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How reliable are *in vitro* IC_{50} values? Values vary with cytotoxicity assays in human glioblastoma cells

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Abstract:

Increasing evidence shows that discrepancies exist among *in vitro* cytotoxicity methods resulting in unreliable drug toxicity profiles. This is particularly criticial for cell lines such as gliomas which are histologically and genetically heterogeneous. The high level of variation in these cells makes comparative analysis difficult and is a severe limitation for the usefulness of high-throughput screening methods. Here we examine variations between four conventional *in vitro* cytotoxicity assays (MTT, Alamar Blue, Acid Phosphatase and Trypan Blue) for assessing the viable cell number following treatment of two human glioblastoma cell lines (U87MG and U373MG) with different chemical agents (carboplatin, etoposide, paraquat). The variations in IC_{50} values between the four assays suggest that even when combining several endpoints such as mitochondrial functions, lysosomal activity, and membrane integrity, a reliable and uniform toxicity profile was not achieved. Because of these variations between cytotoxicity assays using compounds with varying mechanisms of cytotoxicity, then it is possible that the true IC_{50} value of valuable and beneficial compounds for glioblastoma may have been missed through over/underestimation. This highlights the importance of reliability and accuracy in pre-animal models such as *in vitro* models of cytotoxicity for better predictive *in vivo* responses.

Keywords: *In vitro* cytotoxicity assays; glioblastoma cells; chemotherapy; carboplatin; etoposide; paraquat

1. Introduction

The term cytotoxicity is commonly used to refer to the potential of a compound to induce variations in the cellular behaviour and essential processes that subsequently trigger cell death or cause a large decrease in cell survival (Niles and Riss, 2015). Bearing in mind that cells are able to exhibit specific responses as a result of exposure to diverse chemicals or physical stresses, a considerable number of *in vitro* cytotoxicity assays are available. These are generally classified on the basis of the endpoints they measure which usually involve a commom mechanism shared by most cell types. However, some assays may measure specific responses according to a particular cell type, e.g. motility for sperm cells and hemolysis for red blood cells. Currently, the common endpoints used for assessing cytotoxicity include membrane permeability, cellular metabolite content, mitochondrial functions, lysosomal functions and cell death (Mahto et al., 2010). Some features of the most popular *in vitro* methods for evaluating cytotoxicity are shown in Table 1.

One of the stalwarts of *in vitro* chemosensitivity testing has for many years been the colorimetric MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) assay based on the reduction by intracellular dehydrogenases of the yellow tetrazolium salt to purple formazan crystals by viable cells. Originally developed by Mosmann (Mosmann, 1983), the MTT assay had several advantages over the pre-existing technologies of cell counting and radio-labelling of nucleotides. The MTT assay proved to be a quick and convenient technique with lower technical requirements than some of the other methods and was rapidly adopted by investigators (Denizot and Lang, 1986). These advantages also led to the MTT assay being adapted for HTS (high throughput screening) as the process can be fully automated. Other colorimetric and fluorometric assays, such as the Alamar Blue (resazurin reduction), acid phosphatase (*p*-nitrophenol phosphate hydrolysis), XTT (tetrazolium salt reduction) and SRB (sulforhodamine B amino acid complexation) have also been implemented for HTS, each with its own benefits and limitations (Niles et al., 2009,Rajalingham, 2016,Slater, 2001).

The ability to measure the number of viable cells through *in vitro* HTS assays represents an important tool with applications in toxicological safety testing, oncological research, industrial microbiology studies, bioprocess monitoring and drug discovery (Szymanski et al., 2012,Macarron et al., 2011). Recently, increasing evidence has shown some discrepancies between the different methods which has led to either an underestimate or an overestimate of cytotoxicity leading to unreliable toxicity profiles (Rajalingham, 2016). Therefore, it has become critical to establish the reliability of these widely used non-animal methods for assessing the potential cytotoxicity of chemicals and new therapeutic agents. This is particularly important if one takes into consideration the 3Rs ethic, Replace, Reduce, and Refine, where a concerted effort is focused on encouraging researchers to lower the level of reliance upon animal models for screening potential therapeutic compounds in both the discovery and development process (Baumans, 2004).

