Review

Cell Proliferation and Cytotoxicity Assays, The Fundamentals for Drug Discovery

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Abstract: Cell proliferation and cytotoxicity assays are fundamental to drug discovery. This review summarizes prevalent methodologies for assessing cell proliferation and cytotoxicity, including direct cell count, metabolic activity, luminescent labeling, and tri-color viability imaging. The critical determinants that can significantly impact these assay outcomes, such as cellular doubling time, transitional states like quiescence and autophagy, cell cycle stages, metabolic enzyme functions, and genetic variability, are also explored. It is necessary to integrate the commonly used assays with additional analytical techniques to achieve precision in drug discovery. A multi-tiered approach that combines cellular assays with molecular analyses can improve screening processes, reduce false negatives, and increase confidence in the therapeutic potential of lead compounds.

1. Introduction

The determination of cell viability and proliferation is of pivotal importance in the field of drug discovery and development. A variety of reliable, accessible, and scalable assays have been developed to measure cellular responses, including proliferation, viability, and death. Commonly employed assays include monitoring cell number, division, metabolic activity, and DNA/protein synthesis. While numerous assays quantify cell death or inhibition based on viable cell proportions, some measure cytostatic effects, underscoring the significance of selecting the optimal assay method and timing to accurately identify lead compounds.

This paper examines the fundamental methodologies for assessing cell proliferation and cytotoxicity, emphasizing their critical role in drug discovery. We provide a summary of the most prevalent techniques, including direct cell count, metabolic activity assays, luminescent labeling, and tri-color viability imaging. The outcomes of these assays are influenced by a number of factors, including the cellular doubling time, the state of quiescence, autophagy, the cell cycle stages, the metabolic enzyme functions, and genetic variability. The integration of these assays with additional analytical techniques can enhance precision, improve screening processes, reduce false negatives, and bolster confidence in the therapeutic potential of lead compounds.

2. Commonly Used Technologies

Here we summarize some of the most commonly used technologies to determine cell proliferation and cytotoxicity (Table 1). Each of these methods has particular use cases, and the choice of method can depend on various factors including the specific research question, the available equipment, the cell type being studied, and the desired throughput and accuracy of the assay.

Table 1. The advantages and disadvantages of commonly used methods to determine cell proliferation and cytotoxicity.

Assay	Advantages	Disadvantages
Trypan blue exclusion staining	- Direct visualization of viable cells - Relatively quick and easy	- Subjective; requires manual counting - Not suitable for automated high-throughput screening
Methylene blue staining	- Simple, inexpensive, and accessible for most labs - Enables direct visualization of cell morphology and viability	- Subjective interpretation requiring manual counting - Limited sensitivity and not suitable for high-throughput screening
Incorporation of Radioactive Nucleotides	- Directly measures DNA synthesis - Highly sensitive	- Use of radioactivity requires special precautions - Disposal of radioactive waste is necessary
BrdU incorporation	- Specific detection of proliferating cells - Amenable to multiplexing with other markers	- Requires DNA denaturation - Potential health hazard of BrdU
EdU incorporation	- No DNA denaturation needed - Often faster and easier than BrdU	- Can be more expensive than BrdU - Potential health hazard
MTT assay	- Well-established - Colorimetric and easy to perform	- Can be influenced by metabolic activity - Requires cell lysis
XTT assay	- Soluble formazan product - Can be performed without cell lysis	- Less sensitive than MTT in some cell types - Can still be influenced by metabolic activity
WST-1 assay	- Non-radioactive - Water-soluble formazan product	- Can be affected by cellular metabolic differences - Not a direct measure of cell proliferation
LDH assay	- Measures cell membrane integrity - Can be used for high-throughput screening	- Indirect measure of cell death, not proliferation - Interference from endogenous LDH activity
Quantification of ATP	- Directly correlates with viable cell number - Quick and highly sensitive	- Requires cell lysis - Can be influenced by extracellular ATP
CFSE labeling	- Tracks cell division over time - Fluorescently labels cells	- Fluorescence can diminish with each cell division - Requires flow cytometry or fluorescence microscopy
Calcein-AM labeling	- Non-toxic and live-cell permeable - Fluorescent upon esterase activity in live cells	- Only labels live cells - Does not provide information on cell proliferation rate
Real-time cell analysis	- Provides real-time monitoring - Non-invasive and label-free	- Requires specialized equipment - Can be cost-prohibitive for some labs
Clonogenic assay	- Measures the ability of a cell to grow into a colony - Can reflect the reproductive health of cells	- Time-consuming - Requires a significant amount of space in incubators
Triple staining (Calcein-AM, PI, Hoechst) in 3D cell cultures	- Distinct staining of live, dead, and all cells - Suitable for complex tissue models	- Analysis can be complicated - Requires specialized imaging equipment

