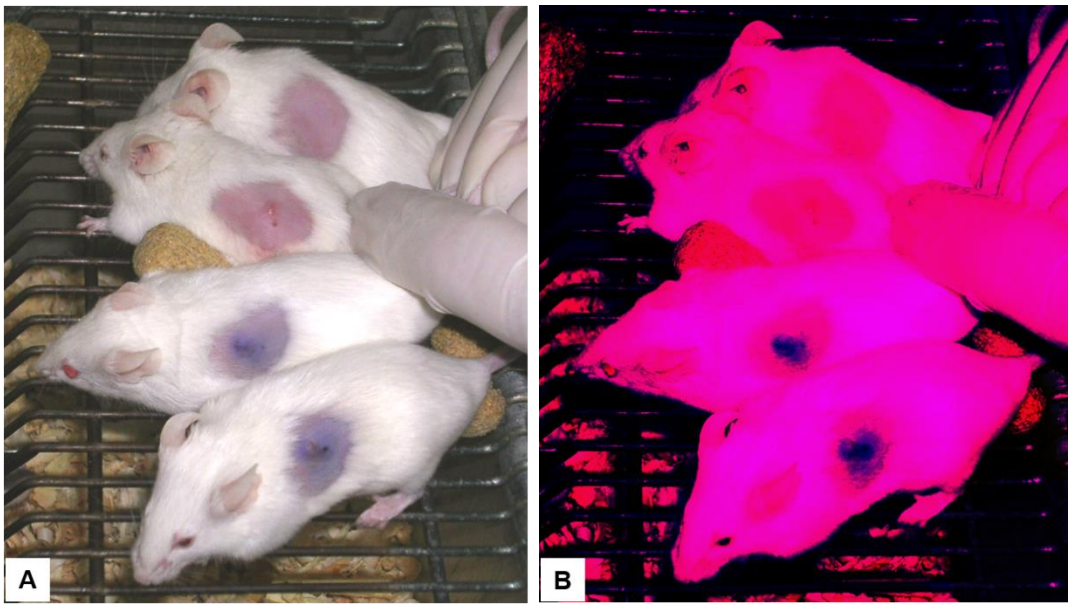

Figure 7.

Table 1. Observed and predicted properties of various tetrazolium salts (& one formazan). Predictions generated using the cited QSAR models. Structure parameters required were obtained as described by Horobin and Rashid-Doubell (2013) and Horobin et al. (2015a).

Compound	Observed interaction with live cells	Predicted property/mechanism (using QSAR model from cited reference/s)	Structure parameters [1]			
			Z	**log P**	**AI [2]**	**HGH [2]**
CTC tetrazolium	Does not enter intact eukaryotic cells.	Weakly lipophilic cation. Strong amphiphilicity retains CTC in plasma membrane, inhibiting dye internalization (Horobin et al., 2013).	1+	0.5	5.1	-4.6
MTT tetrazolium	Accumulates within live cells.	Lipophilic & amphiphilic cation, membrane permeable. Accumulates first in mitochondria then in endoplasmic reticulum (Colston et al., 2003, Horobin et al., 2015a).	1+	1.8	3.8	-2.0
MTT formazan	Accumulates in Golgi apparatus and lipid droplets.	Lipophilic uncharged compound. Accumulates in lipid droplets (Uchinomya et al., 2016) and Golgi apparatus (Rashid-Doubell and Horobin, 1993).	0	4.5	na	na
MTS tetrazolium	No significant accumulation in live cells.	Both free acid & anionic species hydrophilic. Membrane impermeable (Horobin et al., 2015a).	0	-0.3	na	na
			1-	-2.7	na	na
NBT tetrazolium	Accumulates within live cells.	Lipophilic dication. Membrane permeable (Horobin et al., 2015a).	2+	2.3	na	na
WST-1 tetrazolium	Does not enter live cells, reduced at plasma membrane.	Hydrophilic anion. Trapped in plasma membrane due to amphiphilicity, cell entry by flip-flop prevented by very hydrophilic headgroup (Horobin et al., 2013)	1-	-3.6	4.7	-8.3
XTT tetrazolium	Does not enter cell, reduced at plasma membrane.	Hydrophilic anion. Membrane impermeable (Horobin et al., 2013).	1-	-5.7	na	na

[1] Z is electric charge, log P the log of the octanol-water partition coefficient, AI the amphiphilicity index, HGH the head group hydrophilicity.
[2] na indicates not applicable, as molecule is not predicted to be amphiphilic.