Exemplary of this concept is the total ban on animal testing in cosmetics which came into force with the Cosmetic Regulation no. 1223/2009 on March 13th, 2016.

Further complications in the implementation of standardized, commercially available cytotoxicity testing methods, are the variations in response to drugs that may differ not only according to the drug but also according to the cell line being investigated. For example, an important consideration in the context of researching therapies for brain gliomas, is that all gliomas are histologically and genetically heterogeneous (DeAngelis, 2001,Inda et al., 2014), therefore they are also heterogeneous in their therapeutic responses. This high level of variation makes comparative analysis difficult and is a severe limitation for the usefulness of HTS methods. Gliomas derive from neoplastically transformed glial cells that constitute the supportive tissue of the brain, and their growth and proliferation impact brain structure and function (Sizoo et al., 2010). The morbidity and mortality rates of gliomas have remained consistently high due to the heterogeneity of this disease, prognosis is typically poor and no substantial improvements in overall survival have been made over the past four decades (Anderson et al., 2008,Van Meir et al., 2010,Omuro and DeAngelis, 2013). The need for additional therapies is therefore ongoing and the development of more robust modelling systems will aid this progress, enabling the pharmaceutical industry to better target their resources against gliomas.

Previous work in our laboratory on cancer cells has indicated that for all of the previously mentioned assays, results vary between different drugs but yet, they always remain consistently elevated compared to the gold standard Trypan Blue (TB) exclusion method. At present, the simplicity and its low cost maintain the TB exclusion assay as the gold standard method for determining cell numbers and for evaluating cytotoxicity. Although extensively used, through years important limitations have been attributed to this method: it is considered a time-consuming assay and when used for a large number of samples it can provide low precision results (Avelar-Freitas et al., 2014). Furthermore, it is a subjective assay which can however easily be circumvented by blinding the samples to be counted. Additionally, Tramontina and colleagues have shown that exposing live cells to TB for longer than 5 min can cause inappropriate uptake of the dye into viable cells and therefore result in overestimation of dead cells in a sample (Tramontina et al., 2000).

With this background, the aim of this study was to examine variations between four conventional *in vitro* cytotoxicity assays (MTT, Alamar Blue, Acid Phosphatase and Trypan Blue) for assessing the viable cell number following *in vitro* treatment of two human glioblastoma cell lines with different chemical agents. U87MG and U373MG are well established glioblastoma cell lines harbouring a range of different genetic lesions, and that represent a robust and reliable testing model for the comparison of cytotoxicity assays, allowing for both variations in drugs under investigation as well as the susceptibility or resistance of the cell line (Schmidt et al., 2013).

The four *in vitro* assays were compared using three agents that differ in their mode of action in triggering cytotoxic effects. Two anticancer agents were used, Carboplatin and Etoposide, which are mainly apoptotic in nature *via* DNA cross-linking/alkylation and topoisomerase II inhibition, respectively (Avgeropoulos and Batchelor, 1999). As a contrast the herbicide, paraquat, was also investigated since it is a well-known toxin that causes necrotic cell death *via* oxidative stress (Grabie et al., 1993,Pezzoli and Cereda, 2013). By comparing these frequently used enzymatic assays with the gold standard cell counting technique (TB assay), the study aims to provide medical researchers with an assessment of the reliability of these assays and possible pitfalls in some of the most relied upon methods in current usage.

2. Materials and Methods

2.1. Chemicals and reagents

All chemicals, reagents and culture medium were supplied by Sigma Aldrich (St Louis, MO, USA) except where explicitly stated. Etoposide (TOCRIS, Bioscience, Bristol, UK), carboplatin and paraquat dichloride hydrate were dissolved in their appropriate vehicles and diluted to the required concentrations in cell culture medium.

