In Vitro Cytotoxicity Assay for Targeted Radionuclide Therapy: Principles, General Methods and Limitations

By: Tung Nguyen

Contents

INTRODUCTION

Cytotoxicity is an important parameter for in vitro studies of many pharmaceutical lead compounds. By definition, cytotoxicity, refers to drug's lethality towards cells, is what enables therapeutic drugs to eliminate disease. Cytotoxic drugs destroy cell membranes, prevent protein synthesis, and activate apoptosis, among other mechanisms, to kill cells. (1). An evaluation of cytoxicity *in vitro* can predict therapeutic efficacy *in vivo*, enabling cost-effective screening for lead compounds, and insights into molecular mechanisms (1)

Radiotherapy is an important component of cancer treatment with almost 50% of all cancer pateients receiving radiotherapy during their course of illness. (2). Radiotherapy treatment targets tumors with high-energy cytotoxic radiation that eradicates cancer cells (2). Mechanistically, radiation imparts DNA damage to cancer cells, and generates free radicals to worsen DNA damage, which leads to inhibited cellular growth and division. Cancer cells particularly succumb to radiation damage over normal cells due to cancer's characteristically poor DNA repair pathway regulation (2).

There are two types of radiotherapy, external beam radiation therapy and systemic radiation therapy. External beam radiation therapy uses machines, such as a linear accelerator, and gamma knight, to shoot radiation through a patient's body carrying disease. Systemic or radionuclide therapy administers radioactive isotopes inside the body to seek out and destroy target diseased tissue(3). Radionuclides attached to vehicle proteins or small molecules with high specificity towards diseased tissue comprise "targeted radionuclide therapy" and combine the excellent cytotoxicity offered by radioactive decay with the vehicle's pharmacokinetics, customized and personalized for a patient's disease. Targeted radionuclide therapy covers radioimmunotherapy (RIT) and peptide receptor radionuclide therapy (PRRT) (3) which differ in the target receptor and delivery vehicle. *In vivo* studies where candidate radionuclide therapies treat human tumor "xenografts" in mouse models of larger mammals offer the greatest predictive data for efficacy in humans (4). However, these studies can be a costly and time-consuming process that relies on animal sacrifices, and so must be minimized whenever possible. *In vitro* cytotoxicity assays offer an inexpensive and more convenient alternative, which providing additional mechanistic data on a lead compound and the capacity to screen many compounds for a wide variety of commercially available cancer lines (4). The paper will discuss the principle, application, and limitation of five

in vitro assays available for testing radiopharmaceutical cytotoxicity. Protocols for common assays are also included for researchers to refer to in the future.

MTT assay

Principle and Application

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine) assay is a colorimetric assay for assessing metabolically viable cells **(5,6).** Mitochondria in viable cells produce succinate dehyogenase, which reduces the MTT reagent, a tetrazolium salt (yellow), to a purple formazan crystal(5). Spectrophotometers record absorbance at the characteristic formazan wavelength to quantify cell viability in treated cells relative to untreated controls(5,6). For radiotherapies, the MTT assay can measure viability after radiation treatment to generate a radiation survival curve and determine radiosensitivity of established cell lines.

Protocol outline:

The following protocol outline is based on the study done by Muller et al (7) where effects of [^{161}Tb]Tb-PSMA-617 and [^{177}Lu]Lu-PSMA-617 on cell viability were assessed using MTT assay.

Irradiation and plating

The assay is typically performed in 96-well microtiter plates. The initial steps to the assay can vary according to the goal of the experiment and the plating strategy. Plating before irradiation is used to screen sensitivity and efficiency of different treatments, while plating after treatment is used to assess DNA damage (8). For external beam radiation, irradiation is completed using radiation sources such as linear accelerators. Cell viability is assessed at varying radiation doses. For radionuclide therapy, cells are incubated in varying concentrations of the radiopharmaceutical. The control conditions are typically cells incubated in culture media without radioactivity. A washing step is often performed upon completion of the incubation when testing radionuclide therapy compounds. The plates are then placed in an incubator and allowed to grow until large clones are formed. The length of incubation is dependent on the cell line and may take up to 9 days on average (8).

