In Vitro Cell Death Determination for Drug Discovery: A Landscape Review of Real Issues

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ABSTRACT: Cell death plays a crucial role for a myriad of physiological processes, and several human diseases such as cancer are characterized by its deregulation. There are many methods available for both quantifying and qualifying the accurate process of cell death which occurs. Choosing the right assay tool is essential to generate meaningful data, provide sufficient information for clinical applications, and understand cell death processes. In vitro cell death assays are important steps in the search for new therapies against cancer as the ultimate goal remains the elaboration of drugs that interfere with specific cell death mechanisms. However, choosing a cell viability or cytotoxicity assay among the many available options is a daunting task. Indeed, cell death can be approached by several viewpoints and require a more holistic approach. This review provides an overview of cell death assays usually used in vitro for assessing cell death so as to elaborate new potential chemotherapeutics and discusses considerations for using each assay.

KEYWORDS: in vitro, cell death assays, screening drugs

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Introduction

Resistance to death is a major characteristic of cancer cell, hence the emergence of considerable interest in developing therapeutic strategies that could interfere with cellular death decisions. Cell death plays a major role in the homeostasis of tissues and allows the elimination of transformed cells. The deregulation of cell death in cancer implies that effective drugs have to kill cells outright and not only inhibit their growth to provide complete clinical responses. Besides, resistance to cell death during tumorigenesis may also explain tumor relapse following chemotherapeutic treatment. Thus, cell death–related assays are of primary importance in the field of oncology for drug discoveries.[1]

Obviously, cell death is not unequivocal and can occur through many options.[2] Beyond semantic reasons, the ability to distinguish all forms of cell death is also clinically relevant to develop specific therapeutic drugs suitable for every cell death processes. Indeed, as the response to anticancer therapy is modulated by certain forms of cell death, specific modalities of cell death have to be identified by drug discovery assays.[3] Consequently, different in vitro methods for identifying dead cells in cell cultures have been described, including detection of end-stage cell death, irrespective of its type, as well as processes associated with a particular subroutine cell death.[4] If apoptosis, also referred to as programmed cell death, is considered as the most appropriate indicator of cell death, other categories of cell death pathways, including necrosis, autophagic cell death, and anoikis, should be of interest. As a matter of fact, if a recent classification has defined 8 different forms of cell death which may involve various molecular signaling pathways, some researchers describe that at least 11 pathways of cell death exist and that apoptotic cells frequently undergo secondary necrosis under in vitro conditions.[5,6] It appears that apoptosis and necrosis may represent the 2 extreme types of cell death on a wide range of cell death modes. Therefore, it might be difficult to distinguish all of these specific death conditions monolithically, as differences may not be as distinct as it appears. Furthermore, appropriate cell death assays must be rigorously selected for the experimental setting under investigation; choosing the right assay tool and the appropriate end point to measure cell death is a condition sine qua non to generate meaningful data and remains a challenging task.[7] Indeed, cell death can be approached from a variety of viewpoints, including measurement of dead cells (cytotoxicity assay), quantification of live cells (viability assay), and, ultimately, determination of the accurate mechanism involved. Therefore, the plethora of strategies for monitoring cell death requires a selective choice of the best suited cell death assay according to the study aim, but a single method may not be sufficient to determine a type of cell death.[8]

This review provides an overview of cell death assays currently used in vitro for estimation of cell death so as to assess

new potential chemotherapeutics and discusses the ins and outs of these scientific approaches.

