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Analysis of Cyto-Toxic Sensitivity and Comparative Study of 3-(4, 5-Dimethylthiazole-2-Yl)-2, 5-Diphenyltetrazolium Bromide and Crystal Violet Assays Using Death Receptor 5 Monoclonal Antibody

***Kauna Usman[1], Chukwuka O. Opara[2], Hauwa O. Umar[3], Ramatu Salisu[3]**
[1]Department of Pharmacology,
College of Medicine and Health Sciences,
Federal University Dutse,
Jigawa State, Nigeria.
[2]Pharmacy Department,
National Hospital Abuja, Nigeria.
[3]Department of Human Anatomy,
Faculty of Basic Medical Sciences,
College of Medicine and Health Sciences,
Federal University Dutse,
Jigawa state, Nigeria.
Email: kaunau92@gmail.com

Abstract
Colorimetric assays are widely applied in assessing the anticancer activity of compounds. The 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and crystal violet assays are the commonly used colorimetric assays to evaluate the efficacy and interactions of anticancer agents. Due to possible shortcomings, using a single assay may be associated with the risk of erroneous interpretation hence comparisons of the assays is important. The sensitivity of MTT and crystal violet assays to death receptor 5 monoclonal antibody (DR5 mAb) was compared in this study. The assays were evaluated on DR5 mAb treated prostate cancer cells (PC3) and on varying PC3 cell number. Results obtained showed increase in absorbance with increasing cell number while a decreased absorbance was observed on increasing DR5 mAb concentrations with crystal violet assay. MTT assay showed similar pattern for increasing DR5 mAb concentrations except the absorbance values were not as high as crystal violet assays. However, no regular pattern was observed with MTT assay on varying PC3 cell numbers. Up to 86% apoptosis was obtained following haematoxylin staining of DR5 mAb treated PC3 cells.
The result of the study is informative in conducting further research on DR5 mAb for mechanism of cytotoxic activity and in choosing the most suitable assay for evaluating DR5 mAb.

Keywords: 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide, Crystal violet, Death receptor 5 monoclonal antibody, Harris haematoxylin and Prostate cancer cells

INTRODUCTION
Despite recent advances in the detection of early prostate cancer, there are little effective therapies for the advanced or metastatic state of the disease. The death inducing cytokine tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) holds enormous

**Author for Correspondence*

promise as an anti-cancer agent due to its ability to induce apoptosis in tumor cells with minimal adverse effect on normal cells (Trivedi and Mishra 2015). The apoptotic signalling cascade is initiated upon engagement of the death receptors 4 (DR4) and 5 (DR5) to cognate ligands (Suzuki-Karasaki *et al.,* 2014). Upon ligand binding to the death receptors, they aggregate and recruit Fas- associated death domain (FADD), FADD in turn recruits the initiator procaspases 8 and 10, forming a death inducing signalling complex (DISC) where the initiator caspases are auto-activated via proteolysis (Falschlehner *et al.,* 2009). The activated initiator caspases bind and activate the effector caspases 3, 6, 7 triggering the cleavage of the death substrate. DR5 is expressed in a wide variety of tissues including breast, lungs and prostate and it is a transcriptional target for p53 (Surget *et al.,* 2012).

To screen for anti-cancer agents, multiple assays are used to measure the sensitivity of cancerous cells. These assays evaluate cell parameters including ability of the cells to proliferate and cell viability (Sliwka *et al.,* 2016). MTT assay is based on cell proliferation, the principle is based on uptake and reduction of the soluble yellow MTT tetrazolium salt (3-[4, 5-dimethylthiazol-2-yl]-2-5-diphenyl-tetrabromide) by mitochondrial succinic dehydrogenase to an insoluble blue MTT formazin (Vistica *et al.,* 1991). Crystal violet assay is based on the uptake of crystal violet by cell nucleus causing them to stain purple (Uzunoglu *et al.,* 2010). There is need to validate the assay for evaluating the effect of specific cytotoxic agents on cells to avoid erroneous results therefore, the aim of this study was to compare and evaluate the sensitivity of DR5 mAb on PC3 cells using MTT and crystal violet assays.