2.2. Culture of glioblastoma cell lines

Human glioblastoma multiforme cell lines U373MG and U87MG (obtained from ECACC) were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine, in a humidified incubator at 37°C and 5% CO_2. Fresh medium was changed every 3 days and cells were sub-cultured at ~80% confluency.

2.3. Experimental protocol

All experiments were conducted on cells seeded at a density of 2.4 x $10^6/cm^2$ incubated for 24 h post-seeding in 6 cm cell culture dishes and 48 h post-seeding in 96-well microtitre plates. Following the appropriate incubation time, medium was discarded and replaced with fresh medium containing varying concentrations of the compound of interest and incubated for a further 48 h.

2.4. MTT assay

This assay, as previously mentioned, relies on the reduction by intracellular NAD(P)H-dependent dehydrogenases of viable cells, of the soluble and pale yellow tetrazolium salt, MTT, into insoluble and purple formazan crystals, thus reflecting the number of metabolically active cells present in a sample (Berridge et al., 2005).

After 48 h drug exposure, the MTT colorimetric assay was performed on the 96-well plates. Ten µL of MTT solution in PBS (5 µg/mL) were added to each well, including medium only used as negative control, and returned to the incubator for 3 h. Medium was then removed from each well, replaced with 100 µL DMSO for solubilisation of the formazan crystals and the plate was placed on a shaker for 20 min.

Absorbance was read at 570 nm with reference wavelength of 690 nm on a Sunrise Tecan™ microplate reader (Tecan Trading AG, Switzerland). Viable cell count was assessed as a percentage relative to untreated cells.

2.5. Trypan blue exclusion assay

Trypan Blue is a negatively charged, relatively small molecule (960 Da) that is cell membrane impermeable. However, in cells with compromised cell membranes, it is able to penetrate and bind to different intracellular proteins, giving cells a bluish colour readily observable under a microscope. Thus, this assay is able to discriminate in a given population between viable cells and cells with damaged membranes that are usually considered to be dead/dying (Strober, 2015).

After 48 h drug exposure, cells were harvested from 6 cm cell culture dishes by trypsinization, the pellet was resuspended in 1 mL of medium and 100 µL of this suspension was diluted with the appropriate volume of trypan blue for cell counting on an improved Neubauer Haemocytometer (Weber Scientific Internation Ltd, UK). Viable and non-viable cells were counted under light microscopy and are reported as a percentage of the total cell number for each drug treatment.

2.6. Acid phosphatase assay

The acid phosphatase assay developed by Connolly et al. (Connolly et al., 1986) measures the activity of lysosomal acid phosphatases. In viable cells, the synthetic substrate p-nitrophenyl phosphate is hydrolysed to p-nitrophenol by intracellular acid phosphatases, producing a colour change in an alkaline environment that can be measured at 405 nm. This enables a correlation to be drawn between absorbance and viable cell number (Ivanov et al., 2014).

After 48 h drug exposure, medium was removed from all wells and cells were washed twice with PBS. To each well, including medium only used as negative control, 100 µL of acid phosphatase buffer (0.1 M sodium acetate, 0.1% Triton X-100, pH 5) and 5 mM *p*-nitrophenyl phosphate were added to each well and returned to the 37°C incubator for 1 h. The reaction was stopped by adding 50 µL 0.3 M NaOH to each well and the plate read at 405 nm with a reference wavelength of 630 nm on a BioTek Synergy™ HT plate reader (Winooski, VT, USA). Viable cell count was assessed as a percentage relative to untreated cells.

2.7. Alamar blue assay

This assay is based on the reduction by mitochondrial enzymes of viable cells, of the active ingredient of Alamar Blue, resazurin, a blue water-soluble, non-toxic dye, into the fluorescent pink compound, resorufin, which can be quantified using a microplate fluorometer. Colour intensity is hence proportional to the amount of viable cells present in a sample (O'Brien et al., 2000).