Major Pathways of Cell Death and Crosstalk: A Brief Overview

Cell death typically occurs by necrosis, apoptosis, or autophagy. Concerning apoptosis, its characterization implies several criteria, such as changes in the nuclear morphology, especially chromatin fragmentation and condensation, and also the occurrence of apoptotic bodies that contain nuclear material, as well as reduction in cell volume and plasma membrane shrinkage.[9] Intrinsic or extrinsic stimuli can lead to apoptosis, and the 2 different pathways involve specific biochemical manifestations such as the activation of caspases. Caspase 9 and caspase 3 are classically activated in the intracellular pathway with a process of mitochondrial outer membrane permeabilization (MOMP), whereas the extracellular pathway requires the activation of caspases 8 and 3. However, in some cell types, including hepatocytes and pancreatic β cells, the activation of caspase 9 has been described through an MOMP process, leading Galluzzi et al[2] to underline that extrinsic apoptosis is above all activated by extracellular stress signals and is characterized by death receptors that induce a caspase signaling cascade involving caspase 8, caspase 3, or caspase 9. If caspases display a central role in apoptotic mechanism, other families of proteins are also involved, such as SMACs (small mitochondrial-derived activator of caspases), IAPs (inhibitor of apoptosis proteins), and the Bcl-2 family. Death receptor family also plays a central role in the apoptosis machinery through cell surface receptor activation with specific death ligands.[10] The entire process is highly regulated and leads ultimately to massive cell destruction without an inflammatory response, in contrast to immunogenic cell death, a form of cell death also referred as immunogenic apoptosis which can induce an effective antitumor response through the activation of T cells and dendritic cells. Potential immunogenic cell death inducers have been characterized and include some chemotherapies as well as radiotherapy. This cell death variant is defined by the exposure of calreticulin (CALR) on the membrane and the release of other proteins, including members of the heat shock protein family.[11] In parallel, if necrotic cells exhibit common traits in comparison with apoptotic cells, notably some of the cellular changes, including chromatin condensation and apoptotic bodies, are not observed, and necrotic cells stimulate the inflammatory response through the release of intracellular materials into the extracellular milieu. For a long time, necrosis has been regarded only as an uncontrolled cell death process, but the discovery of potential signaling components, such as RIP (receptor-interacting protein) kinases, has led to the concept of programmed necrosis also called necroptosis, a regulated form of necrosis that requires the catalytic activity of receptor-interacting protein kinase 1 (RIPK1) and RIPK3.[12,13]

Regarding autophagy, it is a major, controlled, catabolic mechanism tightly regulated by some autophagy-related genes. Autophagic cell death involves the formation of double membrane which contains vacuoles, and this sequestrated cytoplasm is subsequently digested through the fusion with lysosome vacuoles. However, the specific role of autophagy in the execution of cell death remains controversial and is not fully elucidated yet. Autophagy may constitute an epiphenomenon of cell death that could be analyzed as an ultimate but unsuccessful attempt by cells to cope with stress and re-establish homeostasis.[14] Moreover, concerning recent neologisms introduced to define other putative cell death modalities, only a few of them truly reflect cell death pathways that are biochemically and morphologically distinct from apoptosis and necrosis. Indeed, terms such as mitotic catastrophe, anoikis, and pyroptosis most often refer to cell death mechanisms that are executed by the molecular machinery of apoptosis and necrosis.[15,16] In contrast, ferroptosis, an iron-dependent form of cell death, is morphologically, biochemically, and genetically different from apoptosis, necrosis, and autophagy. This particular form of nonapoptotic cell death can occur in specific pathologic conditions after its activation by an oncogenic RAS-selective lethal small molecule (erastin) and is iron dependent.[17] Finally, even if apoptosis, autophagy, and necrosis display distinct morphologic characteristics and physiologic processes, they do share significant commonalities and more particularly coordinate with each other. As a matter of fact, under some conditions, apoptosis and autophagy can display synergetic effects, whereas autophagy can be activated when apoptosis is suppressed.[18] Consequently, it seems that the existence of several pathways for cell death and the complex relationship between these modalities require the use of different protocols to measure and identify the type of cell death, for quantitative and qualitative analysis. Moving away from a monolithic point of view is necessary as the interrelation between cell death processes appears to be more complex than initially anticipated.