MATERIALS AND METHOD

Experimental design

The study was conducted using PC3 cells purchased from Sigma Aldrich, UK.

PC3 cell culturing

PC3 cells were cultured in Dulbecco's modified eagle's media (DMEM). PC3 cell concentrations of 2500 cells/ml, 1250 cells/ml, 625 cells/ml, 312 cells/ml and 156 cells/ml were plated on 96 well plates while cell concentrations of 250,000 were pipetted into T75 flask. The cells were grown at 37^{o}C in an incubator (Thermofisher, UK) with a humid atmosphere and 5% CO_2 for two weeks.

Cells from the T75 flasks were passaged using phosphate buffer saline (PBS) as wash solution and 0.25% trypsin/EDTA to detach them from flask. Cells were then counted after which 12 ml of cells at 10,000 cells/ml was pipetted into T25 flasks and treated with 1 ml of 2000 ng/ml DR5 mAb each. The flasks were then incubated for 24 hours at 37^{o}C in an incubator with a humid atmosphere and 5% CO_2. PC3 cells in 96 well plates (2500 cells/ml) were treated using 1 ml of DR5 mAb concentrations of 2000 ng/ml, 500 ng/ml, 100 ng/ml and 25 ng/ml after media from the wells were replaced with fresh DMEM. The plates were again incubated at 37^{o}C in an incubator with a humid atmosphere and 5% CO_2 for a week.

Harris haematoxylin staining.

Media in T25 flasks treated for 24 hour with DR5 mAb was emptied into 50 ml tubes. The flasks were washed using 3 ml PBS and the content poured into their respective 50 ml tubes. Trypsin/EDTA was used to detach cells from flask following 2 minutes incubation. The 50 ml tube content was centrifuged at 1000 rpm (approximately 400 g) for 5 minutes to pellet the cells. The supernatant was discarded while the pellets were re-suspended in 1 ml PBS and counted using a haemocytometer (Hausser, UK). Counted cells were diluted to 200,000 cells/ml and 200 µl of the cell suspension and spun in a cytospin at 1000 rpm for 3 minutes

to deposit cells on slides. The slides were then placed in (50:50) acetone /methanol in a coplin jar in the fume cupboard hood for 5 minutes to further fix the cells prior to washing under running water and Tris-buffered saline (TBS) for 3 and 1 minutes respectively. The slides were finally mounted in glycerol/TBS (3:1) and viewed under a light microscope (OMAX, US) using x400 magnification.

MTT assay

DR5 mAb treated 96 well plates from previous procedure were treated with 5 mg/ml MTT solution and incubated for 4 hours at 37ºC. The media was then removed from the wells and 100 µl acid isopropanol added to dissolve the blue crystal of formazin formed in the cell layer. It was further incubated for 30 minutes at room temperature with the plate lid on to prevent the isopropanol from evaporating. A plate reading spectrophotometer (Cole-Parmer, UK) was used to measure the absorbance of each plate at 570 nm afterwards. Untreated plated PC3 cells of varying numbers (2500 cells/ml, 1250 cells/ml, 625 cells/ml, 312 cells/ml and 156 cells/ml) were also analysed using MTT assay as aforementioned.

Crystal violet assay

Cell media from DR5 mAb treated 96 well plates was removed and the cells were washed using 200 µl PBS. Methanol (200 µl) was pipetted into the plates at the fume cupboard hood and allowed to stand for 15 minutes to fix the cells. The methanol was then removed and the wells air dried after which 200 µl crystal violet was pipetted into it while it was left to incubate for 20 minutes. The wells were washed three times using distilled water and then solubilized in 50 µl of 10% glacial acetic acid. This was followed by incubation for 30 minutes and the absorbance of each well read at 570 nm using a spectrophotometer. Untreated plated PC3 cells of varying numbers (2500 cells/ml, 1250 cells/ml, 625 cells/ml, 312 cells/ml and 156 cells/ml) were also analysed

Statistical analysis of data

Data obtained from the study were presented in charts and tables and represented as mean ± standard deviation. Statistical significance was evaluated using one way ANOVA and Dunnett's test where P values of < 0.05 are considered significant.

