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Modification of MTT assay conditions to examine the cytotoxic effects of amitraz on the human lymphoblastoid cell line, WIL2NS

Fiona M. Young [a,*], Wichaya Phungtamdet [b], Barbara J.S. Sanderson [a]

[a] Department of Medical Biotechnology, School of Medicine, Flinders University of South Australia, Bedford Park, SA 5042, Australia
[b] Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

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Abstract

Reported parameters of the MTT assay vary widely, and reflect a need to optimise the assay for different cell types. The MTT assay conditions for the human B-lymphocyte-derived cell line WIL2NS were optimised for MTT incubation and formazan development. The optimised MTT assay was validated by examining the effects of the acaride amitraz on WIL2NS. In pH-buffered media in the absence of cells, MTT formed formazan spontaneously, and absorbance was proportional to both the initial concentration of MTT and the time of incubation at 37 °C. One milligram per millilitre MTT was toxic to WIL2NS cells, but the accuracy of the standard curve was reduced when only 0.2 mg/ml MTT was used. Twenty percent SDS in 0.2 M HCl was preferable to DMSO as a solvent for formazan. Exposure to 0.035% amitraz resulted in a significant reduction in WIL2NS cell numbers after only 2 h of exposure. It was concluded that 0.035% of amitraz has the potential to adversely affect lymphocytes in the systemic blood system in humans, and that an optimised MTT assay was obtained by incubating WIL2NS cells with 0.45 mg/ml MTT for 17 h, followed by addition of acidified SDS for 1 h.

Keywords: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); Amitraz (*N*-methylbis (2,4-xylyminomethyl) amine; WIL2NS lymphoblastoid cell line; Toxicity

1. Introduction

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is commonly used to measure the number of metabolically active cells in in vitro assays. It is based on the conversion of yellow, water-soluble MTT to the purple, water insoluble endproduct, formazan, by mitochondrial dehydrogenase. The amount of formazan formed is proportional to the number of metabolically active cells (Mossman, 1983). The key steps in the assay are to incubate cells with MTT, extract the formazan crystals from the cells, dissolve the formazan and measure absorbance at 570 nm (Plumb et al., 1989).

The MTT assay is used frequently, but the reported conditions and parameters of the assay vary widely. The concentrations of MTT reported range from 0.45 mg/ml (Mossman, 1983; Garn et al., 1994) to 2 mg/ml (Ho et al., 1997). The amount of formazan produced is dependent upon a number of parameters including the initial concentration of MTT used, and on the physiological attributes of the cell line (Plumb et al., 1989; Vistica et al., 1991; Van de Loosdrecht et al., 1991).

Incubation times for MTT vary from 0.4 to 6 h (Twentyman and Luscombe, 1987), and the methods

* Corresponding author.

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for extracting and dissolving formazan have been variously reported as using propanol (Denizot and Lang, 1986), 2-propanol and 1 M HCl (Mueller et al., 2004), DMSO, or 0.04 N HCl in isopropanol (Green et al., 1984; Pieters et al., 1990) or 20% SDS in 50% dimethyl formamide (Garn et al., 1994; Hansen et al., 1989). However, mixtures of organic solvents and media do not effectively dissolve formazan, and the addition of organic solvents to media containing serum also results in the precipitation of serum proteins, which interfere with the absorbance readings (Denizot and Lang, 1986; Green et al., 1984). To avoid these problems, plates have been centrifuged or aspirated to remove supernatants containing MTT and media before the addition of organic solvents such as DMSO (Wang and Zheng, 2002) or DMSO and ethanol (Sladowski et al., 1992). SDS has also been added to reduce serum protein precipitates (Green et al., 1984).

Intensity of signal and reproducibility of results within the MTT assay are related to cell homogeneity and metabolic activity, and these parameters vary according to the cell lines being used (Vistica et al., 1991; Plumb et al., 1989). Since the human lymphoblastoid cell line, WIL2NS, has proved useful in toxicological studies (Humpage et al., 2000; Shield and Sanderson, 2004), we have defined the optimal MTT assay conditions for this cell line.