Cell Viability, Cytotoxicity, and Cell Proliferation: Fundamental Intricacies

Common and necessary terminology

The assessment of new potential chemotherapeutics and drug screening involve cell viability and cytotoxicity assays to identify and define safety thresholds for subsequent clinical use. Such assays rely on several cell functions, including enzyme activity, cell membrane activity, cell adherence, adenosine triphosphate (ATP) production, coenzyme production, and nucleotide uptake activity. If a compound interferes with cellular attachment, causes dramatic morphologic changes, affects cell growth rate, or leads to a reduction in overall viability, it is considered to be cytotoxic.[19] However, cytotoxicity reflects the cell-killing property of a chemical compound and is not associated with a specific cell death pathway. Cell viability can be defined as the number of healthy cells in a sample, without any distinction between cells which are actively dividing or quiescent ones, whereas cell proliferation is the measurement of the

number of cells that are dividing in a culture. Viability assays aim to measure cellular maintenance and survival parameters. Thereby, a myriad of assays have been designed and used so as to measure viability or cytotoxicity in vitro, including, in particular, classical dye inclusion or exclusion and colony formation assays.[20]

Vital dyes and colony formation assays

Vital dyes, which are fluorescent or colored molecules that distinguish between living and dead cells, provide conclusive tests to assess cell death in vitro. The vital dyes most frequently used include exclusion dyes that cannot cross intact plasma membranes, such as 4′,6-diamidino-2-phenylindole or propidium iodide, for instance, and thus only label dead cells. The dye exclusion assay with trypan blue is widely used in routine laboratory work. Blue-stained cells are dead cells and the percentage of viable cells is calculated as ratio of viable and total number of enumerated cells. Hemocytometer and classic light microscopes are commonly used for cell counting. Cytofluorometry is routinely used in co-staining protocols, and fluorescence microscopy facilitates the identification of dead cells by visual inspection.[21] By contrast, other dyes such as calcein acetoxymethyl ester (calcein-AM) can be used to selectively label living cells, thanks to their lipophilic properties that allow them to penetrate easily into cells; subsequently, they are hydrolyzed by intracellular esterases with a generation of fluorescent and plasma-membrane–impermeant products which are retained exclusively by living cells. If permeability assays remain a quick and inexpensive method to assess cell death and provide robust artifact-free information, it is unable to discriminate between different cell modes. Furthermore, concerning dyes with lipophilic properties, the enzymatic activity of intracellular esterases may be affected by cell death–unrelated phenomena. Another disadvantage of such permeability assays concerns the issue of cell death with an intact membrane, as most cytotoxic agents are not intracellular; thus, it could underestimate cellular damage. Concerning colony formation assays, it is based on the number of cells which form colonies in vitro. Cells are seeded at low densities, and the number of colonies is counted after a growth period. Clonogenic assays constitute the most reliable method for assessing cell viability. However, it tends to become harder and time-consuming when the experiment implies several samples.[22] It is also used to assess cell proliferation despite the fact that the measurement of DNA synthesis is often preferred for studying proliferation as it is easier. Indeed, the method involves labeled DNA precursors that are introduced into cells before the measurement of DNA incorporation, after incubation. The occurrence of cell division in vitro is associated with DNA incorporation through a proportional relationship.

Intracellular protein release–based assays

One of the most reliable methods for studying cell death is to measure the leakage of cellular components from compromised cultured cells when membrane integrity is altered, and especially measurement of intracellular proteins (most often enzymes) in cell culture supernatants. The presence of biomarker activity is typically proof positive for the presence of cytotoxicity, hence the frequent use of the assays for drug discovery experiments. For instance, lactate dehydrogenase (LDH) has long been used as a marker of cell death for in vitro models as LDH is released through the broken cell membrane after cell death.[23] Kits for the fluorometric or colorimetric detection of LDH are commercially available. Adenylate kinase and glucose-6-phosphate dehydrogenase are other cellular enzymes that can also be used as cell death markers even if a loss in their activity can occur during cell death assays, unlike LDH; that is why cell death assays based on LDH activity are more effective than other enzyme-based cell death assays. Lactate dehydrogenase activity can be indirectly measured by subjecting the sample to a coupled enzymatic chemistry reagent containing lactate, oxidized nicotinamide adenine dinucleotide, diaphorase, and an appropriate redox dye such as resazurin, which produces either a change in absorbance or a shift in the fluorescence profile. Methods have improved and a significantly easier assay has been described which allows same-well measurement of LDH without the need for sampling. This simplified assay suitable for high-throughput screening (HTS) protocols is now routinely used as it is inexpensive and can be easily implemented in 96- or 384-well plates.[24] However, a major inconvenience of these techniques is that physicochemical factors such as variations in the culture medium, ie, pH, may affect the activity of these enzymes. Subsequently, enzyme activity may decline with time in the extracellular milieu.[25] Besides, it is unable to discriminate between different cell death modes, and detection may be aggravated by morphologic changes in dying cells.