RESULTS

Comparative study of MTT and crystal violet assays on varying PC3 cell number

MTT and crystal violet assays were carried out on varying PC3 cell numbers to analyse the sensitivity of the assays on the cells.

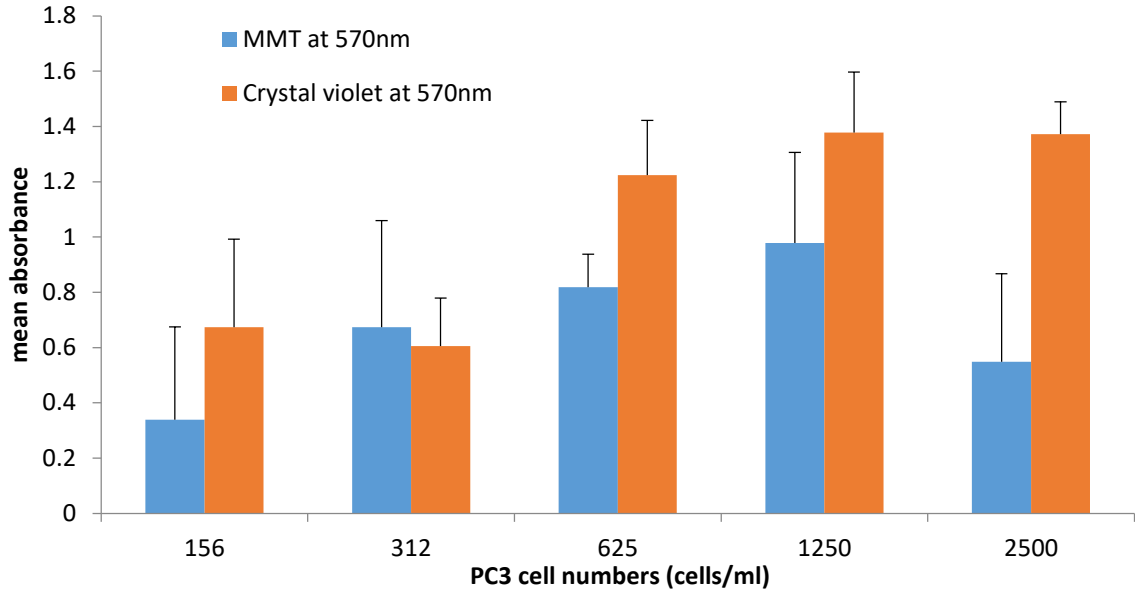

Figure 1: Effect of MTT and crystal violet assays on PC3 cells.

Crystal violet assay showed higher absorbance for all cell numbers except for 312 cells/ml compared to MTT assay, while there was increasing absorbance with increase in cell number for MTT assay there was no regular pattern for crystal violet assay.

