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Research paper

Pitfalls of the MTT assay: Direct and off-target effects of inhibitors can result in over/underestimation of cell viability

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ABSTRACT

The MTT assay (to a less degree MTS, XTT or WST) is a widely exploited approach for measuring cell viability/drug cytotoxicity. MTT reduction occurs throughout a cell and can be significantly affected by a number of factors, including metabolic and energy perturbations, changes in the activity of oxidoreductases, endo-/exocytosis and intracellular trafficking. Over/underestimation of cell viability by the MTT assay may be due to both adaptive metabolic and mitochondrial reprogramming of cells subjected to drug treatment-mediated stress and inhibitor off-target effects. Previously, imatinib, rottlerin, ursolic acid, verapamil, resveratrol, genistein nanoparticles and some polypeptides were shown to interfere with MTT reduction rate resulting in inconsistent results between the MTT assay and alternative assays. Here, to test the under/overestimation of viability by the MTT assay, we compared results derived from the MTT assay with the trypan blue exclusion assay after treatment of glioblastoma U251, T98G and C6 cells with three widely used inhibitors with the known direct and side effects on energy and metabolic homeostasis — temozolomide (TMZ), a DNA-methylating agent, temsirolimus (TEM), an inhibitor of mTOR kinase, and U0126, an inhibitor of MEK1/2 kinases. Inhibitors were applied shortly as in IC_{50} evaluating studies or long as in studies focusing on drug resistance acquisition. We showed that over/underestimation of cell viability by the MTT assay and its significance depends on a cell line, a time point of viability measurement and other experimental parameters. Furthermore, we provided a comprehensive survey of factors that should be accounted in the MTT assay. To avoid result misinterpretation, supplementation of the tetrazolium salt-based assays with other non-metabolic assays is recommended.

1. Introduction

The diverse high-throughput colorimetric, fluorimetric and luminescence-based assays were developed and optimized to measure viability of cells directly in 96-well plate format using a multiwell scanning spectrophotometer (Weyermann et al., 2005). The tetrazolium salt-based assays (MTT, MTS, XTT or WST) are one of the most widely exploited approaches in cancer research for measuring cell proliferation, viability and drug cytotoxicity, which nowadays prevail over more strict but time-consuming methods such as the trypan blue exclusion or thymidine titration assays. In living cells, the water-soluble yellow dye MTT is reduced to a dark purple (blue-magenta) colored formazan precipitate, which can be analyzed colorimetrically after dissolving in an organic solvent (*e.g.*, dimethyl sulphoxide or isopropanol). MTT is cell membrane-permeable due to a net positive charge, whereas MTS, XTT and WST-1 have a net negative charge and largely cell-impermeable. Moreover, the MTS, XTT and WST-1 tetrazolium salts require an intermediate electron acceptor for reduction and form water-soluble formazans (no need in an organic solvent) (reviewed in Berridge et al., 2005). The MTT-based viability assay was developed by Mosmann who revealed that the MTT-formazan production was proportional to the number of metabolically viable cells (Mosmann,

Abbreviations: AhR, aryl hydrocarbon receptor; AMPK, AMP-activated kinase; ARG, abl-related gene tyrosine kinase; BK, the large-conductance voltage- and calcium-activated K^+ channel; ATP, adenosine triphosphate; COX1, cyclooxygenase 1; CSF1R, macrophage colony stimulating factor receptor; CYP1A, cytochrome 4501A; DDR, discoidin domain receptor; eGFP, enhanced green fluorescent protein; ER, estrogen receptor; FKBPs, FK506-binding proteins; GSK3β, glycogen synthase kinase 3β; HECNU, N-(2-chloroethyl)-N-nitroso-N′-2-hydroxyethylurea; hERG, the human ether-a-go-go-related gene K^+ channel; JNK1α1, c-Jun N-terminal kinase 1α1; c-KIT, stem cell factor receptor; LDH, lactate dehydrogenase; MAPKAP-K2, mitogen-activated protein kinase-activated protein kinase 2; MSK1, mitogen- and stress-activated protein kinase 1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTIC, (5-(3-methyltriazen-1-yl)imidazole-4-carboxamide); MTT, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); NQO2, NAD(P)H dehydrogenase, quinone 2; PDGFR, platelet-derived growth factor receptors; PDK1, 3-phosphoinositide-dependent protein kinase-1; PKA, cAMP-dependent protein kinase; PKB/AKT, protein kinase B; PRAK, p38-regulated/activated protein kinase; ROS, reactive oxygen species; Sirt1, sirtuin 1; WST-1, sodium 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium)-1,3-benzene disulfonate; XTT, sodium 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxanilide; formazan, (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan).