MTT Assay

After the incubation period, MTT reagent is added to each well and allowed for 30 minutes incubation at 37oC. The remaining MTT solution is removed and Dimethyl sulfoxide (DMSO) is added to dissolve the formazan crystals. A microplate reader is used to obtain absorbance readings of DMSO extracts at 560 nm. Absorbance readings obtained from the reader are correlated to the number of cells, in the phase of exponential growth. To quantify cell viability, the absorbance reading of the test samples can be expressed as the percentage of the absorbance of the control samples as it represents 100% viability.

Limitation:

One major limitation to the MTT assay is the lack of sensitivity. Recent literature data has shown that MTT reduction is an inadequate test for cell viability and yields false results (8). The test compound may directly interact with MTT, or mitochondrial dehydrogenase activities, ultimately overestimate the absorbance readings (9,10). MTT assay may not be applicable in assessing radiopharmaceutical compounds with a vehicle molecule that interact with mitochondrial dehydrogenase (eg, PET tracer for mitochondria-functional imaging). In addition, previous studies have also found that MTT reagent may exhibit cytotoxic effects to cells and can cause dramatic morphological changes during formation of formazan crystals demonstrated in **Figure 1** (1,11).

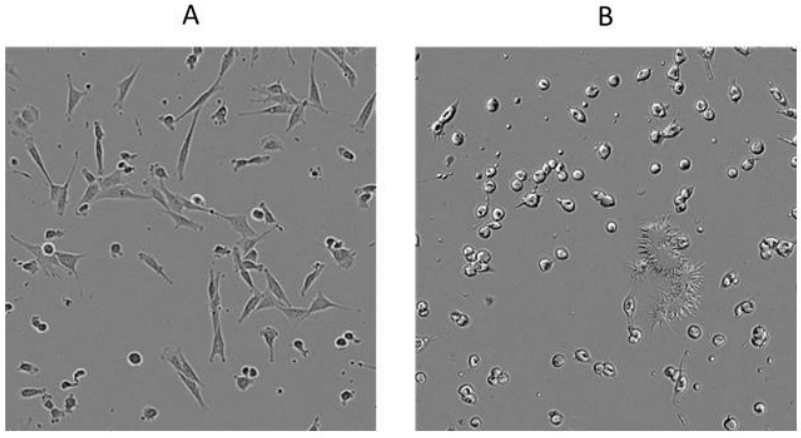

Figure 1: Change in NIH3T3 cell morphology after exposure to MTT (0.5 mg/mL). Panel A shows cells photographed immediately after addition of MTT solution. Panel B same cells photographed after 4 hours of exposure to MTT (1).

Comet assay

Principle and application:

Developed by Ostling and Johanson, comet assay, or single-cell gel electrophoresis (SCGE) is a standard method for evaluating chemical genotoxicity *in vitro*(12,13). Genotoxicity is the property of the radiopharmaceutical that cause damage to genetic information in the cancer cells. The assay quantifies early DNA damage at the single cellular level (12,13). The assay lyses cells with detergent and high salt concentrations following embedding cells in agarose to immobilize DNA for subsequent electrophoresis (12,13). Single-strand DNA breaks, double strand DNA breaks and breaks resulting from abasic sites are detected under alkaline conditions. Relaxed loops and DNA fragments travel further in an agarose gel than undamaged DNA in electrophoresis (12,13). Visualization of comets are based on fluorescence microscopy and quantification are done through software analysis. Compared to other traditional methods of DNA damage assessment, comet assay is direct, sensitive, inexpensive, and relatively simple (12).

For radiopharmaceutical compounds, comet assay reliably measures genotoxicity. Few examples include [^{225}Ac]Ac-DOTA(0)-Phe(1)-Tyr(3)-octreotide (DOTA-TOC) for alpha therapy, [^{177}Lu]Lu-DOTA-TOC for beta therapy, and [^{18}F]2-fluor-2-deoxy-D-glucose (FDG) and [^{68}Ga]Ga-PSMA-11 for positron-emitting radiotracers (14).

Protocol outline:

The following is the general workflow for comet assay used to evaluate DNA damage caused by radiopharmaceutical listed above as outlined by Schmeiser et al (14), although many variations exist in the literature. **Figure 2** is the scheme of the comet assay procedure.