After 48 h drug exposure, 10 µL Alamar Blue reagent (1 mg/mL resazurin sodium salt in PBS) was added to each well, including medium only used as negative control, and incubated for a further 4 h to promote the reaction and the subsequent pink colour generation by metabolically active cells. The

absorbance was then read on a BioTek Synergy^{TM} HT plate reader (Winooski, VT, USA) at 530 nm with a reference wavelength of 590 nm. Viable cell count was assessed as a percentage relative to untreated cells.

2.8. Statistical methods

All experiments were repeated independently a minimum of three times, unless stated otherwise and data are expressed as mean ± standard error of the mean (SEM). IC_{50} values were analysed using GraphPad Prism 7 using non-linear sigmoidal curve fitting with the normalized response or the Excel add-in ed50v10. Statistical differences were determined using the Student's t-test with $p < 0.05$ considered as statistically significant.

3. Results and Discussion

3.1 Changes in viable cell number associated with different in vitro assays following exposure to chemical compounds

In the past two decades, the use of *in vitro* methods has become the most used alternative to animal experiments for evaluating hazardous effects induced by chemicals and for predicting *in vivo* human toxicity (Doke and Dhawale, 2015). Specifically, cell-based *in vitro* assays are considered major tools largely used for preliminary screening and ranking of new chemical entities through the drug discovery and development process (Eisenbrand et al., 2002). In the present study, variations between four conventional *in vitro* cytotoxicity assays in terms of the number of viable cells on two human glioblastoma cell lines in the presence of carboplatin, etoposide and paraquat were investigated (Fig. 1). The results show that in the majority of cases there are clear inter-assay differences for both cell lines since the toxicity profiles differ for the three individual compounds. A clear dose-response effect was observed for all assays and for all three compounds in the U87MG cell line (Fig. 1A-C). However, in U373MG cells an appreciable dose response-effect was observed for all assays only in the case of paraquat (Fig. 1F) and carboplatin with the exception of the Alamar Blue assay (Fig. 1D). No dose-response effect was observed in the presence of etoposide using the Alamar Blue and MTT assays (Fig. 1E).

The mean IC_{50} values reported in Table 2, defined here as the concentration of drug required for 50% inhibition of viable cell number *in vitro*, obtained from each individual cytotoxicity curve, also reflect these differences where a wide variation in values was observed. In all cases, the TB exclusion assay showed the highest sensitivity for detecting cytotoxicity giving the lowest IC_{50} values for all the compounds compared to the other three enzyme-based methods and in both cell lines, indicating that this assay might over-estimate the actual toxicity of the compounds. These differences were always statistically significant except in the case of U373MG cells in the presence of paraquat assessed with the Acid Phosphatase assay. Instead, the IC_{50} values obtained for all the compounds using the Alamar Blue assay were, in general, the highest in both cell lines compared to the other three test methods, indicating that it has the lowest sensitivity in terms

of viable cell number determination. For instance, the IC_{50} of etoposide in both cell lines was less than 60 μM using the Trypan Blue exclusion assay, however, the Alamar Blue assay failed to produce an IC_{50} value in either cell line up to concentrations of 300 μM. Additionally the Acid Phosphatase assay also showed high IC_{50} values in both cell lines being ~240 μM – much higher than the values produced by the gold standard direct observation which returned IC_{50} values 4-fold less. Results such as these in a HTS setting for glioblastoma, may direct an automatic system to disregard a potentially beneficial compound as being without and hence to misjudge the full benefits of compounds that are being screened.