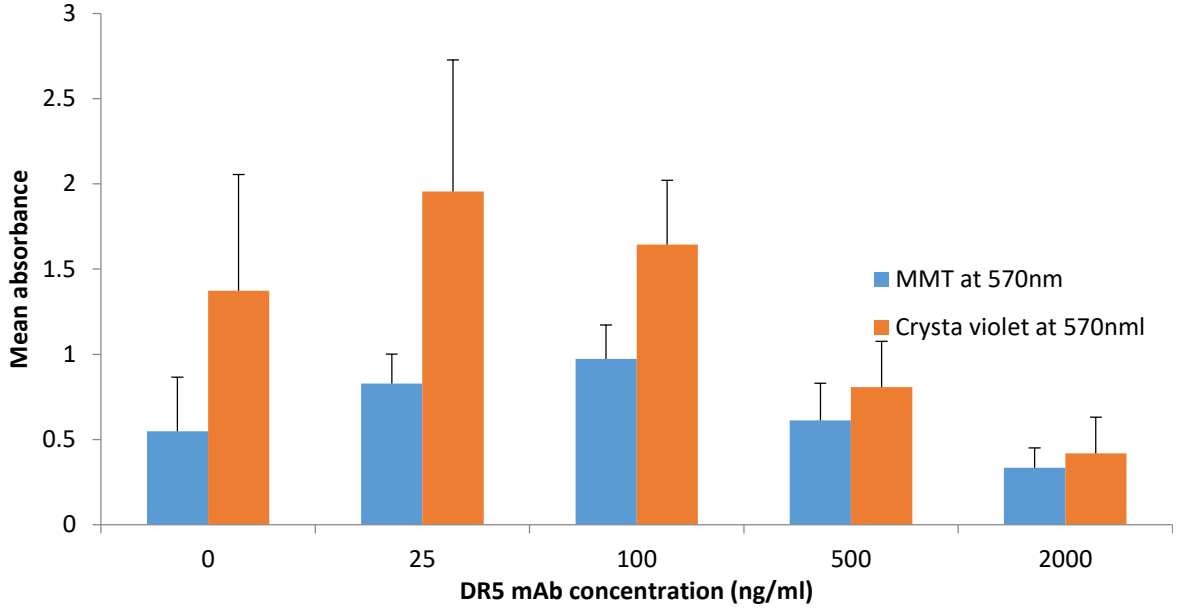

Figure 2: DR5 mAb dose-dependent sensitivity to PC3 cells using MTT and crystal violet assays.

To evaluate the susceptibility of DR5 mAb on PC3 cells, sensitivity analysis was performed with varying DR5 mAb concentrations using MTT and crystal violet assay. Higher absorbance was seen with crystal violet assay than MTT assay at all concentrations. DR5 mAb showed significant cyto-toxic activity with both assays. Using the data above, the extent of cell killing is determined by the percentage survival as reported by Patel *et al*, (2009).

Table 1: Percentage survival of PC3 cells following treatment with varying DR5 mAb concentrations.

DR5 mAb Concentration (ng/ml)	Percentage survival MTT assay (%)	Percentage survival Crystal violet assay (%)
0	100.00	100.00
25	150.90	142.40
100	177.50	119.80
500	111.60	58.80
2000	61.00	30.60

DR5 → mAb-monoclonal antibody, MTT → 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide.

The higher the concentration of DR5 mAb, the lower the percentage of cell survival. Highest percentage of survival was observed at 100 ng/ml and 25 ng/ml DR5 MAb concentration for MTT and crystal violet assays respectively.

Table 2: apoptotic effect of DR5 mAb treated PC3 cells from Harris haematoxylin staining.

Cells	DR5 mAb treated cells	Untreated cells
Total number of counted cells.	100	100
Number of apoptotic cells observed.	86	57
Number of survival cells observed.	14	43
Percentage apoptosis.	86	57

Harris haematoxylin stain was used to evaluate the extent of apoptosis based on the cells' morphological changes including membrane blebbing, chromatin and nuclear fragmentation. Subjected to the same conditions, the percentage of apoptosis was higher on DR5 mAb treated cells than the untreated ones.