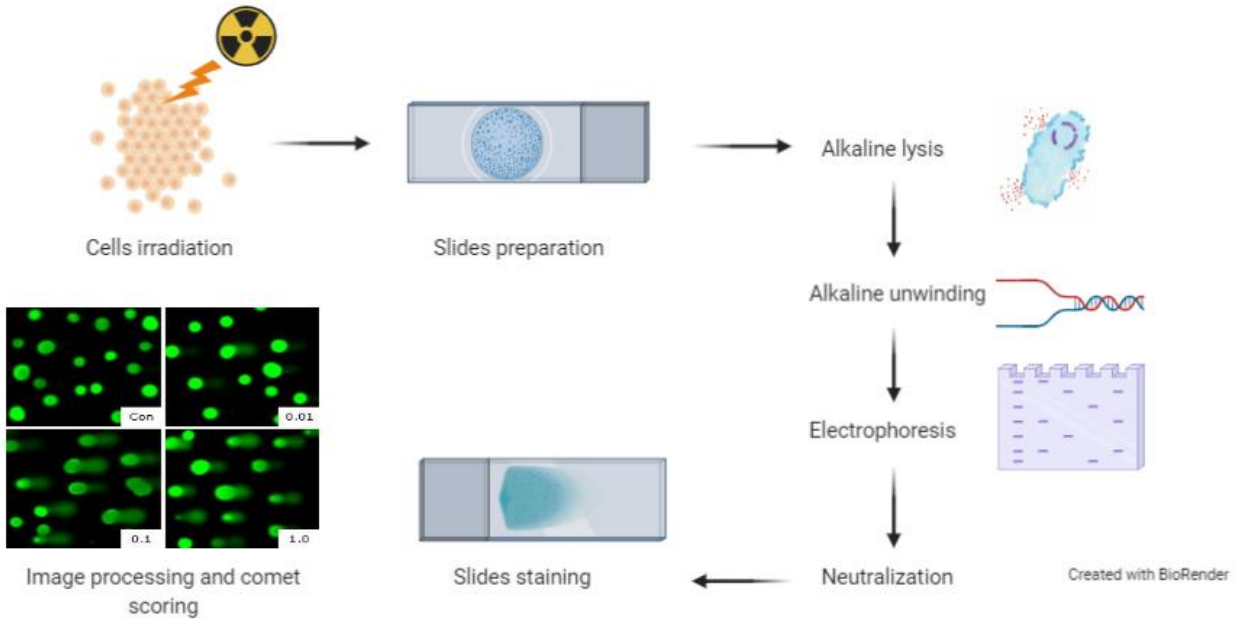

Figure 2: Schematic representation of critical steps in the alkaline comet assay made with BioRender.

Sample preparation

The candidate radiopharmaceutical is dissolved in Tris buffer with varied activity concentration and added to the human cell samples. Schmeiser et al. used an activity concentration ranging from 1 to 50 kBq/ml for alpha-particle-emitting radiotracer [^{225}Ac]Ac-PSMA-617, 10 to 400kBq/mL for beta-particle-emitting radiotracers [^{177}Lu]Lu-PSMA-617, and 5 to 40 MBq/ml for positron emitting radiotracers [^{18}F]FDG and [^{68}Ga]Ga-PSMA-11 (14). Samples are mixed and left at a -20^{o}C for 24 hours. Cell samples are then mixed with low melting (LM) point agarose and incubated for 1h at 4oC. LM agarose is in liquid state at 37^{o}C whereas typical agarose is solid at regular room temperature (12). Raising the temperature over 37^{o}C may result in cell death, and denaturation of DNA, thus LM agarose must be used for this assay (12,13). The agarose mixed sample is immobilized on a CometSlide. Cells are then treated with a lysis buffer to remove membranes and histones from the DNA.