2.1. Evaluating Cell Growth

The most straightforward technique for evaluating cell growth is the use of trypan blue exclusion staining, which is the preferred approach for determining viable cell counts using dye exclusion [1]. Viable cells reject

the blue dye, thus permitting a precise measurement of cell vitality [2]. Typan blue staining has been used in numerous research studies over the past several decades, including investigations into the viability of polymorphonuclear cells (PMNs) treated with a novel cis-dichloroplatinum (II) complex. [(Qu)$_2$PtCl$_2$] [3].

Cell growth can be gauged by monitoring the incorporation of radioactive nucleotides like [3H] - thymidine into the DNA of cells, with subsequent detection by autoradiography [4]. An alternative strategy involves the employment of 5-bromo-2′-deoxyuridine (BrdU) as a substitute for thymidine [5]. To identify cells that have integrated BrdU into their genetic material, one employs a monoclonal antibody that targets BrdU, in conjunction with a secondary antibody linked to either an enzyme or a fluorescent dye [6]. The BrdU incorporation assay allows for the determination of the proliferative state of cancer cells with greater sensitivity than is possible with other methods. This assay is a typical approach for the screening of anti-tumor agents, such as magainin II [7,8].

An enhanced substitute for conventional BrdU assays is the use of 5-ethynyl 2′-deoxyuridine (EdU), which leverages click chemistry for the fluorescent labeling of DNA synthesis [9]. It is a commonly used methodology for quantifying cell availability, as exemplified by the investigation of the proliferation of estrogen-responsive MCF-7 breast cancer cells [10]. The introduction of EdU into a population of healthy cells leads to its incorporation into the DNA during active replication phases. The Cu-catalyzed click chemistry facilitates swift coupling of fluorescent tags to EdU, enabling a precise and quantifiable evaluation of cell proliferation [11]. These assays are designed in various formats, making them suitable for a range of applications including high-throughput screening [12], flow cytometry [13], microscopic examination [14], and studies conducted in living organisms [15]. Moreover, the availability of four distinct fluorescent tags with peak excitation wavelengths at 488, 555, 594, and 647 nanometers enhances the ability to perform multiplex experiments by pairing them with various fluorescent labels.

2.2. Evaluating Metabolic Cell Growth

Analytical methods that investigate metabolic activity are informative for assessing cell growth, survival, and cytotoxic responses. Metabolically functioning cells are capable of converting tetrazolium compounds, such as MTT, XTT, and WST-1, into vibrant formazan derivatives. This reduction is a hallmark of metabolically active cells. A surge in metabolic processes is indicative of cell proliferation, whereas contact with toxic agents typically leads to a reduction in such activity.

MTT, chemically known as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide or Thiazolyl Blue, is a tetrazolium salt that is soluble in water [16]. Previous studies have demonstrated the efficacy of the MTT assay in assessing cytotoxicity in the context of multidrug-resistant human leukemic cells [17]. It forms a yellow-tinted solution when dissolved in media or salt solutions devoid of phenol red. The MTT's tetrazolium ring is cleaved by cellular dehydrogenase enzymes, yielding an insoluble formazan of a purple hue (Figure 1). This precipitated formazan is solubilized using solvents like isopropanol, and its concentration is then determined spectrophotometrically through optical density measurements [18].
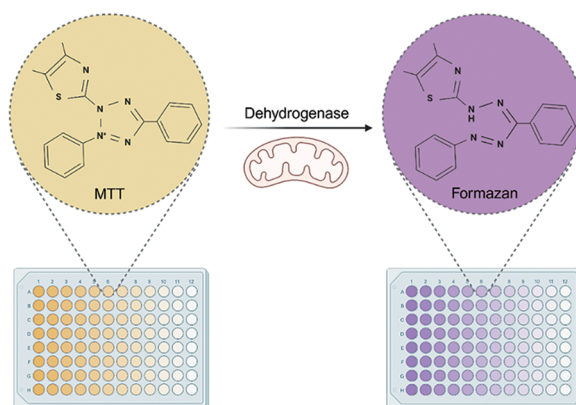