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1983). Since the pioneer report on MTT reduction by the mitochondrial electron-transporting chain (Slater et al., 1963), the mitochondrial complex II/succinate dehydrogenase has been frequently erroneously considered a major intracellular site of MTT reduction. However, it is now documented that mitochondrion is only one of the MTT reduction sites, whereas non-mitochondrial, cytosolic and microsomal MTT reduction makes the major contribution to an overall reduction. MTT reduction occurs throughout a cell by oxidoreductases, which utilizes NADH/NADPH. Superoxide may also contribute to intracellular MTT reduction, whereas the cell surface oxidoreductases are responsible for extracellular MTT reduction (Berridge et al., 2005).

Changes in the activity of dozens of the mitochondrial and non-mitochondrial oxidoreductases, cellular metabolic and energy perturbations, and oxidative stress may significantly impact the MTT assay readout. A level of glucose, its uptake rate and rate of glycolysis, a level of lactate, pyruvate, and NADH/NADPH were shown to influence MTT reduction (Table 1). Berridge et al. (2005) deduce that the MTT assay (as well as MTS, XTT or WST-based assays) "do not actually measure the number of viable cells or their growth but rather an integrated set of enzyme activities that are related in various ways to cell metabolism". Furthermore, the factors changing a rate of endo-/exocytosis and intracellular trafficking of MTT-formazan may also affect MTT reduction (Table 1). Thereby, one should be concerned by the possibility that a degree of viability change judged from the observed changes in MTT reduction between control and experimental cells can be over/underestimated due to the influence of experimental manipulations on the parameters mentioned above rather than due to a real viability decrease.

Significant over/underestimation of cell viability by the MTT assay (or other tetrazolium salt-based assays) was shown for a number of the well-characterized inhibitors and proteins in comparison to the alternative approaches such as the trypan blue exclusion assay, thymidine titration, propidium iodide staining assay, *etc.* (Table 2). For example, rottlerin has been widely used as a specific inhibitor of protein kinase C delta (PKCδ) since 1994 (Gschwendt et al., 1994). However, the late studies revealed that rottlerin did not block recombinant or immunoprecipitated PKCδ. In contrast, rottlerin inhibited PRAK and MAPKAP-K2, produced a substantial inhibition of JNK1α1, MSK1, PKA, PDK1, PKB/AKT, and GSK3β as well as non-kinase enzymes, including β-lactamase, α-chymotrypsin, and malate dehydrogenase, and directly activated several types of K+ channels (BK and hERG) (Soltoff, 2007). Rottlerin treatment dissipated the inner mitochondrial membrane potential, accelerated electron transfer and increased dehydrogenases activity, oxygen consumption, and NADH oxidation (Maioli et al., 2009). Unsurprisingly, rottlerin caused overestimation of viability in the MTT assay (Maioli et al., 2009).