Comet Assay

CometSlides are then transferred to a horizontal electrophoresis unit filled with an alkaline electrophoresis buffer. Alkaline conditions help unwind and denature the DNA. The unwinding of two DNA strands by alkaline denaturation around the break is essential to reveal the break. Alkaline condition makes comet tails more pronounced and extends the useful range of damage that can be detected including single and double strand break, alkali-labile sites and DNA single-strand breaks associated with incomplete excision repair sites (13,16). Neutral condition on the other hand, is mostly used to detect double-stranded DNA breaks. Although, the evidence from the literature showed that neutral conditions will result in decreased sensitivity (16). Although, the evidence from the literature showed that neutral conditions will result in decreased sensitivity (13,15,16).

DNA samples are allowed to unwind for 20 minutes before performing electrophoresis. Prior to fluorescence microscopy, the dried agarose is stained with a DNA-binding dye for 15 minutes at room temperature in the dark. Dye such as SybrGreen is preferred as they are easy to handle and readily available (17).

Quantitative Analysis:

Slides are visualized through fluorescence microscopy. There are different approaches to analyze comets. Collins et al. outline 4 approaches (18):

- Manually measuring the length of comet tail on a photomicrograph or using graticule
- Image analysis using a charge-coupled device camera linked to a computer.
- Categorize comets by visual inspection
- Automated systems that search for comets and automatically perform the analysis

Primary comet assay descriptors include %DNA in tail, tail length, tail moment, or visual score, number of comets analyzed per sample (18). Overall levels of DNA migration are essential information when reporting comet results. (16,18) Increased comet tail formation is an indication of DNA damage. Comet tail moment is used to quantify the extent of DNA damage in individual cells. Tail moment is the product of the fraction of total DNA in the tail and the tail length (tail

moment= tail length * % of DNA in the tail). Tail moments can be automatically calculated through a computer system as an average for the selected cells.

Limitation:

One of the limitations is the small number of samples that can be run in a single electrophoresis (19). This sample size can be improved through the use of an eight-welled glass slide (19,20). Another limitation pertains to assay variability across research groups, who use of different protocols (19). This problem can be partially addressed by the inclusion of reference standards in all experiments (21). References standards can be cells treated with toxic agent to yield a known amount of DNA damage, or untreated (negative controls) (21). Manual scoring of comet tails can be a tedious process especially when sample size is increased. The scoring may also be biased by differences in experience or interpretation of the researchers. Although an automatic scoring option is available, it has not been entirely successful to date. When assessing breaks produced by ionizing radiation, the dynamic range of absorbed dose of comet assay is quite limited, between a fraction of 1 Gy and 10 Gy. After 10 Gy, the distance between breaks is about 1 megabase, making it misleading to think in terms of DNA fragmentation (22). The dynamic range is conveniently physiological; DNA breaks within this range are repairable, thus cytotoxicity does not need to be considered as a possible cause of DNA damage (22).

Clonogenic assay

Principle and application

Human tumor clonogenic assay developed by Hamburger and Salmond is widely used in the field of radiation biology in the last 50 years (23,24). Radiation induces cellular reproductive failure as a cell killing mechanism (25). The assay is based on the ability of a single cell to grow into a colony (23). The initial study generated the first radiation-dose response curve for X-Ray irradiated HeLa mammalian cell (23). Cells are plated at low density and subjected to varying doses of irradiation. Reproductive viability between control untreated cells and cells that have undergone radiation treatment are assessed in this assay. In addition, the assay is also widely used in evaluating radiation sensitivity of different cell lines. Though, it is not semi-automatable and rapid, making it less advantageous than the MTT assay (23).

Protocol outline

The irradiation and plating protocol for the clonogenic assay is similar to the protocol for MTT assay. When performing the clonogenic assay, plating density of cells can have an impact on the outcome of the experiment (26). Generally, plating densities take into consideration the situation that the end point analysis is determined optically by counting clones. High plating density may cause cell coalescence, therefore making it impossible to count single colonies. Plating density may vary depending on the cell line(8,26).