Figure 1. Principle of MTT assay. The yellow MTT is oxidized by dehydrogenase enzymes in the mitochondria of living cells into purple formazan.

XTT assays offer a variant to the MTT method. The XTT cleavage product is inherently water-soluble, which obviates the necessity for a subsequent dissolution step [19]. XTT undergoes transformation into formazan within living cells through an intricate cellular reaction, this transformation is reflective of the NAD (P)H production via the glycolytic pathway [20]. For example, the XTT assay is used to assess the anti-proliferative impact of selenium compounds on colon cancer cells [21]. The formazan dye amount is in direct relation to the count of metabolically functioning cells in the sample.

The WST-1 assay employs the stable tetrazolium salt known as WST-1, which is bio-reduced, predominantly at the cell surface, to a soluble formazan product. This reduction process is largely dependent on the glycolytic pathway's NAD(P)H production within viable cells [22]. The resultant formazan dye quantity is in direct proportion to the number of metabolically active cells within the culture [16]. For example, the WST-1 assay was employed to assess the antitumor efficacy of magainin II on a range of cancer cell lines [8].

The Lactate Dehydrogenase (LDH) release assay stands as a fundamental approach for determining cellular membrane integrity, a hallmark of cytotoxicity. This method is a reliable tool for the analysis of cytotoxic lymphocyte-mediated lytic activity in acute myeloid leukemia blasts [23]. LDH, a stable cytosolic enzyme present in all cell types, is released into the culture supernatant upon membrane compromise or cell lysis [24]. The assay exploits the enzyme's ability to catalyze the conversion of lactate to pyruvate, concomitant with the reduction of a tetrazolium salt into a red formazan product [25]. Spectrophotometric measurement of formazan reflects the enzymatic activity of LDH and, by extension, the proportion of damaged cells [26]. This assay offers a sensitive and rapid assessment of drug-induced cytotoxicity, facilitating its widespread application in cytotoxicity screenings and evaluation of anticancer agents.

2.3. Assessing Cell Viability via Luminescence

Quantification of ATP, which signifies active metabolism in cells, is an efficient method for determining viable cell numbers. The Luminescent ATP Detection Assay leverages luciferase from fireflies to oxidize D-luciferin in a reaction that emits light, the intensity of which reflects the ATP concentration within the cells [27] (Figure 2). The luminescence-based cell viability assay can be used to rapidly detect cisplatin resistance in ovarian carcinoma [28]. The procedure for this assay is streamlined, involving a direct application of the detection solution to the cultured cells in serum-enriched media, removing the need for labor-intensive steps such as cell rinsing, media discarding, or repetitive transfers. The assay's high sensitivity enables the detection of individual cells or as little as 1nM of ATP, in an optimized buffer condition [29]. By correlating ATP levels with cell viability, this method is extremely versatile, applicable in a range of contexts from cell survival analysis to proliferation and cytotoxicity studies [30–32].
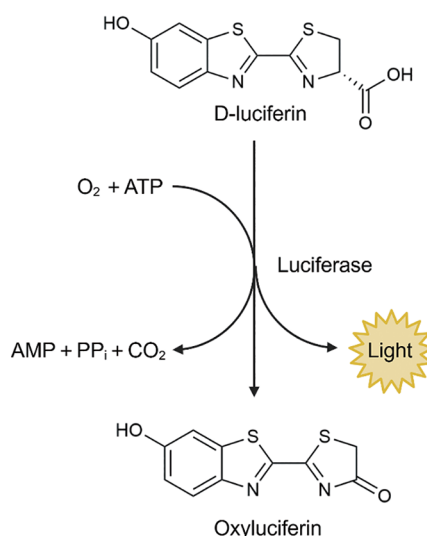

Figure 2. Principle of luminescent ATP detection assay. The luminescent substrate D-luciferin is oxidized to oxyluciferin by luciferase from fireflies in the presence of oxygen and ATP produced by cell lysis, and this reaction is accompanied by the production of light.