Metabolism-based assays

Adenosine triphosphate is produced by living cells and is a sine qua non condition to cellular life. During the cell death process, the potential to synthesize ATP decreases, whereas endogenous cytoplasmic adenosine triphosphatases remove any remaining ATP. Therefore, cell viability can be measured by counting the ATP amount from cultured cells which is a valid marker of viable cells, under certain experimental conditions.[26] Sensitive quantification of intracellular ATP is achieved by luciferase-based assays which are amenable to HTS studies. It is based on the ability of firefly luciferase to generate a luminescent signal. Hall et al developed mutant forms of luciferase, which were stable to environmental extremes as well as homogeneous reagent for measuring ATP directly from cultured cells. The luminescent ATP detection provides many advantages and has become the technique of choice for measuring cell viability in HTS laboratories: it is both easy and non time-consuming, whereas test compounds do not interfere with fluorescent assay methods. Moreover, the ATP assay is the most sensitive microplate assay available for

detecting viable cells in culture as less than 10 cells per well can be labeled.[27,28] However, it should be recognized that decreased intracellular ATP concentrations may result from nonlethal perturbations, including cessation of proliferation (senescence or contact inhibition, for instance) and inhibited mitochondrial respiration. Thus, the measurement of ATP is not always directly linked with cell viability.[25] Alternatively, the measurement of metabolic activity can be achieved using tetrazolium salt as dead cells cannot metabolize the substance. The incubation of viable cells with tetrazolium salt leads to the production of colored formazan product, associated with metabolic activity. Subsequently, colorimetric assays MTT, XTT, or WST-1 will be used to measure cell survival. Unfortunately, similar to the ATP-based assays, several factors may inhibit mitochondrial reductases, and consequently, the production of colored formazan product may not provide unequivocal data on cell viability.[29] Finally, as ATP-based and MTT-based assays are highly susceptible to metabolic interferences and may generate false-positive results, their results must be validated in a secondary time with cell death markers. Metabolism-oriented tests remain useful to get preliminary information but cannot identify cell death modes and discriminate between cytotoxic and antiproliferative effects.

Cell Death–Oriented Assays: An Anchorage Point for Drug Discovery

Apoptosis assays: caspase activation, membrane alterations, DNA fragmentation, and mitochondrial changes

A broad range of chemotherapies work by interfering with the mechanisms of cell death, and inducing apoptosis remains a promising strategy for cancer drug discovery. Using apoptosis as an end point underlies several novel approaches of cell death assays. Apoptosis involves caspase activation, cleavage of cellular protein substrates, cleavage of DNA into size fragments, and formation of apoptotic bodies. Therefore, all these characteristics represent potential targets for novel specific drugs. Despite the fact that caspase 8 and caspase 3 might be also involved in alternative nonlethal functions, as suggested by Yi et al, the detection of apoptosis using caspase enzymes remains reliable and widely used.[30] The occurrence of caspase activity in a cell during apoptosis leads to the cleavage of specific substrates that can later be easily detected as a marker of caspase activation. Indeed, several antibodies are available and can be used against a myriad of caspase substrates, including PARP-1 (poly-ADP-ribose-polymerase-1), for instance, through immunoblot analysis, immunofluorescence, and flow cytometry. Furthermore, the detection of early apoptosis may be possible, thanks to antibodies that only detect the caspase-cleaved form of a substrate protein with site-specific cleavage.[31,32] Alternatively, caspase activation can be assessed by other reagents that are compatible with HTS applications, such as exogenous proluminescent or fluorogenic caspase substrates. A real-time assessment of caspase activity may then be allowed, thanks to substrates that are bifunctional compounds, and fluorescent biosensors can be introduced into cells.[33] Thereby, studying caspase activity remains a reliable and specific approach for assessing apoptosis.[34] Among the characteristics of apoptosis, the exposure of phospholipid phosphatidylserine (PS) on the cell membrane in response to proapoptotic stimuli represents a major feature of the caspase-dependent process in healthy cells. Therefore, a PS-binding protein such as annexin-V is experimentally used to detect PS exposure. Several annexin-V derivatives associated with different fluorochromes can be used for apoptosis measurement using multicolor flow cytometry or fluorescence microscopy. To distinguish apoptotic cells from nonapoptotic cells, annexin-V is usually combined with cell-impermeable dyes, such as propidium iodide, as apoptotic cells are annexin-V positive but with intact plasma membranes. It remains a sensitive and rapid method to assess apoptosis, but we must keep in mind that PS exposure can also occur in cell death–unrelated conditions, especially when T lymphocytes are activated.[35-37]