Imatinib treatment induced a dose-dependent increase in MTT reduction that was inconsistent with proliferation and apoptosis assays, which on the contrary demonstrated the reduced cell number and increased apoptosis (Sims and Plattner, 2009). The direct and off-target effects of imatinib may explain this phenomenon. Imatinib was designed to target BCR-ABL fusion tyrosine kinase in patients with chronic myeloid leukemia (CML). A "magic bullet" effect of imatinib due to its high rate of CML cure in an early phase of disease was ascribed to specific inhibition of oncogenic BCR-ABL tumor "driver", and this afforded strong grounds for introducing the "oncogene addiction concept", according to which tumor cells are dependent on a single activated oncogenic protein or pathway to maintain their malignant properties (Santos et al., 2011; Sawyers, 2009). However, imatinib also targets the receptor tyrosine kinases (c-KIT, PDGFR, CSF1R, and DDR) and non-receptor tyrosine kinases (BLK, ARG, LCK, and LYN), as well as RAF kinase family members (BRAF and CRAF) and the oxidoreductase NQO2 at the therapeutic concentrations (Anastassiadis et al., 2011; Breitkopf et al., 2010; Davis et al., 2011; Hantschel et al., 2008; Kitagawa et al., 2013; Packer et al., 2011; Steegmann et al., 2012). Imatinib treatment significantly influenced metabolic enzyme activities, caused changes in cellular glucose metabolism, and led to an increase in the mitochondrial activity and energy balance in a cell line-dependent manner with contrast differences between imatinib-sensitive and resistant cells (Barnes et al., 2005; Breccia and Alimena, 2009; Gottschalk et al., 2004; Klawitter et al., 2009; Kominsky et al., 2009).

A significant discrepancy in cell viability between the MTT assay and direct cell counting or propidium iodide staining assay was observed upon ursolic acid treatment (Es-Saady et al., 1996). Ursolic acid stimulated reactive oxygen species (ROS) generation, glutathione depletion, opening of mitochondrial permeability transition pores, and ATP decline (Lu et al., 2014). Resveratrol treatment increased formazan formation in different leukemia cell lines but not prostate carcinoma cells (Bernhard et al., 2003). Resveratrol was documented to activate AMPK–SIRT1 pathway, act as ER modulator, has an inhibitory effect on cAMP phosphodiesterases, COX1 and F_0F_1 ATPase, and promotes ROS generation. Both inhibition and activation of mitochondrial complex I by resveratrol treatment was controversially observed (Bitterman and Chung, 2014).

Table 1
A medium composition, cell growth state, concentration and consumption rate of energy supply metabolites may influence MTT reduction rate.

Tested factor/substance	Main observations	Ref.
Conditioned medium	Formazan production by cells was less efficient in conditioned medium (medium, which supported cellular growth for several days) than in fresh medium. Changes in pH of conditioned medium but not in glucose content partially explained differences. Additional factors were suggested to be involved	Jabbar et al. (1989)
Fetal calf serum	Underestimation was observed for cells cultured in 10% serum but not in </= 5%. No difference in total mitochondrial activity was observed	Zhang and Cox (1996)
Glucose and NAD(P)H	A glucose concentration decrease in culture medium or cellular NAD(P)H concentration decrease was accompanied by concomitant decrease in MTT-formazan production. The kinetics of formazan formation and the degree of saturability significantly varied among tested cell lines	Vistica et al. (1991)
Glucose	MTT reduction was acutely sensitive to cytochalasin B and 2-deoxyglucose, which inhibit glucose uptake through plasma membrane glucose transporters, and to inhibitors of glycolysis such as iodoacetamide	Berridge et al. (2005)
Glucose, lactate and pyruvate	Glucose was a good substrate for MTT reduction by both neurons and astroglia. In glucose-free medium, MTT reducing activity in neurons was enhanced by pyruvate and the reducing activity of lactate was equivalent to that of glucose. In contrast, lactate and pyruvate were the poor substrates for astroglial MTT reduction. Acetate and malate caused negligible effects. Glycolysis played an important role in MTT reduction by astroglia, whereas mitochondrial oxidative metabolism — in neurons	Takahashi et al. (2002)
Growth state	The growth state of cells, exponential or confluent, has moderate but significant influence on MTT reduction	Liu and Dalgleish (2009)
Serum albumin	In a concentration-dependent manner, human and bovine albumins increased formazan formation *via* direct chemical interaction with MTT or XTT. Free SH-groups of cysteine residues were implicated	Funk et al. (2007)
Cholesterol	Cholesterol/methyl-β-cyclodextrin complex decreased MTT reduction, enhanced exocytosis of formazan, and promoted a cell type-dependent increase in oxygen consumption. No cytotoxicity of cholesterol itself was revealed in trypan blue and propidium iodide assays. Treatments that decrease or increase cellular free cholesterol levels inhibited or promoted, respectively, formazan exocytosis	Ahmad et al. (2006) and Liu et al. (1998)