To monitor cellular division, researchers may utilize 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE). Inside the cell, CFSE is enzymatically processed to yield a fluorescent marker. The succinimidyl group couples with the cell's internal proteins' primary amines. With each cell division, the fluorescent signal is divided between the daughter cells, thus permitting the tracking of cell division rates through flow cytometry [33]. CFSE is particularly useful for investigating the proliferation of lymphocytes, including T cells [34].

The technique of live/dead simultaneous staining employs fluorescent markers for the concurrent identification of live and dead cells. Calcein-AM, a dye that easily integrates into cellular membranes, is non-fluorescent by itself; however, once inside living cells, esterases convert it into Calcein, which shines bright green upon excitation at 490 nm and emits at 515 nm [35,36].

2.4. Real-Time Cell Analysis

Real-time cell analysis (RTCA) technology is an innovative approach to monitoring cellular events. It was first used as a novel real-time cell electronic sensing system in 2008 [37]. This non-invasive, label-free methodology utilizes microelectronic sensor arrays integrated into the bottom of cell culture plates. Cells in proximity to the sensors alter the electrical impedance across the sensor surface, a change that is recorded in real-time [38]. This dynamic monitoring offers a comprehensive view of cell health, including proliferation, spreading, cytotoxicity, and cell adhesion, over the entire course of an experiment. RTCA is particularly valuable for kinetic analysis of cellular responses to pharmacological agents, delivering a temporal resolution unattainable by endpoint assays. One particular example is the use of RTCA to measure the effects of antibiotics on biofilm inhibition and induction [39].

2.5. Clonogenic Assay (Colony Formation Assay)

The Clonogenic assay, or colony formation assay is a definitive test for determining the long-term effects of cytotoxic agents on the survival and proliferation of cancer cells. This assay is predicated on the ability of a single cell to grow into a colony. Following treatment with a test agent, cells are seeded at low densities and cultured for an extended period, allowing for the development of colonies. The colonies are then fixed, stained, and quantified [40]. The number of colonies reflects the fraction of cells that retain the capacity to proliferate, giving insight into the effectiveness of the drug in eradicating tumor-initiating cells [41]. The clonogenic assay was applied to detect tumor cells in peripheral blood stem cells from breast cancer patients [42]. The Clonogenic assay is highly regarded for its prognostic relevance in cancer therapeutics, providing essential data on the potential of a drug to prevent relapse and promote long-term remission.

2.6. Triple Staining in 3D Cell Cultures

For both 3D and traditional 2D cultures, a tri-chromatic cell viability imaging protocol is effective. This method enables the concurrent fluorescent labeling of live cells using Calcein-AM, dead cells with Propidium Iodide (PI), and all cells using Hoechst 33342. Through the action of intracellular esterases, Calcein-AM, which has an affinity for calcium, yields a green fluorescence, pinpointing the metabolically active and viable cells [35]. PI, on the other hand, can only penetrate cells with compromised membranes, binding to the DNA within and producing a vivid red fluorescence, while it is actively excluded by the membranes of living cells [43]. Lastly, Hoechst 33342, which is a DNA stain that is minimally cytotoxic, emits a blue fluorescence and is utilized to count the total cell population [44]. The use of 3D cell culture to simulate the tumor microenvironment in the case of nuclear staining allows for the rapid screening of anti-cancer compounds [45].

3. Factors to be Considered

To obtain inhibition of a drug candidate on cell growth, the strategy of reducing the ratio of live cells from 100% is commonly used. However, due to the nature of some commonly used methods and the complexity of how cells respond to extracellular stimuli, simply determining the ratio of live to dead cells may not accurately reflect the effect on cell proliferation and death. For example, the results of cell death

following treatment with a drug candidate may be affected by factors such as doubling time, intermediary states such as quiescence and autophagy, cell cycle phase, metabolic enzyme activity, and genetic background.