Clonogenic assay:

Prior to colony counting, the cells are rinsed and stained. Using the count, the plating efficiency (PE) and survival fraction (SF) can be calculated using the following equations (8):

$$PE = \frac{\#\ of\ colonies\ formed}{\#\ of\ cells\ seeded} \times 100\%$$

$$SF = \frac{\#\ of\ colonies\ formed\ after\ irradiation}{\#\ of\ cells\ seeded \times PE}$$

The end result is cell survival curve with radiopharmaceutical concentration as the independent variable and survival fraction on the y-axis as outlined in **Figure 3.**

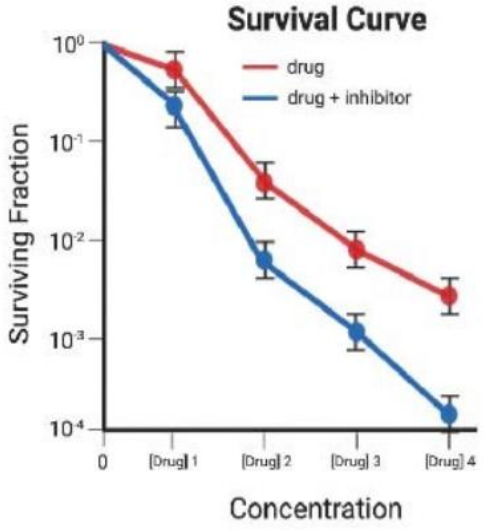

Figure 3: The ideal survival curve obtained at the end of clonogenic assay. Concentration of radiopharmaceutical is the independent variable plotted on the x axis and the surviving fraction is plotted on the y-axis as the dependent variable.

Limitation

Clonogenic assay remains the gold standard for testing cell response to potential radiotherapy drugs (27). However, there are major limitations pertaining with throughput and reproducibility. Depending on the cell line, this assay can take up to 9 days to complete (8). The counting colony process is done manually through human visualization, and prone to bias. Overestimating cell counts may present experimental variation, limiting the reproducibility of the assay. According to Wiesenthal and Lippman, clumpy colonies may create a bias in the assay during the counting process. As noted, clumping artifacts are often miscounted by researchers, and often resulted in false negatives (27). Lastly, clonogenic assay is only limited to adherent cells, and not all adherent cells are capable of forming colonies in vitro at low cell density where cell-to-cell contacts and self-produced growth factors are limited (29).

Annexin V Assay

Principle and Application:

In addition to radiation-induced reproductive failure, radiation can induce apoptosis as a cell killing mechanism. Cells undergoing apoptosis as an immediate consequence of radiation damage usually die in interphase within a few hours of irradiation, irrespective of and without intervening mitosis (30).

Under normal conditions, cells maintain an asymmetric distribution of phospholipids in the two leaflets of the cellular membranes with phosphatidylserine (PS) facing the cytosolic side (31). In contrast, for cells undergoing apoptosis, membrane asymmetry is lost, resulting in the exposure of PS at the outer leaflet of the plasma membrane and externalization of PS (31). Annexin V assay detects externalization of PS on cells, enabling quantification of apoptotic cells. The assay can detect differences in cell death by necrosis or apoptosis as well as be capable of live-cell imaging (31).

Vandenbulcke *et al*. used Annexin V assay for apoptosis cell scoring after exposure to radioimmunotherapy compound, ^{213}Bi-rituximab (32). Apoptosis was scored by flow cytometric analysis of cells stained with Annexin V, and the scores were expressed as % excess in irradiated samples over spontaneous apoptosis in non-irradiated samples.

Limitation:

One limitation is the fact the annexin V staining must be performed on live cells (33). As a result, the cell number may be restricted. Although cells undergoing apoptosis may externalize PS, describing annexin V positive cells as apoptotic can be misleading (34). Cells lacking Xpr8, which are common in some cancers do not lose plasma membrane asymmetry undergoing apoptosis, and defective autophagy may result in false negative results (34). Failure to ensure that cells retain an intact plasma membrane is a common error in assessment and reporting of annexin V data. Annexin V will bind to cells regardless of the conditions if plasma integrity is lost.