DNA fragmentation during apoptosis is a caspase-mediated mechanism and leads to specific internucleosomal DNA double-strand breaks with fragments of multiple of 180 base pairs in size. Historically, a gel electrophoresis of genomic DNA was used for demonstrating internucleosomal DNA fragmentation as apoptotic cells display a characteristic DNA ladder, whereas necrotic cells show a smear of randomly degraded DNA, but it has been superseded by faster and easier methods. As a matter of fact, determination of DNA content and number of apoptotic cells is now preferentially investigated using flow cytometer with DNA-binding fluorochromes, such as propidium iodide, through the detection of hypodiploid nuclei. Another method widely used to study and measure DNA fragmentation is the terminal dUTP nick end labeling technique based on the formation of endonuclease-generated DNA breaks that are enzymatically labeled by terminal transferase with uridine triphosphate derivatives coupled with fluorochromes; secondarily, an immunoperoxidase reaction allows the detection. DNA fragmentation is then evaluated quantitatively by fluorescence microscopy or by flow cytometry. One has to be vigilant about the risk of false-positives linked to necrotic cells, and this method should be paired with additional assays.[38,39] Mitochondrial membrane depolarization (MMD) occurs at early apoptotic stages and before the primary morphologic and biochemical alterations. Therefore, membrane potential monitoring can provide information about kinetics of apoptotic processes. A variety of potentiometric dyes, with both flow cytometry and fluorescence microscopy, can be used for the measurement of the mitochondrial membrane potential. However, the hypothesis of MMD as an early marker of mitochondrial alterations is largely controverted, and the decrease in the transmembrane potential might rather be considered as a marker of mitochondrial damage that may take

place in late apoptosis and also during necrosis.[40] Beyond MMD, the MOMP process can also be investigated through the release of apoptogenic factors, including cytochrome c, Smac, or endoG, from the mitochondria to the cytosol. Finally, if apoptosis was initially characterized by specific morphologic features, we must keep in mind that microscopic analysis of cells is still a major way for studying apoptosis, for instance, through microscopic analysis of chromatin condensation after DNA staining.

Autophagy assays

Autophagy inhibitors are considered as potent chemosensitizers and might therefore be introduced into combination regimens for optimal anticancer therapies. Methods to measure autophagy include electron microscopy for quantifying autophagosomes, immunoblotting for detecting the lipidation of microtubules-associated protein 1 light chain 3 (also known as LC3), or immunofluorescence microscopy for monitoring the redistribution of LC3 from a diffuse pattern to a punctate one.[41] Complementary techniques are necessary as the autophagosomal accumulation of LC3 can be influenced by lysosomal acidification. For instance, another way is to assess the autophagic flux, thanks to several methods: pulse-chase, immunoblotting, or luciferase-based methods for monitoring the degradation of autophagic substrates. A luciferase coupled with LC3 has recently been used to develop a real-time HTS-compatible assay for monitoring the autophagic flux. Moreover, firefly luciferase has been shown to constitute a preferential autophagic substrate and is hence a trustful marker of autophagic degradation that may be applicable to HTS studies. In addition, recent developed assays allow determining the phosphorylation status of substrates from critical autophagy regulator kinases, such as mTORC1 and UNC51-like kinase 1. Besides, several of these assays have been recently adapted for HTS. Finally, these methods should be used to identify the underlying molecular mechanisms of autophagy more than quantifying its levels.[42–45]