3.1. Doubling Time and Cell Cycle

The doubling time is the time it takes for a cell or group of cells to double in number. Most cancer cells grow quite rapidly, and the doubling time in culture is generally 2 to 3 days. If cells are seeded to 50% confluence in a culture well plate, untreated cells will double in 2 days, while drug-treated cells' doubling time will be over 2 days, which means the cells keep growing after treating but slower untreated cell, shows that the drug is simply inhibiting growth rather than inducing cell death (Figure 3). In addition, it is important to consider whether the drug candidates induce quiescence or the G_0 phase of the cell cycle. Quiescence is a reversible state in which cells retain the ability to re-enter the cell cycle when the drug is withdrawn or when appropriate stimuli are applied. Cells in quiescence are typically in the G_0 phase of the cell cycle, indicating that they are not actively proliferating (Figure 4). The purpose of chemotherapeutic agents is to bring non-dividing cells out of the G_0 phase, making them more susceptible to subsequent cycles of chemotherapy. This allows the chemotherapeutic agents to be incorporated into the DNA double strand, resulting in DNA damage and subsequent cell death [46]. However, some drug candidates appear to only inhibit cell growth rather than induce cell death. Cancer cells in the dormant state can develop mutations, drug resistance, and metastasis, making therapy even more challenging [47].
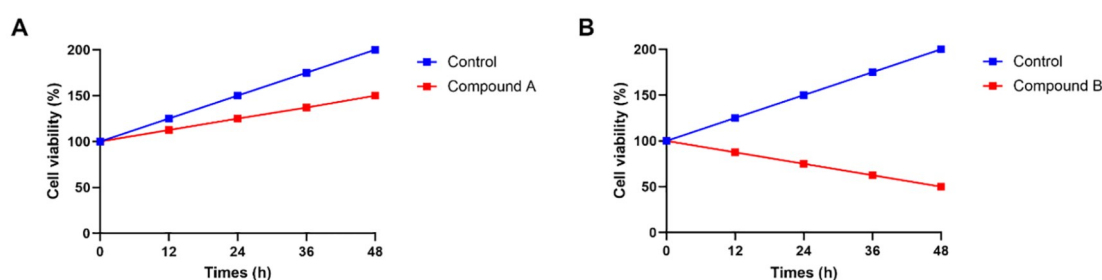

Figure 3. Diagram of cancer cell proliferation when treated with different compounds. (**A**) The effect of cell proliferation inhibition on cell doubling time was investigated by treating cancer cells in the presence or absence of cell proliferation inhibitor compound A. (**B**) The effect of cytotoxicity on cell doubling time was investigated by treating cancer cells in the presence or absence of compound B. The X axis is the time of co-culture of drugs and cancer cells; at 48 h, the compound is removed.
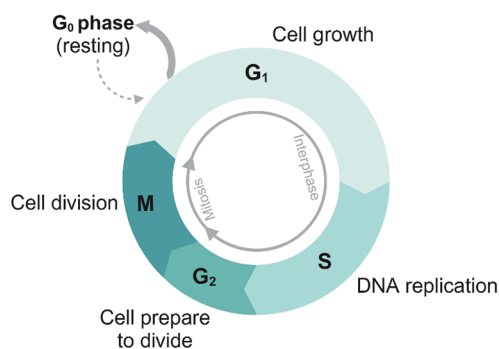

Figure 4. Diagram of typical cell cycle. The typical cell cycle consists of four phases, including G_1 phase, S phase, G_2 phase, and M phase. G_1, S and G_2 phases are collectively referred to as interphase. Cell grows in G_1 phase; DNA replication occurs in S phase; cell prepares for mitosis in G_2 phase. Cell division occurs during the M phase. Cells in G_0 phase leave the cell cycle and stop dividing, remaining in a quiescent state.

