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Comparison of three cytotoxicity tests in the evaluation of the cytotoxicity of a spermine analogue on human breast cancer cell lines

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Abstract

Using three cytotoxicity assays, we have investigated the effect of the spermine analogue N^1,N^{11}-diethylnorspermine (DENSPM) on four human breast cancer cell lines with different known genetic lesions. Cells were seeded in 96 well plates and DENSPM was added 24 h later to give final concentrations from 0.1 to 100 μM. At 24, 48 and 72 h of treatment, the protein content was determined with a modified Lowry assay. Mitochondrial activity was determined with the AlamarBlue™ and MTT assays. These two assays differ with respect to where in the electron transport chain the reduction of the substrate takes place. Treatment with increasing concentrations of DENSPM resulted in differential responses in the four cell lines. There was a good of agreement between the protein content and the MTT assay showing increased negative effect with increased dose of DENSPM. The AlamarBlue™ assay on the other hand showed a stimulation of substrate reduction compared to control at DENSPM concentrations that were inhibitory according to the protein content and MTT assay. Thus, the data clearly show that the MTT and AlamarBlue™ assays are not equivalent. Importantly, the AlamarBlue™ assay presumably also reflects cytoplasmic reduction of the substrate through DENSPM-induced mechanisms.

Keywords: Apoptosis; Cell proliferation; Human breast cancer; Mitochondrial activity; Spermine analogue

1. Introduction

Investigation of cytotoxicity is very important in many contexts. Today there are a number of methods, which measure different endpoints and are based on microwell assays. Two such common assays are the MTT and AlamarBlue™ assays.

In the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is added to the medium of the cells in the microwell. MTT is taken up via endocytosis by living cells and reduced to blue formazan crystals inside the cell via mitochondrial nicotinamide adenine dinucleotide phosphate (NADH) dependent dehydrogenases. The amount of formazan generated is assumed to be directly proportional to the cell number when using homogenous cell populations (Berridge and Tan, 1993; Denziot and Lang, 1986; Fricker and Buckley, 1996; Husøy et al, 1993; Mosmann, 1983; Nocari et al., 1998; Plumb et al., 1989; Takahashi et al., 2002; Yamaguchi et al., 2002). After removal of the MTT containing medium, the formazan crystals in the cells are then dissolved by addition of e.g. dimethyl sulphoxide (DMSO) and the absorbance is monitored.

Abbreviations: DENSPM, N^1,N^{11}-diethylnorspermine; DMSO, dimethyl sulphoxide; FCS, fetal calf serum; IC_{50}, inhibitory concentration causing 50% inhibition of maximum response; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NADH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; SD, standard deviation

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The AlamarBlue™ assay is based on a similar mechanism. The AlamarBlue™ solution, containing an oxidation-reduction indicator (resazurin) (O'Brien et al., 2000), is added to the culture medium of the cells in the microwell assay. The oxidized resazurin (blue, non-fluorescent) indicator is taken up by cells and is reduced to resosurfin (pink, highly fluorescent at 590 nm) intracellularly by the action of the mitochondrial electron transport chain. In contrast to MTT, AlamarBlue™ is assumed to take up electrons at the end of the electron transport chain at the level of oxygen. The difference in the level of reduction of MTT and resazurin is because they have different redox potentials. The fluorescence intensity monitored in the well, still containing AlamarBlue™, reflects the extent of metabolic activity of the cells and is assumed to be directly proportional to the cell number (Ahmed et al., 1994; Gloeckner et al., 2001; Goegan et al., 1995; Nocari et al., 1998). Thus, the MTT and AlamarBlue™ assays should theoretically give the same results although a greater part of the mitochondrial transport chain takes part in the AlamarBlue™ reduction. An important advantage of the AlamarBlue™ assay, compared to the MTT assay, is that it allows further cell cultivation or other tests (e.g. protein determination) after assay readout. The AlamarBlue™ assay permits a continued aerobic ATP production while the MTT assay does not.

A means to determine the cell number that is not based on metabolic activity is to measure the protein content. To determine the protein content, we have used the Lowry assay (Lowry et al., 1951; Peterson, 1979; Sapan et al., 1999; Stevens, 1992) modified to 96 well plates.

In this report, the AlamarBlue™ and MTT assays, together with the Lowry assay, were used to investigate the effect of the polyamine analogue N^1,N^{11}-diethylnorspermine (DENSPM) on MCF-7, HCC1937, SK-BR-3 and L56Br-C1 human breast cancer cell lines. The polyamines putrescine, spermidine and spermine are crucial for normal cell proliferation, where increased levels of intracellular polyamines occur in rapidly proliferating cells. Inhibition of polyamine biosynthesis, followed by polyamine depletion, causes a decreased rate of cell proliferation and finally growth arrest (Thomas and Thomas, 2001). Polyamine depletion may also result in apoptosis (Ha et al., 1997; Hegardt et al., 2003; Huang et al., 2003; Kramer et al., 1997; Nitta et al., 2001, 2002). A number of polyamine analogues that inhibit polyamine biosynthesis and stimulate their catabolism have been synthesized. They do not take over the function of the naturally occurring polyamines. These polyamine analogues have been shown to have antitumor activity in experimental breast, prostate, melanoma and lung cancer models (Frydman et al., 2003a,b; Huang et al., 2003; Porter et al., 1993; Zagaja et al., 1998). DENSPM has been evaluated in Phase I clinical trials and Phase II studies have been initiated (Hahm et al., 2002). We show that combined data from the MTT, AlamarBlue™ and Lowry assays can be used to study DENSPM-induced effects on cell proliferation, apoptosis, mitochondrial activity and also on cytoplasmic events.

2. Materials and methods

2.1. Materials

Growth medium components were purchased from Biochrom, Berlin, Germany. Tissue culture plastics were purchased from Nunc, Roskilde, Denmark. AlamarBlue™ was purchased from BioSource, Camarillo, CA, USA. MTT was purchased from ICN Biomedicals Inc., Aurora, OH, USA. Phosphate-buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.3) was purchased from Oxoid Ltd., Basingstoke, Hampshire, United Kingdom. DMSO and Folin-Ciocalteu Phenol reagent were purchased from Merck KGaA, Darmstadt, Germany. The MCF-7 (HTB-22), HCC1937 (CRL-2336) and SK-BR-3 (HTB-30) cell lines were purchased from American Type Culture Collection, Manassas, VA, USA.

2.2. Chemicals

N^1,N^{11}-Diethylnorspermine (DENSPM) was purchased from Tocris Cookson Ltd., Bristol, United Kingdom. The water-soluble substance DENSPM was dissolved in PBS to give a stock solution of 2 mM. The stock solution was sterile-filtered and stored at −20 °C. The stock solution was further diluted in complete cell culture medium to give the final concentrations.

2.3. Cell culture

All cell lines were cultured at 37 °C, in a humidified incubator with 5% CO_2 in air. The human epithelial adenocarcinoma breast cancer cell line MCF-7, which has a wildtype *p53* gene, was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), non-essential amino acids, insulin (10 μg/ml), penicillin (50 U/ml) and streptomycin (50 mg/ml). The human adenocarcinoma breast cancer cell line SK-BR-3, which has a mutated *p53* gene, was cultured in the same medium as MCF-7 cells, but without the addition of insulin. The human carcinoma breast cancer cell line HCC1937 (Tomlinson et al., 1998), which has mutated *p53* and *BRCA1* genes, was cultured in MEM α-medium supplemented with 10% heat-inactivated FCS, non-essential amino acids, gentamycin (0.1 mg/ml), epidermal growth factor (20 ng/ml) and insulin (10 μg/ml). The human