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MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets

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ABSTRACT

Although MTT is widely used to assess cytotoxicity and cell viability, the precise localization of its reduced formazan product is still unclear. In the present study the localization of MTT formazan was studied by direct microscopic observation of living HeLa cells and by colocalization analysis with organelle-selective fluorescent probes. MTT formazan granules did not colocalize with mitochondria as revealed by rhodamine 123 labeling or autofluorescence. Likewise, no colocalization was observed between MTT formazan granules and lysosomes labeled by neutral red. Taking into account the lipophilic character and lipid solubility of MTT formazan, an evaluation of the MTT reaction was performed after treatment of cells with sunflower oil emulsions to induce a massive occurrence of lipid droplets. Under this condition, lipid droplets revealed a large amount of MTT formazan deposits. Kinetic studies on the viability of MTT-treated cells showed no harmful effects at short times. Quantitative structure–activity relations (QSAR) models were used to predict and explain the localization of both the MTT tetrazolium salt and its formazan product. These predictions were in agreement with experimental observations on the accumulation of MTT formazan product in lipid droplets.

Introduction

At present colorimetric assays using the tetrazolium salt thiazolyl blue, also termed MTT, after **m**ethyl-**t**hiazolyl-**t**etrazolium (Mosmann, 1983) are widely used for assessment of cytotoxicity, cell viability, and proliferation studies in cell biology (Horobin and Kiernan, 2002; Berridge et al., 2005; Van Meerloo et al., 2011). The method has been extended and improved by several authors (Denizot and Lang, 1986; Gerlier and Thomasset, 1986; Carmichael et al., 1987; McHale and McHale, 1988; Edmondson et al., 1988; Plumb et al., 1989; Niks and Otto, 1990; Heo et al., 1990; Rubinstein et al., 1990; Merlin et al., 1992; Cedillo-Rivera et al., 1992; Hussain et al., 1993; Thom et al., 1993; Marshall et al., 1995; Sieuwerts et al., 1995; Chiba et al., 1998).

MTT gives a yellowish aqueous solution which, on reduction by dehydrogenases and reducing agents present in metabolically active cells, yields a water insoluble violet-blue formazan (Fig. 1A). The lipid soluble formazan product may be extracted with organic solvents and estimated by spectrophotometry. It is currently widely thought that the amount of MTT formazan is directly proportional to the number of living cells (Van Meerloo et al., 2011), however such conclusions have been seriously questioned (Etxeberria et al., 2011).

Ever since early applications of the method as a viability assay for cultured cells (Mosmann, 1983), MTT has been considered to be reduced by the activity of mitochondrial dehydrogenases in living cells. Consequently it has been assumed that the sites of reduction, and of formation of the formazan precipitate, were the mitochondria. In particular it has been claimed that the mitochondrial succinate dehydrogenase of viable cells reduced MTT to the corresponding formazan (Saravanan et al., 2003).

Unfortunately, numerous authors refer to these assumptions and continue to suggest that (a) MTT is reduced in the mitochondria, or (b) that the MTT assay is a suitable indicator of mitochondrial function, allowing the evaluation of mitochondrial activity (Huet et al., 1992; Nikkhah et al., 1992; Van de Sandt et al., 1993; Gieni et al., 1995; Bednarska et al., 1998; Heinzelmann-Schwarz et al., 2003; Walker et al., 2004; Bruzell et al., 2005; Feuerstein et al., 2009; Du et al., 2010; Van Meerloo et al., 2011). Regarding assumption (a), mitochondria do not show reducing properties, but rather oxidizing power. Thus the classical Janus green B staining of mitochondria in living cells is due to the maintenance of the dye's colored oxidized form in this organelle, whereas it is reduced to the colorless leucobase elsewhere in the cytoplasm (Lazarow and Cooperstein, 1953). With regard to assumption (b), although an important source of reducing power in the cell is the reduced

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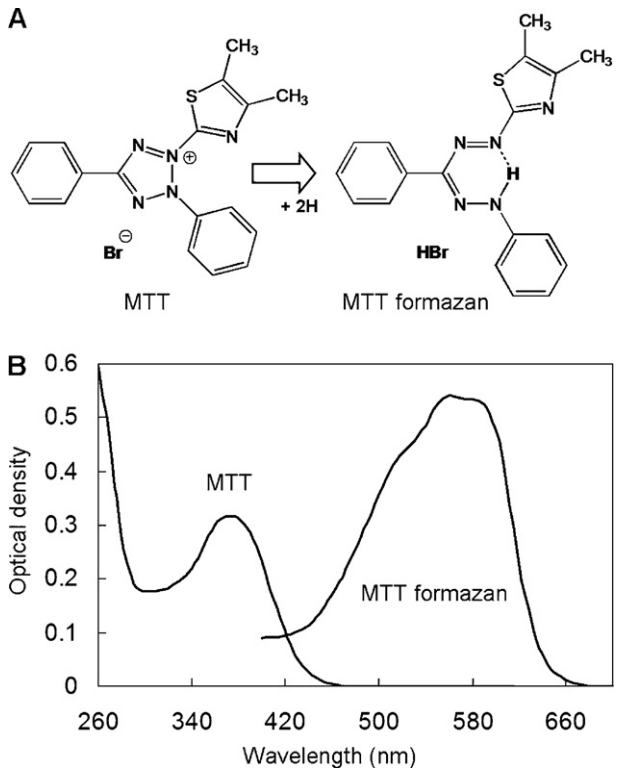

Fig. 1. (A) Chemical structure of MTT and its reduced formazan product; (B) absorption spectra of MTT in distilled water and MTT formazan in sunflower oil, both at the same concentration (0.016 mg/ml).

nicotinamide adenine dinucleotide (NADH) coenzyme generated in the mitochondria, the use of MTT reduction as a direct assay for mitochondrial activity would be only a highly indirect method.