To validate the MTT experimental method, we have used it to investigate the effect of amitraz on WIL2NS cells. Amitraz is an insecticide and acaricide commonly used to prevent tick and mite infestation (Hollingworth, 1976) of cattle (Eamens et al., 2001; George et al., 1998), dogs (Elfassy et al., 2001), pigs and other animals. It is also sprayed onto cotton and hops (Weichel and Nauen, 2003) from aircraft and ground sprinklers, and is used to control psylla infestations of pears (Schaub et al., 2002). Amitraz is used at a concentration of 0.025% in dipping baths to control the cattle tick, *Boophilus microplus*, which is a major pest of cattle in Australia (McDougall and Lewis, 1984), but the powder which workers use to make up the dip solution contains 50% amitraz active ingredient. Dog owners are advised to use amitraz-impregnated (0.025%) collars or a weekly rinse (0.05%), although owners are warned that they may experience migraine headaches and asthma attacks (Shaw and Foster, 2000). Fourteen children admitted to hospital after dermal exposure to amitraz (12.5–0.25%) exhibited bradycardia or tachycardia, myosis and hyperglycemia (Kalyoncu et al., 2002) and other reports also concluded that amitraz can be absorbed through skin and enter the systemic blood system (Ulukaya et al., 2001; Aydin et al., 1997). Amitraz binds to α2-adrenergic receptors (Altobelli et al., 2001) and exerts downstream effects, but apart from this, little is known about the toxic effects of amitraz in humans (Yaramis et al., 2000).

2. Methods

2.1. Chemical reagents

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and SDS (sodium dodecyl sulfate) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl) was purchased from BDH Laboratory Supplies, Poole, England. A stock solution of amitraz (*N*-methylbis (2,4-xylyminomethyl) amine, Chem Service, USA) in methanol (10 mg/ml) was stored at room temperature in the dark.

2.2. Cell culture conditions and MTT assay optimisation

The human non-cancerous, non-adherent human male B-lymphocyte-derived cell line, WIL2NS, was obtained from the ATCC (CRL-8155, Rockville, MD, USA) and maintained in RPMI 1640 (Trace Biosciences, Castle Hill, NSW, Australia), with 5% fetal calf serum (CSL, Parkeville, Vic., Australia) at 37 °C in a 5% CO_2 fully humidified incubator.

All MTT assays were carried out in 96-well round-bottom plates. MTT was dissolved in distilled water (5 mg/ml), filter sterilised and stored in 1 ml aliquots at −18 °C. Different concentrations of MTT were made by diluting further in culture medium (1:10, 1:4, 1:2) then adding 80 μl to wells containing 100 μl of cell suspension, giving final concentrations of 0.22, 0.45 and 1 mg/ml of MTT. The calculations used to determine MTT concentration in this study differ from the calculations used in some other reports. For example, in this study, 80 μl of a 1 mg/ml MTT solution was added to 100 μl of cell suspension, producing 0.08 mg MTT in 180 μl, a concentration considered equivalent to 0.45 mg/ml.

To describe the interaction between MTT and culture medium, wells without cells were filled with 100 μl RPMI ± fetal calf serum (FCS, 5% or 10%), or DMEM ± FCS, or EMEM ± FCS (DMEM and EMEM from Gibco BRL, Invitrogen Life Sciences, Mount Waverley, Vic., Australia) or phosphate buffered saline (PBS), or water. MTT (80 μl) was added to all wells to give a final concentration of 0.45 mg/ml, and wells were incubated from 6 to 96 h at 37 °C, before incubating with 80 μl 20% SDS in 0.02 M HCl for 24 h. The absorbance of each well was determined by using an automatic plate reader (Model 550 with Microplate Manager Software; Biorad) with a 570 nm test wavelength and a reference wavelength of 655 nm (Abe and Matsuki, 2000). These experiments were conducted in six replicates, on three different occasions ($n = 3$).

The optimal concentration of MTT, and the optimal duration of MTT incubation, were determined by plating WIL2NS cells in triplicate standard curves on four