Detection of necrosis, ferroptosis, and immunogenic cell death

One of the best methods to study necrosis is the use of morphologic changes that can occur after death cell, thanks to light or electron microscopy. Indeed, the term necrosis can be defined as a mass of dead cells and does not depend from a specific cell death pathway. Specific characteristics of necrotic cells have been described, including nuclear swelling, chromatin flocculation, loss of nuclear basophilia, breakdown of organelle function, and cytolysis through swelling, which leads to membrane break.[46] Nonetheless, if secondary necrosis represents the end point of all cell death modalities, primary necrosis, by contrast, constitutes an entire cell death mechanism with specific characteristics. It is difficult to identify specific markers of necroptosis as most of the biochemical processes that characterize necroptosis can also take place during apoptotic cell death, and the best way to detect necroptosis remains the analysis of a crucial kinase activation: receptor-interacting protein 1 (RIP1), which is on the crossroad of death signaling pathways. Its activation can be measured by enzymatic assay or by studying its phosphorylation on S161. The use of pharmacologic inhibitors such as Necrostatin-1 that inhibits RIP1 activity may be useful to identify necroptosis.[47] Another commonly used assay for necrosis detection measures the extracellular release of high-mobility group box 1 (HMGB1), a nonhistone chromatin-binding protein that normally regulates transcription, and kits are available based on enzyme-linked immunosorbent assay tests for quantification of HMGB1. A recent biomarker for necrotic cells has also been described: peptidylprolyl isomerase A, also known as cyclophilin A (CYPA), seems to be specifically released from necroptotic cells. Enzyme-linked immunosorbent assay kits for the quantitative measurement of CYPA are also commercially available, and drugs that bind and inhibit CYPA are being explored and might be used to develop CYPA-oriented assays. Besides, CYPA-oriented assays and HMGB1 assays should be adapted to HTS assays for detecting necrotic cell death. At present, data concerning molecular mechanisms of necroptosis are scarce and require further research.[48,49] Concerning the detection of ferroptosis, cells which undergo this form of cell death exhibit specific morphologic features such as smaller mitochondria with increased density that can be easily identified. Besides, the occurrence of ferroptosis can be confirmed using ferroptosis inhibitors such as Ferrostatin-1.[17] Finally, regarding immunogenic cell death, current research focuses on the development of inducers through vaccination assays with immunodeficient mice reconstituted with a human immune system and simultaneous detection of CALR exposure, ATP secretion, and HMGB1 release for assessing cell death. Guidelines for the detection of immunogenic cell death have recently been published.[50]

Advantages and drawbacks of methods used for assessing cell viability, apoptosis, autophagy, and necrosis are presented in Table 1.

Role of Cell Death Assays in Drug Discovery Screening Programs

A better understanding of cell death mechanisms is a sine qua non condition for improving the process of drug discovery. In a recent review, Rello-Varona et al[51] claim that the correct evaluation of cell death in every experimental setting related to cancer research is a must and advocate for a real cell death characterization so as to understand therapeutic failure, treatment-related side effects, and even provide susceptibility markers. Furthermore, this crucial assessment leads to chemotherapeutic approaches for targeting specific cell death pathways, and several novel drugs are being currently tested in

Table 1. Advantages and drawbacks of methods used for assessing cell viability, apoptosis, autophagy, and necrosis.