In addition to NADH, there are other reducing intracellular agents such as ascorbic acid, dihydrolipoic acid, cysteine, α-tocopherol, glutathione, *etc.* (Meister, 1994; Liu et al., 1997; Schafer and Buettner, 2001; Moini et al., 2002; Packer and Cadenas, 2007; Winterbourn and Hampton, 2008) that can also reduce MTT efficiently. Interestingly, some tissue phospholipids were shown to reduce MTT, possibly on account of endogenous reducing agents such as ubiquinone or vitamin E (Tranzer and Pearse, 1963) or after additional hydroquinone treatment (Carmichael, 1968).

Biochemical evidence indicates that MTT is mainly reduced in the cytoplasm by NADH (and in lesser amount by NADPH) and dehydrogenases associated to the endoplasmic reticulum (ER) (Berridge et al., 1996, 2005), endosome/lysosome vesicles (Bernas and Dobrucki, 2002), and plasma membrane (Bernas and Dobrucki, 2000). It has been claimed that the MTT formazan gives rise to extracellular deposits of needle-shaped crystals by exocytosis (Liu et al., 1997; Isobe et al., 1999; Abe and Saito, 1999; Molinari et al., 2005; Hatz et al., 2007; Diaz et al., 2007). The view that MTT formazan is eliminated by exocytosis is, however, not supported by other studies (Bernas and Dobrucki, 2004). Measurement of the brightness of formazan crystals under polarized light was proposed for assessing drug-induced cytotoxicity in tumors (Colangelo et al., 1992). The viability of bacterial cultures was also evaluated by resonance light scattering of formazan crystals using a fluorescence spectrophotometer (Xiao et al., 2007).

Unfortunately, morphological characterization of the MTT reduction within cells has rarely been documented. Microscopic descriptions are scarce, and the precise nature of MTT formazan granules is still debated. MTT was claimed to be reduced in cytoplasmic vesicles (Liu et al., 1997), the product being deposited as cytoplasmic formazan granules (Hatz et al., 2007), which increased cell refringency (Huet et al., 1992). On account of light scattering imaging studies, small (<3 μm) intracellular deposits were described as MTT formazan crystals (Bernas and Dobrucki, 2004). Consistent with its lipophilic character, the localization of MTT formazan in cellular lipids has also been recently reported (Diaz et al., 2007).

The present paper investigates at which cellular site MTT is first reduced to formazan, and where this formazan is then accumulated. In particular, we sought to identify the structures and the nature of the processes being demonstrated within cells by MTT reduction. The site of MTT formazan localization was studied in living cells by direct microscopic observation and by colocalization with organelle-selective fluorescent probes. In addition, evaluation of the MTT reaction after treatment of cells with sunflower oil emulsions to induce a massive occurrence of lipid droplets (Stockert et al., 2010; Horobin and Stockert, 2011), and also kinetic studies on the viability of MTT-treated cells, were carried out. Finally, quantitative structure–activity relations (QSAR) models were used to predict and explain the localization of both the MTT tetrazolium salt and its formazan product. These predictions were compared to the experimental observations. For a general introduction to the QSAR modelling approach see Horobin (2001, 2010).

Since the report of Diaz et al. (2007) is contrary to so much prior published work, we carried out the detailed investigation described here, using bright-field microscopy, colocalization studies and predictive QSAR methods. As will be seen, we too conclude that localization is in lipid droplets and not in mitochondria.

Materials and methods

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) was purchased from Sigma–Aldrich (St. Louis, MO, USA; 98% purity). To analyze the oil solubility and spectroscopic properties of MTT formazan product, MTT was dissolved in distilled water (1 mg/ml) and then ascorbic acid was added to a final concentration of 4 mg/ml. The dark violet-blue flocculent precipitate of MTT formazan was washed and resuspended in distilled water. The suspension was mixed (1:1, v/v) with sunflower oil and agitated, and the oil phase finally diluted with more oil (1:60, v/v). Spectrophotometric studies were made using a Shimadzu (Columbia, MD, USA) UV–Vis 1601 spectrophotometer.

HeLa cells were grown on 22 mm square coverslips placed into 35 mm culture dishes using Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin and 1% (v/v) 0.2 M L-glutamine (complete DMEM; all products from Gibco, Paisley, Scotland, UK). Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and the culture medium was changed daily. Experiments were done during the exponential phase of cell growth.

A stock MTT solution (1 mg/ml in distilled water) was prepared immediately prior to use, and filtered through a 0.22 μm Millipore® filter. The culture medium of each cell culture was replaced by 1.5 ml of complete DMEM, to which 0.1 ml of the MTT stock solution was added (final MTT concentration: 62.5 μg/ml). Cells were incubated for 1 to 4 h at 37 °C, after which the medium was removed and the culture washed with phosphate buffered saline (PBS). For the viability assay, the formazan product was dissolved in 1.5 ml dimethylsulfoxide (DMSO) and the absorbance measured at 540 nm with a plate reader (SpectraFluor, Tecan, Männedorf, Switzerland). Cell survival was expressed as the percentage of formazan absorbance. Results were the mean values and standard deviation (SD) from at least three different experiments in triplicate.