Autophagy is another state in which cells either grow or die, in which cells degrade damaged organelles and dysfunctional components to survive under stimuli. Critical factors such as 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFK-2/FBPase 3, PFKFB3) and scribble planar cell polarity protein (SCRIB)

that mediate autophagy could promote tumor cell proliferation when the stimuli are removed [48]. Drugs that induce autophagy would also appear to inhibit growth when quantified by the proliferation assays mentioned above.

Duration of treatment is also an important factor in determining the sensitivity of cells to candidate compounds. Programmed cell death pathways must pass the signal in a highly ordered fashion, which means that cells take time to die. Therefore, treatment durations longer than a doubling time may better reflect the effect on cell proliferation or death than shorter durations.

Clonogenic assays could be incorporated for low-cost validation of screening-based results, which essentially test the effect on cell growth and death at the single cell level in a relatively long step [40].

3.2. Enzyme Activity and Cell Viability

The growth of cells targeted by candidate compounds that affect mitochondrial enzymes may be affected by the type of assay chosen. For example, the half maximal inhibitory concentration (IC_{50}) for cisplatin can differ by approximately 10-fold when measured by the MTT assay, which measures mitochondrial dehydrogenase activity, versus trypan blue staining of dead cells [49].

Methods that measure enzyme activity to reflect cell viability may also be affected by the fact that metabolism is usually increased in the fraction of cells that are dying. For example, NAD(P)H-dependent dehydrogenase enzymes measured by the MTS assay and esterase measured by calcein-AM dyes may be lower than the actual level that could inhibit proliferation.

3.3. Mutations and Genetic Background

Cancer cells are highly heterogeneous, with mutation rates as high as 96% for driver mutations and 80% for loss-of-function mutations in tumor suppressor genes in solid tumors [50,51]. The epidermal growth factor receptor alone carries approximately 22,500 variants in human lung cancer [52]. Mutations would have a major impact on the activity of drug candidates, especially for the majority of anticancer drugs that target DNA synthesis or repair. For example, mutation and half-life of p53 are closely related to the susceptibility to cisplatin resistance [53]. Mutations in the multidrug resistance (MDR) gene are associated with resistance to several first-line chemotherapeutic agents, such as the topoisomerase I inhibitor topotecan [54]. The addition of cancer cell lines with different genetic backgrounds could help identify drug sensitivity profiles and accelerate treatment development [55], especially cancer cells expressing genes directly related to drug resistance [56]. The NCI-60 human tumor cell line screen contains kidney, prostate, ovary, colon, breast, brain, lung cancer, and melanoma and leukemia, which greatly mimic the complexity of tumor and widely used in the development of anticancer drugs [57].

Innovative methods such as machine learning can help reduce noise and error rate, increase robustness and accuracy in cancer drug development [58]. While omics studies, especially genomic study, with increased sensitivity, robustness and lower cost, also provide signatures of gene mutation and drug sensitivity across large scale of cancer cell lines for existing drug types [59]. The benefits of using these innovative technologies are particularly evident when determining the efficacy of drug combinations. Using these technologies, the combination of irinotecan with the checkpoint kinase 1 (CHEK1) selective inhibitor rabusertib was found to be particularly promising to address the unmet clinical need for the treatment of colorectal cancer patients with MSS or KRAS-TP53 double mutation [55].

4. Conclusion and Future Directions

In pharmacological research, proliferation and cytotoxicity assays are essential for early-stage drug evaluation as they provide insight into compound effects on target cells. However, these assays offer limited mechanistic understanding and cannot distinguish between types of cell death or cell cycle arrest mechanisms. Therefore, it is necessary to integrate these assays with additional analytical techniques to achieve precision in drug discovery. Flow cytometry can distinguish between apoptosis and necrosis, which enhances the interpretation of cytotoxic effects. High-content screening provides morphological context, allowing for a better understanding of cell health beyond mere survival. Additionally, incorporating 'omics' analyses can further broaden the understanding of compound impact by revealing perturbations in cellular

pathways. A multi-tiered approach that combines cellular assays with molecular analyses can refine screening processes, reduce false negatives, and advance lead compounds with increased confidence in their therapeutic potential.

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