Concerning with the sensitivity of the assays used here, the inhibitory concentrations values (IC_{50}) were considered as the metric of interest for establishing the reliabilities among the different methodologies. In contrast to the enzyme-based assays performed in 96-well formats, in the present study Trypan Blue Assay was the *in vitro* assay with the highest sensitivity for detecting cytotoxicity in both cell lines after treatment with carboplatin, etoposide and paraquat. Since it was introduced in 1960, the use of Trypan Blue has been considered a direct *in vitro* assay for quantifying cell numbers, and additionally, due to the uptake of the blue dye by cells with compromised membranes, this method offers the advantage over others of distinguishing between dead/dying and live cells (Tran et al., 2011). At present, this assay is still considered as the least expensive requiring no specialized or expensive equipment and the easiest to perform, despite its low-precision when it is used for a large number of samples (Kim et al., 2011). Automatic cell counting techniques are highly attractive alternatives to the Trypan Blue exclusion method of determining cell number, however technical requirements and high financial barriers to entry means that this approach is often not available to research organisations with restricted budgets, such as Universities or small/start up biotechnology companies.

The MTT assay is generally believed to be driven exclusively by the succinate dehydrogenase in the mitochondria of metabolically active cells which reduce it into its coloured formazan product. However, several studies have recently indicated that other intracellular enzymes such as NAD(P)H-dependent oxidoreductases can also be responsible for the tetrazolium salt reduction (van Meerloo et al., 2011) and that damaged mitochondria may still be able to reduce tetrazolium salts. Based on the premise that MTT reduction is proportional to the number of viable cells, evidence suggests that this non-specific dye reduction could lead to underestimate results of cytotoxicity (Hamid et al., 2004, Martin and Clynes, 1993). Indeed, Jo et al. recently reported that the MTT assay was unreliable for assessing cytotoxicity induced by different ethanol concentrations on primary glioma cells GBL-13 and GBL-15, stating that a better toxicological sensitivity was obtained when U87MG and U373MG were used (Jo et al., 2015). Rivera and Miller found that Neutral Red uptake which measures lysosomal integrity was more sensitive for measuring the changes in C6 glioma cells viability after 6 h of dinitrobenzene exposure in contrast with the MTT assay, which did not show any response within the range of concentrations used (Rivera and Miller, 2008).

The MTT assay also proved to be less sensitive than the Trypan Blue assay in human hepatoma cells (HepG2) for detecting radiation-induced cell viability (Chung et al., 2015). Interestingly, in our study the IC_{50} for carboplatin detected by the MTT assay in U87MG cells (223±23 μM) compares favourably with the toxicological data described by Pendylala and Creaven who reported a similar IC_{50} value after 48 h of carboplatin exposure on the same cell line (Pendyala and Creaven, 1993).

Over the last 50 years, the use of the Alamar Blue assay has gained acceptance for measuring cell proliferation and cytotoxicity due to its versatility and simplicity, combined with the advantage of measuring viable cell number over time and allowing multiple analytic assays to be performed on the same samples, unlike the MTT assay (O'Brien et al., 2000). However, contrary to the evidence proposing the Alamar Blue assay as being one of the most sensitive *in vitro* cytotoxic assays, our results suggest that this is not always the case, since we observed the lowest sensitivity in both glioblastoma cell lines and for all three drug models. This finding could possibly be due to these particular cell lines and hence peculiar to this study. Although this method has been recognised for giving reproducible cytotoxicity profiles, some inherent limitations have recently been highlighted. Similar to the MTT reduction, resazurin reduction to resorufin may not be entirely restricted to changes in mitochondrial function suggesting that microsomal enzymes also play a role, leading to an underestimation of the potential cytotoxicity induced by chemicals (Gonzalez and Tarloff, 2001). An important consideration of these metabolic assays is that the reduction of the substrates which is affected by changes in intracellular metabolic activity may not have a direct effect on the overall number of viable cells since these assays cannot distinguish between quiescent and actively dividing cells (Quent et al., 2010).