H2AX Assay

Principle and Application:

Phosphorylation of the histone H2AX is a well characterized marker of DNA double-strand breaks (DSBs). Phosphorylated H2AX rapidly accumulates over megabase domains at the sites of DSB, and can be microscopically visualized as discrete nuclear foci (35,36). Immunofluorescence using specific primary antibodies and secondary antibodies coupled with fluorescent dye can be used to detect these nuclear foci. H2AX induction is one of the earliest events detected in cells following exposure to radiation (36). There is a close correlation between H2AX foci and DSB numbers and between the rate of foci loss and DSB repair, providing a sensitive assay to monitor DSB repair in individual cells using physiological doses.

y-H2AX assay is widely used in assessing DSB in external irradiation studies, but not as common in systemic irradiation from radiopharmaceuticals. Even less common, H2AX assay is rarely used for in vitro studies, but more common for *in vivo* immunohistological staining studies. Nevertheless, there are studies that utilized the assay for monitoring effects of radionuclide therapy in vitro. For example, Bailey et al utilizes y-H2AX assay to monitor formation and persistence of DNA damage and cytotoxicity in MDA-MB-468 cancer cell line when exposed to the auger electron radiotherapy agent ^{111}In-DTPA-hEGF targeting human epidermal growth factor (37). The study concluded that y-H2AX assay maybe a useful biomarker to predict and monitor the outcome of treatment with radiopharmaceutical.

Limitation:

The assay represents an indirect monitor of DSB formation. One major limitation of this assay is pertaining to the subjective process involved during the scoring process. The process is often done by manually, thus scoring may differ depending on the skill of the researcher. In addition, the radiation absorbed dose range is limited to 3-4 Gy for foci scoring background (33).

CONCLUSION AND FUTURE DIRECTION:

There are several radiopharmaceuticals that eliminate diseased cells through selective induction of cytotoxicity. Cytotoxicity assays are essential for *in vitro* evaluation of these potential radiopharmaceuticals. *In vitro* assays are cost efficient and more convenient compared to in vivo studies. As an example, genetoxicity testing for unscheduled DNA synthesis in animal model cost an average of $32,000, whereas the in vitro testing only cost $11,000 (38). Results generated from *in vitro* studies can help predict the outcome of in vivo studies, ultimately saving time and resources. Although the options are limited, there are other aspects of radiopharmaceutical cytotoxicity that can potentially be assessed in the future.

Matador assay:

Matador assay is a novel cytotoxicity assay developed by Matta et al recently in 2018 (39). The assay is a luciferase based, a cytosolic sequestered variant of reporters. Luciferase of interest is expressed in target cells, such that it is retained within the healthy cells but is released from dead and dying cells (39). It is based on the concept that loss of cell membrane integrity results not only in the release of cytosolic sequestered reporters into the surrounding medium but also in the greater and faster penetration of the reporter substrate into the cell where it can react with many reporters still trapped inside the cell (39). The major advantage is the longer half-life, brightness, and stability of the marine luciferase. In addition, the assay is non-radioactive, thus will not interfere with the activity level from the radiopharmaceutical.

Compared to the MTT assay, the Matador assay provides real-time measurement of cell viability/cytotoxicity. It is also more sensitive and holds advantages for miniaturization and automation. The assay is best used in assessing cytotoxicity induced by several immunotherapy, including, NK cells, chimeric antigen receptor (CAR) T cells therapy, antibodies, and bispecific T

cell engager (39). As they are applicable to immunotherapy, there is a potential that this assay will be beneficial in evaluating cytotoxicity of radioimmunotherapy agents.

Reactive Oxygen Species Assay

Radiation generates reactive oxygen species (ROS), such as H_2O_2 and superoxide, which interact with cellular molecules and cause indirect damage to cells (40). The abnormal accumulation of exogenous ROS in response to ionizing radiation may cause lipid peroxidation, DNA mutations, and protein denaturation (41). Thus, quantifying ROS generated upon exposure to the radiopharmaceutical may provide cytotoxicity information of the drug. There are various fluorometric test kits available for quantification of ROS. For instance, H_2DCF-DA is a chemical reduced form of fluorescein. Once H_2DCF-DA has diffused into cells, it is deacetylated by cellular esterase to a non-fluorescent compound and rapidly oxidized by ROS into highly fluorescent DCF (42). Fluorescence intensity can then be monitored using flow cytometry, fluorometer, and fluorescence microscope (42). However, it is important to note that mitochondria also produce endogenous ROS under normal condition (43). Thus, a control sample of healthy cells may be required to quantify the baseline ROS in the cells.

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