METHODS	ADVANTAGES	DISADVANTAGES
Cell viability		
Vital dyes	Routine laboratory work with cytofluorometry and fluorescence microscopy Robust artifact-free data	Lack of specificity concerning the type of death Inappropriate for cell death with an intact membrane Lipophilic properties of certain dyes interact with cell death–unrelated process
Colony formation assays	Reliable method	Time-consuming Laborious Only for a limited number of samples
Intracellular protein release assays (LDH)	Kits for fluorometric or colorimetric detection are commercially and easily available Simplified assay suitable for high-throughput screening protocol Implementation in 96- or 384-well plates	Physicochemical factors affect the activity of the measured enzymes Lack of specificity concerning the type of death
Metabolism-based assays (ATP)	Easy Non time-consuming Technique of choice Tests compounds do not interfere with fluorescent assay methods	Variations in intracellular ATP concentrations can result from nonlethal perturbations Risk of metabolic interferences and false-positive results Preliminary information only
Apoptosis		
Caspase activation	Reliable and specific approach Several antibodies available Early detection Real-time assessment Routine laboratory work with immunoblot analysis, immunofluorescence, and flow cytometry	Caspases can be involved in alternative nonlethal functions
Membrane alterations (PS exposure)	Several annexin-V derivatives associated with different fluorochromes available Sensitive Rapid Flow cytometry or fluorescence microscopy	Lack of specificity PS exposure can occur in cell death–unrelated conditions
DNA fragmentation	Fast and easy Flow cytometer	Risk of false-positives Preliminary information
Mitochondrial changes MMD	Early detection Kinetics data Several potentiometric dyes available	Largely controverted
MOMP process	Time-saving Sensitive Specific	
Autophagy		
Quantification of autophagosomes Detection of the lipidation of microtubules-associated protein 1 light chain 3 Monitoring the redistribution of LC3 Assessment of the autophagic flux	May be applicable to HTS studies Routine laboratory work (electron microscopy, immunoblotting, immunofluorescence microscopy) Qualitative data	Complementary techniques are necessary Influence of lysosomal acidification on LC3 No quantitative data
Necrosis		
Morphologic changes Measurement of RIP1 activation Measurement of HMGB1 Measurement of CYPA	Light or electron microscopy ELISA kits available	Scarce specific markers Not adapted to HTS assays

Abbreviations: ATP, adenosine triphosphate; CYPA, cyclophilin A; ELISA, enzyme-linked immunosorbent assay; HMGB1, high-mobility group box 1; HTS, high-throughput screening; LC3, light chain 3; LDH, lactate dehydrogenase; MMD, mitochondrial membrane depolarization; MOMP, mitochondrial outer membrane permeabilization; PS, phosphatidylserine; RIP1, receptor-interacting protein 1.

clinic, mostly in phase I and II trials. To develop new drug combinations, a further understanding of the accurate effect of chemotherapy on the death signaling pathways is still required.[52] Cell death remains a central basis in every therapeutic approach regarding cancer. Recently, in the specific field of antisarcoma therapy, the assessment of cell death mechanisms has been published, and the authors highlight the fact that sarcoma research needs the implementation of a better determination of cell death mechanisms to understand chemoresistance and cell death immunogenic potential. Several clinical trials that evaluate target therapies in sarcomas regarding apoptosis and mitotic catastrophe are still ongoing.[53] Concerning the innovative concept of immunogenic cell death, the induction of this form of death through nanocarrier-based drug delivery systems may lead to potential therapy in pancreatic cancer, hence the need to possess strong cell death assays to explore the potential role of immunogenic cell death in nanomedicines.[54]

Discussion

Understanding every molecular signaling pathway involved in cell death and elaborating potential drugs that could interfere with these specific mechanisms are the main framework for progress. An insight into cell death processes is a condition sine qua non to develop more effective and specific therapeutic approaches. Several in vitro methods for assessing cell death exist and studies largely recommend not using a single method, for having both quantitative and qualitative data. Cells can die by different modes, which are sometimes difficult to distinguish, and it is thereby essential that the measurement of cell death involves complementary methodologies. Indeed, the main drawback of apoptosis-specific and necrosis-specific assays is that neither of these assays can provide unequivocal information on the occurrence of one specific cell death modality compared with the other. Similarly, reliable method for the detection of autophagy has not yet been developed. Another important point concerns the timing of experimentation as apoptosis often occurs very quickly, whereas necrosis is much slower; thus, the use of additional clonogenicity and viability assays is necessary so as to correctly assess cell death pathways.[21] Finally, progress has been made concerning cell death–based HTS approaches leading to the identification of promising compounds, and the combination of increasingly sophisticated microscopic methods should accelerate the discovery of cell death modulatory drugs.

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Author Contributions

BM wrote the first draft of the manuscript. J-BG, AV, SE, DA, and CR contributed to the writing of the manuscript and jointly developed the structure and arguments for the paper. NM and CR-L agreed with and made critical revisions and approved the final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

The authors have read and confirmed their agreement with the ICMJE. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. The external blind peer reviewers report no conflicts of interest.

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