Contrary to the MTT and Alamar Blue assays, the Acid Phosphatase method estimates the number of viable cells by detecting alterations of the lysosomal membranes. Lysosomes are multifunctional organelles well known for containing a great quantity of hydrolytic enzymes including Acid Phosphatase. An increase in the amount of this enzyme due to lysosomal labilisation induced by different chemical insults has been considered as a marker of cellular damage (Yang et al., 1996). However, some studies have postulated that variations in Acid Phosphatase activity can be affected by the cell density and cell cycle distribution which consequently lead to artefacts in the fluorescent signal generated (Martin and Clynes, 1993). This assumption was later confirmed by a study undertaken by Friedrich and colleagues who observed lower cellular signal intensities in confluent human colon carcinoma cell lines (Friedrich et al., 2007). In our study, the Acid Phosphatase assay appears to be the most suitable assay for detecting differences among the two cell lines when a toxicant such as paraquat is used.

3.2 Different cytotoxic responses detected between the two glioblastoma cell lines upon exposure to chemical compounds using different assays

The two glioblastoma cell lines, U373MG and U87MG differ in their cytotoxic responses according to the chemical compound used. The IC_{50} values reported in Table 2 indicate that U87MG cells are generally more sensitive to the two anti-cancer drugs, carboplatin and etoposide than U373MG cells since the IC_{50} values are lower. Indeed, these two cell lines differ in their expression of the multi-drug resistance associated protein (MRP) where it is almost 4-fold more expressed in U373MG cells compared to U87MG cells which accounts for the increased resistance observed against etoposide and other drugs with respect to U87MG cells (Mohri et al., 2000). Furthermore, U373MG cells differ from U87MG cells in that they have mutant p53 and phosphatase and tensin homolog (PTEN), whereas U87MG, normally considered to be drug-sensitive, harbour a wild-type p53 gene but without expressing the functional protein to any measurable extent due to Mdm2 overexpression which destabilizes it (Weller et al., 1998). Interestingly, the IC_{50} values for U87MG cells are not too dissimilar from each other when comparing the two drugs for each individual assay, except for the MTT assay where a higher IC_{50} value was obtained in the presence of carboplatin (248 μM vs 141 μM). This suggests that both drugs are just as effective at similar concentrations on U87MG cells. These considerations can also be deduced from the cytotoxicity profiles in Fig. 1A and 1B. On comparing the IC_{50} values of U373MG cells obtained with the two anti-cancer drugs, the results show that these cells are more resistant to carboplatin than to etoposide regardless of the large variability in the IC_{50} values obtained with the different assays. In accordance with our results, variability between cytotoxicity assays using cell counting, Alamar Blue and Acid Phosphatase assays and volume determination has also been reported on three-dimensional stem cell neurospheres after etoposide treatment (Ivanov et al., 2014). Similarly, Gajski et al., (Gajski et al., 2016) observed inter-assay differences in human glioblastoma A1235 cells treated with the biotoxin, bee venom (BV). By using the MTT, Crystal violet and Trypan blue exclusion assays, they found that at higher concentrations of BV tested, the Crystal violet assay was very sensitive compared to the other two assays which were more comparable with each other.

With regards to cytotoxicity induced by a non-anticancer agent, such as the toxin, paraquat, it is interesting to observe that U373MG cells, normally considered to be more drug-resistant than U87MG cells (Siegelin et al., 2009,Ravizza et al., 2004,Mohri et al., 2000) appear to be less resistant in the presence of a toxicant. This reduced sensitivity with respect to U87MG cells has also been observed by Datta et al. when testing the resistance of these two cell lines against cisplatin (Datta et al., 2004). This can be inferred by both the lower IC_{50} values and by the cytotoxicity profiles (Fig. 1C and 1F). The differences between the two cell lines can also be seen by the profiles reported in Fig. 2A-C, which compare the effect of each individual drug using the assay which resulted the most sensitive one for discerning the differences between them, excluding the TB assay. While paraquat indeed showed potent cytotoxic effects and lower IC_{50} values than the other compounds tested, especially in the more drug-resistant cells U373MG, it also showed the same discrepancies as the chemotherapeutic compounds when each method was tested against each other

as seen in Table 2. As mentioned above, the Acid Phosphatase assay (Fig. 2A) appears to be particulary sensitive for discriminating the differences in cytotoxicity to toxicants among the two cell lines since there is large gap in the IC_{50} values (Table 2). Instead, for the anti-cancer agents, the Acid Phosphatase assay is the least ideal one for determining their drug sensitivities, since it showed the smallest variability in IC_{50}. In these cases, the MTT assay appears to be the most sensitive one for determining differences in drug-resistance to anti-cancer agents. Although the Alamar Blue assay shows the greatest variability in IC_{50} values in the case of carboplatin, as pointed above, it appears to over-estimate cytotoxicity.