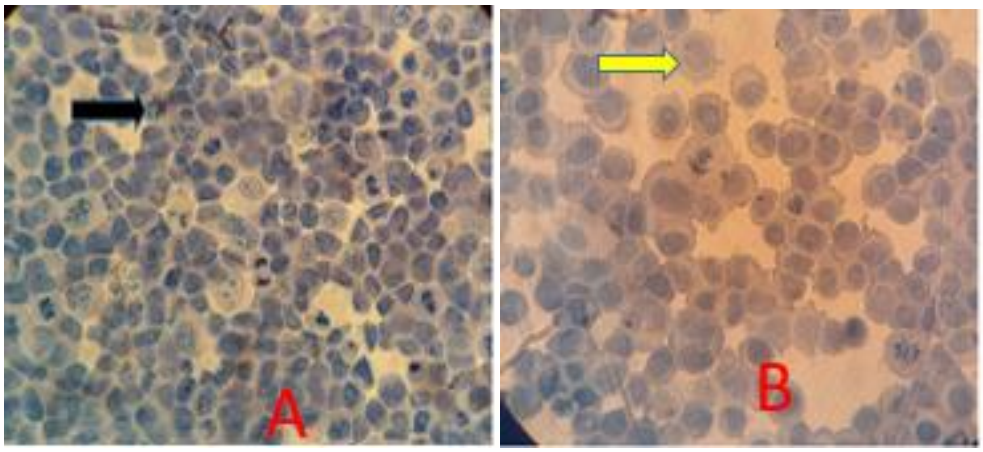

Plate I: Untreated (A) and DR5 mAb treated (B) PC3 cells prior to Harris haematoxylin staining; at X400 magnification.
Key: ➡ healthy cells, ⇨ apoptotic cells

Membrane blebbing, condensed nucleus and mitosis are seen in both cells but more of these features were observed in the DR5 mAb treated cells than the untreated cells.

DISCUSSION

DR5 mAb is known to induce apoptosis of various tumor cell lines through the DR5 receptor by binding and activating the TRAIL receptor through their death domains (Edagawa *et al.*, 2014). The death domain is known to be very significant in transmitting death signals from the cell surface to the intracellular signalling pathway (Daniel *et al.*, 2001). Apoptotic signals once received are known to trigger apoptosis through two pathways, the intrinsic and extrinsic pathways (Fulda and Debatin, 2006). The intrinsic pathway is mediated by non-receptor mediated stimuli that produce intracellular signals, these are mitochondrial initiated events (Elumalai *et al.*, 2012). The extrinsic pathway is mediated by trans-membrane

receptor interactions (Ashkenazi, 2008). Once the initiator caspases are activated, the execution phase of apoptosis is triggered. The activation of the caspase cascade is the most important part of cell signalling triggered by TRAIL and it is the basic function of DR5. Apoptosis is characterised by morphological and biochemical changes which was evident following Harris haematoxylin staining of cells. Plate IA and IB showed cells with condensed nucleus and membrane blebs. However, there was increased apoptosis with DR5 mAb treated than untreated cells. These changes are due to the activation of cytoplasmic endonuclease that degrades nuclear materials and proteases further degrading the cytoskeletal and nuclear proteins by the executioner caspases (Hengartner, 2000; Sliwka *et al.*, 2016).

At higher DR5 mAb concentrations, PC3 cells showed increased sensitivity to the agent as decreased absorbance were recorded with both assays. Both assays revealed lower absorbance to higher concentrations of the agent which is indicative that the DR5 mAb is cytotoxic. However, crystal violet assay demonstrated higher sensitivity to the agent on the cells showing that crystal violet assay is a more sensitive assay between the two assays adopted in the study. Literature has shown that MTT assay has decreased sensitivity in the presence of compounds that uncouple mitochondrial respiratory chain as they enhance formazin crystal (Bruggisser *et al.*, 2002). The pH of the cell medium as well as D-glucose concentrations have also been reported to give false negative results with MTT assays (Wang *et al.*, 2010). Regarding the irregular patterns obtained, non-specific staining is reported to occur in cells after reaching confluence (Chiba *et al.*, 1998). Improper solubilisation of glacial acetic acid and difference in their optical properties are also known to cause disparity in absorbance of both assays. Other factors that may decrease the sensitivity of a cytotoxic agent is the presence of decoy receptors (DcR1, DcR2 and osteoprotogerin) that bind to DR5 mAb to form non-functional complexes thus, competing with the DR5 receptors (Mantovani *et al.*, 2001).

CONCLUSION

In conclusion, PC3 cells were significantly sensitive to DR5 mAb however higher sensitivity was demonstrated by crystal violet assay compared to MTT assay.

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