These outcomes are of particular concern in the context of drug discovery and development, especially given the trend towards large scale reliance upon HTS as a primary research tool. If, as suggested by these results there are large scale variations between cytotoxicity assays across a range of compounds with varying mechanisms of cytotoxicity, then it is possible that the true IC_{50} value of valuable and beneficial compounds for glioblastoma may have been missed through over/underestimation. This highlights the importance of reliability and accuracy in pre-animal models such as *in vitro* models of cytotoxicity.

Variations between methods and utilised materials are to be expected in the course of scientific enquiry. However, improving the accuracy and reliability of the screening methods applied to broad screening of potential anti-cancer compounds, constitutes an imperative target for all major pharmaceutical companies, as well as the smaller players in the drug discovery and development industry. Hence research efforts should be directed to correct and optimize the assays and methods of investigation that are being used in order to maximise the benefits of research. Reduction in costs in terms of correct decision making early in the process, will hopefully lead to reductions in the failure rate of inappropriate compounds entering expensive and involved clinical trials.

4. Conclusions

In conclusion, the comparison between the four different *in vitro* assays used for assessing the cytotoxicity on two different glioblastoma cell lines, suggests that even when combining several endpoints such as mitochondrial functions, lysosomal activity, and membrane integrity, a reliable and uniform toxicity profile was not achieved. Indeed, variations between IC_{50} values were seen in all experiments with differences observed between testing methods, cell lines and cytotoxic agents under investigation. A thorough knowledge of the advantages and limitations of each *in vitro* assay prior to use may help avoid underestimating or overestimating toxicological results and could lead to better predictive *in vivo* responses. Since many cytotoxic studies have been and are being conducted using patient-derived primary glioblastoma cells to perform patient-specific research, one needs to carefully interpret the cytotoxic results if obtained from the first generation MTT assays and to run other cytotoxic assays in parallel in order to produce more reliable and accurate cytotoxic results.

Competing interests

The authors declare no conflict of interest.

Author contributions

HMW designed the study. JS, AD and ED performed the experiments ED, JS, AD and HMW interpreted the data and wrote the manuscript. HMW interpreted the data and revised the manuscript. All authors have read and approved the final version.

Funding

E.D. was funded by an internal research grant from the Polytechnic University of the Marche provided by MIUR (Italian Ministry of University and Research).

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Legends to Figures

Figure 1. Comparison of different assays on viable cell number of U373MG and U87MG glioblastoma cells. Cells were exposed to increasing concentrations of either Carboplatin, Etoposide or Paraquat for 48 h and cell survival was assessed using different assays: Acid Phosphatase (▲), MTT (•), Alamar Blue (■), Trypan Blue (▼). The results are expressed as the percentage of viable cells compared to the control. Data are presented as mean ± SEM of at least 18 values from at least 3 independent experiments, except for (*) which represent the mean ± SEM of 12 values from two independent experiments.

Figure 2. Comparison of different drugs on viable cell number of glioblastoma cells. U373MG (•) and U87MG (■) cells were exposed to increasing concentrations of Paraquat, Etoposide, Carboplatin for 48 h and cell survival was assessed using different assays: Acid Phosphatase (A), MTT (B), Alamar Blue (C).

The results are expressed as the percentage of viable cells compared to the control. Data are presented as mean ± SEM of at least 18 values from at least 3 independent experiments.